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Synthesis of Novel 9-*O*-Substituted Berberine Derivatives and Evaluated for Their Anticancer Activity

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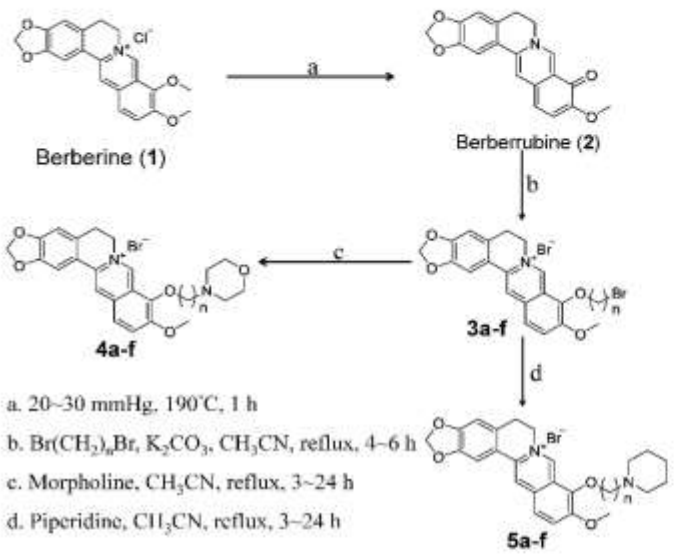
INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent form of cancer affecting human and has become the second leading cause of death from cancer in Taiwan. Most serious hepatoma cells have high proliferative activity and progress to give rise to invasion and metastasis. For HCC patients, The most widely chemotherapy against HCC is doxorubicin. However, these modalities do not substantially improve the 5-year survival because of their severe side effects like hepatic dysfunction and heart damage. Thus, suitable treatment strategy is necessary for improving the prognosis of the disease. berberine (**1**) is a natural alkaloid isolated from *Berberis* species has biological and pharmacological activities, such as anticancer. However, because of its hydrophilic in nature, berberine has a low inhibiting effect on suppressing cancer cell growth. So, we modified berberine to decrease its hydrophilic. Among these compounds, compound **4f** and **5f** have much better anti-cancer ability than berberine to DLD-1, SW480 and HepG2 cell lines. In conclusion, compound **4f** and **5f** may be potent anticancer agents.

MATERIALS AND METHODS

Chemistry

Scheme I: Synthesis of berberine derivatives.



a. 20~30 mmHg, 190°C, 1 h

b. $Br(CH_2)_nBr$, K_2CO_3, CH_3CN, reflux, 4~6 h

c. Morpholine, CH_3CN, reflux, 3~24 h

d. Piperidine, CH_3CN, reflux, 3~24 h

Table 1. Yields and physical properties of berberine derivatives.

Compound	Formula	M.W.(mole/g)	cLog*P*	Yield (%)
4a	$C_{26}H_{29}N_2O_5^+Br^-$	529.42	-0.35	80.00
4b	$C_{28}H_{33}N_2O_5^+Br^-$	557.48	0.29	70.00
4c	$C_{29}H_{35}N_2O_5^+Br^-$	571.50	0.82	59.34
4d	$C_{31}H_{39}N_2O_5^+Br^-$	599.56	1.88	25.05
4e	$C_{33}H_{43}N_2O_5^+Br^-$	627.61	2.93	58.37
4f	$C_{35}H_{49}N_2O_5^+Br^-$	655.66	3.99	45.52
5a	$C_{27}H_{31}N_2O_4^+Br^-$	527.45	0.87	14.31
5b	$C_{29}H_{35}N_2O_4^+Br^-$	555.50	1.50	7.90
5c	$C_{30}H_{37}N_2O_4^+Br^-$	569.53	2.03	24.00
5d	$C_{32}H_{41}N_2O_4^+Br^-$	597.58	3.09	22.44
5e	$C_{34}H_{45}N_2O_4^+Br^-$	625.64	4.15	64.47
5f	$C_{36}H_{49}N_2O_4^+Br^-$	653.69	5.21	73.30

RESULTS

Table 2. Cell viability of berberine and its derivatives in three cancer cell lines for 48 h .

Compound	n	IC_{50} (μM) HepG2 liver	SW480 colon	DLD-1 colon
Berberine	-	7.99 ± 1.77	8.68 ± 0.58	14.26 ± 9.98
4a	3	>20	>20	>20
4b	5	16.58 ± 3.82	15.39 ± 1.76	18.45 ± 0.03
4c	6	12.29 ± 2.27	8.33 ± 3.99	17.15 ± 2.39
4d	8	2.47 ± 0.35	1.44 ± 0.47	7.82 ± 1.19
4e	10	0.43 ± 0.08	0.51 ± 0.16	1.84 ± 0.28
4f	12	0.25 ± 0.05	0.41 ± 0.27	1.48 ± 0.03
5a	3	12.57 ± 1.71	>20	>20
5b	5	14.94 ± 0.37	14.07 ± 1.74	>20
5c	6	6.26 ± 1.59	11.30 ± 2.87	17.82 ± 1.09
5d	8	3.67 ± 1.16	1.18 ± 0.04	8.78 ± 2.38
5e	10	0.72 ± 0.06	0.58 ± 0.16	3.21 ± 0.05
5f	12	0.37 ± 0.07	0.41 ± 0.38	1.55 ± 0.12

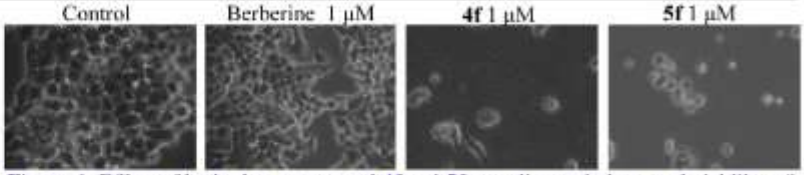


Figure 1. Effect of berberine, compound **4f** and **5f** on cell morphology and viability of hepatocellular carcinoma HepG2 cells for 48h.

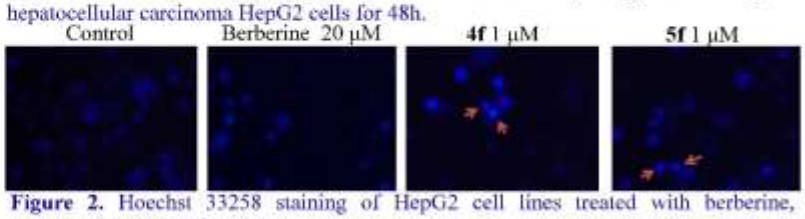


Figure 2. Hoechst 33258 staining of HepG2 cell lines treated with berberine, compound **4f** and **5f** for 24 h.

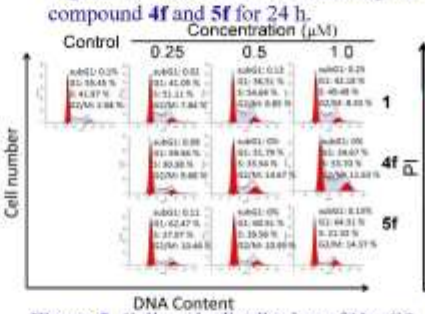


Figure 3. Cell cycle distribution of HepG2 cells treated with berberine and compound **4f** and **5f** for 24h.

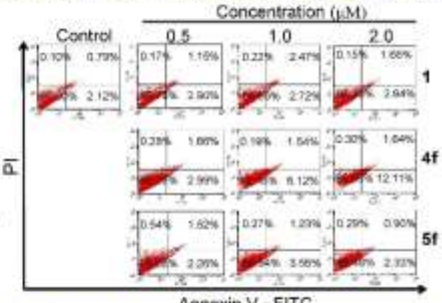


Figure 4. Apoptosis analysis of HepG2 cells treated with berberine and compound **4f** and **5f** for 48 h.

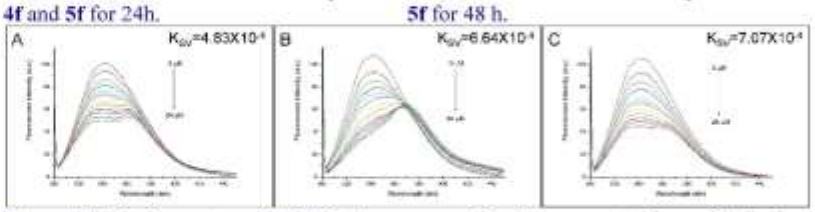


Figure 5. Emission spectra of HSA in the presence of various concentrations of berberine (A), compound **4f** (B) and **5f** (C).

CONCLUSIONS

In this study, we synthesized two series of new 9-*O*-substituted berberine derivatives **4a-f** and **5a-f**. We found that these compounds which are more hydrophobic have much stronger anticancer abilities than berberine. Thus, we revealed that 9-*O*-position lipophilic substituted berberine derivatives **4f** and **5f** could be potential candidate for new anticancer drug on hepatocellular carcinoma.

探討光滑念珠菌(*Candida glabrata*)感染泌尿道膀胱上皮細胞的環氧化酶-2 (cyclooxygenase-2)誘導之訊息傳遞路徑

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Introduction

上皮細胞分布於人體皮膚及腔道(如口腔、泌尿道等)黏膜表層的地方，主要功能為具有保護的作用，可以隔絕外來致病原，是人體的第一道防線。一旦微生物侵入宿主的上皮細胞，會誘導上皮細胞產生免疫相關反應，以抵禦致病菌的侵犯。念珠菌是人類身體的正常菌叢，為一種伺機性感染真菌，常分布於口腔、消化道、呼吸道及生殖泌尿道的黏膜上。但由於免疫功能不全者，體內的免疫功能降低，或是念珠菌大量增生時，原本共生菌就會成為致病菌而造成各種疾病。而在致病性真菌中，念珠菌屬於最常見的致病性真菌。在住院病人中，有10%的尿道感染是由念珠菌所引起，其中非白色念珠菌 (non-*albicans Candida* species)占了一半，而*Candida glabrata*則為非白色念珠菌中最常見的菌種。一般認為，菌絲的形成與念珠菌的致病力有關。但*C. glabrata* 在一般環境下不會形成菌絲，在早期被認定為致病力弱的共生菌，但由於*C. glabrata*對多種Azole類藥物具有抗性，因此在臨床上Azole類抗真菌藥物普遍使用下，導致*C. glabrata*感染人數與日俱增，目前在臨床上*C. glabrata*的感染逐漸被重視。

Aim

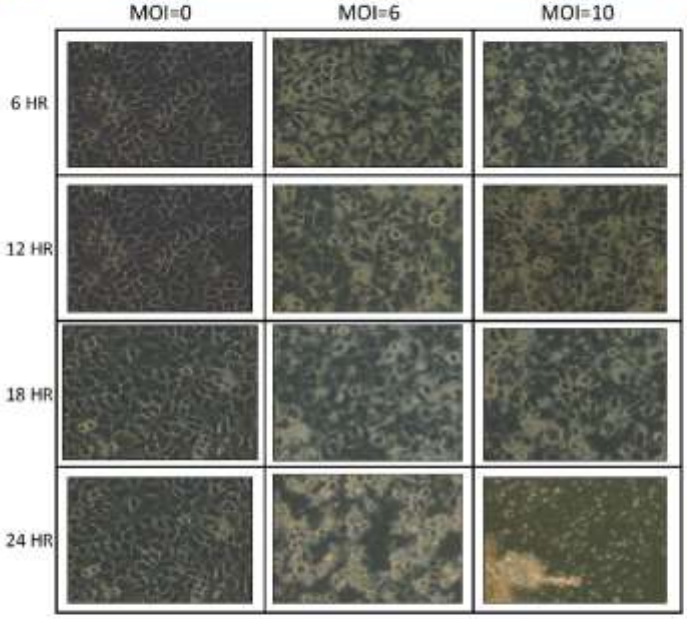
探討光滑念珠菌(*C. glabrata*)感染泌尿道膀胱上皮細胞後，誘導細胞的環氧化酶-2 (cyclooxygenase-2, COX-2)基因表現之胞內訊息傳遞路徑，幫助我們了解念珠菌感染人類膀胱上皮細胞的相關機轉。

Materials and methods

*C. glabrata*和TSGH 8301細胞株共培養 → ◆ Microscope ◆ Western blot

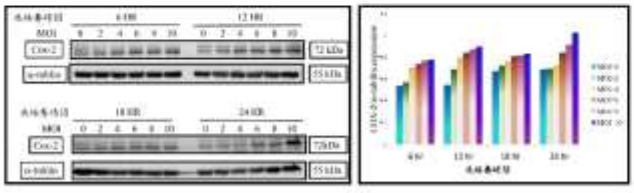
Results

顯微鏡下，受*C. glabrata*感染的膀胱上皮細胞的生長型態。(400X)



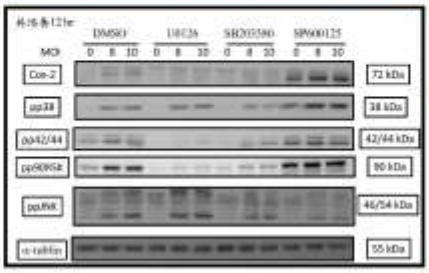
- TSGH 8301細胞株受*C. glabrata* 感染後，細胞表面會有*C. glabrata*黏附。
- *C. glabrata* 感染TSGH 8301細胞株的時間越長、MOI越高，對細胞的傷害越大。

*C. glabrata*感染TSGH 8301細胞株後，COX-2蛋白表現量上升。



- TSGH 8301細胞株與*Candida glabrata*共培養時間越長、MOI越高，細胞的COX-2蛋白表現量上升越趨明顯。

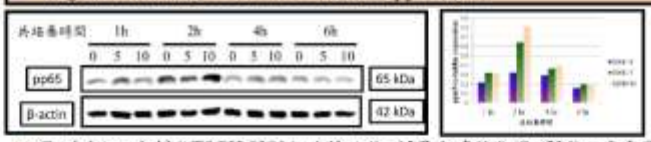
C. glabrata 刺激TSGH 8301細胞經由ERK-MAPK和p38-MAPK pathway，誘導COX-2蛋白表現上升。



- U0126：MEK1/2 inhibitor
- SB203580：p38 inhibitor
- SP600125：JNK inhibitor

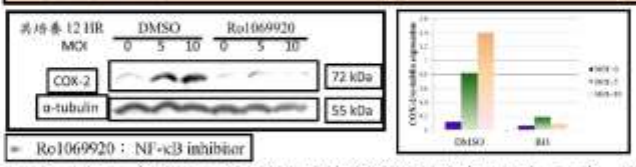
- *C. glabrata* 會刺激TSGH 8301細胞株的COX-2蛋白以及MAPK pathway的相關蛋白磷酸化表現量上升。
- 加入MEK1/2、p38 inhibitor後，COX-2蛋白表現會被抑制，推論*C. glabrata* 會刺激細胞經由ERK-MAPK和p38-MAPK pathway，使COX-2蛋白表現量上升。
- 加入JNK inhibitor後，COX-2蛋白不被抑制，推論*C. glabrata* 不經由JNK-MAPK pathway，使COX-2蛋白表現量上升。

C. glabrata 刺激TSGH 8301細胞活化pp65蛋白。



- *C. glabrata* 會刺激TSGH 8301細胞株，使p65蛋白磷酸化(Ser536)。代表*C. glabrata* 可能會刺激細胞經由NF-κB pathway產生某些反應，而刺激2小時，p65蛋白磷酸化程度最高，2小時後即降回原值。

C. glabrata 刺激TSGH 8301細胞經由NF-κB pathway，誘導COX-2蛋白表現上升。



- Ro1069920： NF-κB inhibitor

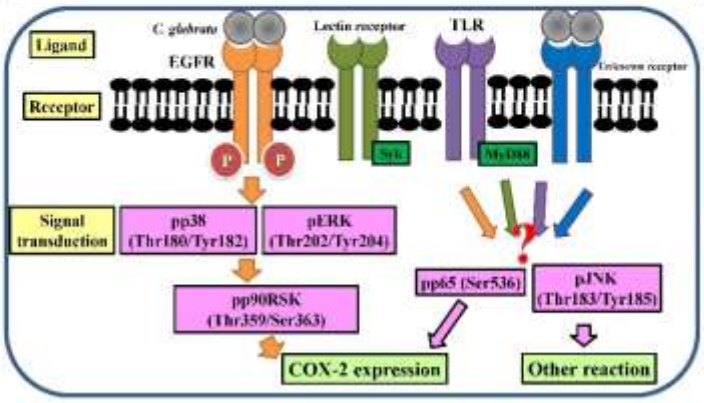
- *C. glabrata* 會刺激TSGH 8301細胞株的COX-2蛋白表現上升，而前一個結果證明*C. glabrata* 會活化NF-κB pathway相關蛋白—— p65，使其磷酸化，而加入NF-κB inhibitor後， COX-2蛋白表現會被抑制，推論*C. glabrata* 會刺激細胞經由NF-κB pathway，使COX-2蛋白表現量上升。

C. glabrata 刺激TSGH 8301細胞的EGFR，誘導COX-2蛋白表現上升。

- Syk inhibitor
- ST2825：MyD88 inhibitor
- PD168393：EGFR inhibitor

- *C. glabrata* 會刺激TSGH 8301細胞株的EGFR，誘導細胞經由ERK-MAPK和p38-MAPK pathway，促使COX-2蛋白表現上升。

Conclusion



葡萄糖濃度對新型隱球菌生物膜生成能力的影響

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摘　　要

台灣為隱球菌腦膜炎的主要流行區之一，其致病菌*Cryptococcus neoformans* (新型隱球菌) 是一種具莢膜的酵母菌，通常存在於土壤、鳥類排泄物和腐爛有機物中，經呼吸道感染人體而引起肺部疾病。若患者免疫功能低下，病菌會透過循環系統散播全身，並透過血腦屏障侵入中樞神經系統引發腦膜炎。葡萄糖醛酸木糖甘露聚糖為*C. neoformans*莢膜主成分之一，並且與其生物膜的形成有關。生物膜的形成將病菌保護在多糖層中，使之不受抗真菌劑作用而造成臨床上治療的困難。*C. neoformans*依其血清型可分為*C. neoformans* var. *grubii* (血清型A)、*C. neoformans* var. *neoformans* (血清型D) 和*C. neoformans* var. *gattii* (血清型B、C)；*C. neoformans* var. *grubii*佔全世界隱球菌感染症的70%，會感病病人隱球菌感染大多由此血清型造成；*C. neoformans* var. *neoformans*佔全世界隱球菌感染症的9%，臨床上常見於急性感染。但較少造成腦膜炎；*C. neoformans* var. *gattii*好發於免疫力正常的人，容易造成腦部病變和肺部感染。本計畫中分析彰化基督教醫院隱球菌腦膜炎患者腦脊髓液檢體分離之隱球菌，利用L-canavanine glycine bromothymol blue agar區分*C. neoformans*和*C. gattii*，並利用polystyrene培養盤及硝化纖維膜貼附菌體培養進行生物膜形成活性分析。分析臨床分離菌株生物膜形成能力後，發現*C. neoformans*形成生物膜之能力易受到葡萄糖濃度影響，高濃度葡萄糖培養液會促進菌體生成生物膜。本研究將利用生物膜形成分析，進一步以人工骨髓液培養來模擬*C. neoformans*感染病人過程中，生物膜形成之過程以及葡萄糖濃度之影響。

材 料 與 方 法

菌種

本研究所使用的59株*C. neoformans*臨床菌株，是從彰化基督教醫院經抗真菌劑治療之隱球菌腦膜炎確診患者之CSF檢體中分離培養，並經莢膜染色鏡檢證實，且以PCR增幅其ITS區域片段確認。

培養基

根據Luis R. Martinez等人之研究，minimal medium (20 mg/ml thiamine、26 mM glycine、20 mM $MgSO_4 \cdot 7H_2O$、58.8 mM KH_2PO_4、30 mM glucose) 可促使*C. neoformans*形成生物膜，因此本研究以minimal medium 進行體外生成生物膜定量分析系統之建立。此外，以含有4 mM及10 mM葡萄糖之minimal medium分別模擬正常人及糖尿病患者腦脊髓液中葡萄糖濃度，欲探討在糖尿病患者中*C. neoformans*形成生物膜之能力是否會有差異。研究中，為了模擬*C. neoformans*在中樞神經系統的感染，使用人工腦脊髓液 (ACSF) (119 mM NaCl、26.2 mM $NaHCO_3$、2.5 mM KCl、1 mM NaH_2PO_4、1.3 mM $MgCl_2$、10 mM glucose) 進行*C. neoformans*體外生物膜定量分析。

體外生成生物膜定量分析系統──結晶紫染色分析法

在12孔盤中，分別加入含有不同葡萄糖濃度的minimal medium，並分別加入10^7 cfu培養至晚對數期的*C. neoformans*，於37℃靜置培養48小時後，將孔中的菌液全部取至微量離心管中，離心後以無菌水清洗離心管底部菌體，並加入結晶紫進行染色，結晶紫染色後離心去除多餘的結晶紫溶液，並加入無菌水清洗未結合在菌體上的結晶紫，最後加入75%酒精將菌體上之生物膜脫色，短暫離心後取上層酒精溶液以微孔盤分光亮度儀 (Anthos 2010, Anthos labtec instruments) 測量OD_{595}的吸收光讀值，扣除背景值 (75%酒精) 後即為生物膜生成指標。

以CGB agar鑑別*C. neoformans*和*C. gattii*

根據Tesfaye Belay等人的研究，*C. gattii*可將canavanine作為唯一碳源，而*C. neoformans*則無法，因此本研究以L-canavanine glycine bromothymol blue agar (CGB agar) (Glycine、L-canavanine sulphate、Bromthymol blue、Thiamine HCl、KH_2PO_4、$MgSO_4$) 從臨床菌株中區分出*C. neoformans*和*C. gattii*。

結　　果

以CGB agar鑑別*C. neoformans*和*C. gattii*

將*C. neoformans*臨床菌株以CGB agar培養48小時後，*C. gattii*會將L-canavanine代謝產生鹼性物質，使bromthymol blue變為藍色，為陽性反應。在figure 1中，A為陰性反應，B為弱陽型，皆為*C. neoformans*。在本研究中，已鑑定的9株臨床菌株為*C. neoformans* (table 1)。

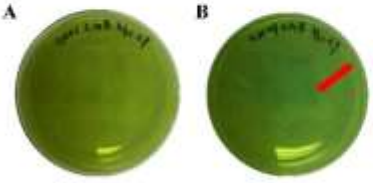

Figure 1. 以CGB agar 鑑別*C. gattii*與*C. neoformans*。A為CN01，反應為陰性；B為CN09，紅色箭頭處有微弱的陽性反應 (weak reaction)。

Table 1. 以CGB agar 鑑別*C. gattii*與*C. neoformans*。

	Positive	Weak reaction	Negative
Strain	-	09	01、05、06、10、11、12、13、14、

體外生成生物膜定量分析系統──結晶紫染色分析法

將*C. neoformans*以含30 mM葡萄糖之minimal medium培養48小時後，生物膜形成情形如 figure 2所示，可觀察到CN06生物膜形成量較CN01多。以結晶紫染色後測量OD_{595}的吸收光讀值，其值扣除背景值 (75%酒精) 後即為生物膜生成量，結果如figure 3。在figure 3可看到CN06生物膜形成量較CN01多，與肉眼觀察結果一致，且隨著minimal medium中葡萄糖濃度增加，生物膜形成量也隨之增加。

將*C. neoformans*以不同葡萄糖濃度之ACSF培養160小時後，亦可觀察到ACSF中葡萄糖濃度增加，生物膜形成量也隨之增加 (figure 4)。

Figure 2. *C. neoformans*以含30 mM葡萄糖之minimal medium培養48小時生物膜形成情形。A為CN01；B為CN06。

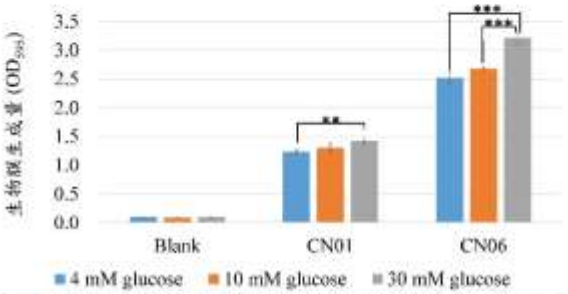

Figure 3.結晶紫染色法分析不同葡萄糖濃度之minimal medium對*C. neoformans*臨床菌株生物膜形成之影響。

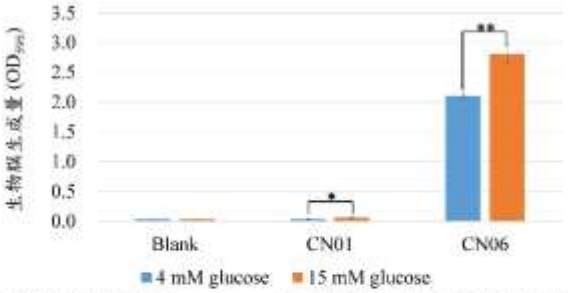

Figure 4.以結晶紫染色法分析*C. neoformans*在不同葡萄糖濃度的ACSF中生物膜生成。

結　　論

1. 以結晶紫染色分析法進行*C. neoformans* 臨床菌株體外生成生物膜定量分析，其結果具有統計上之差異，成功建立體外生成生物膜定量分析系統。
2. 在含有不同葡萄糖濃度的minimal medium或ACSF中，隨著葡萄糖濃度增加生物膜生成量有增加的趨勢，推測高濃度之葡萄糖培養液會促進*C. neoformans*生成生物膜。

Analysis of Proinflammatory Response and Mechanism in Human Urinary Epithelial Cells Infected by *Candida albicans*

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Introduction

Candida albicans (*C. albicans*)

- *C. albicans* are normal constituents of the normal oral, urinary tract and gut flora in ~50% of the population and are also the most common fungal pathogen that can cause a variety of mucosal diseases with significant morbidity (Candida and Candidiasis / Philadelphia: Mosby Elsevier, 2008).
- According to statistics, the majority of fugal infections are caused by the Candida species, and the most proportion is *C. albicans*, which accounting for 72.3% of the pathogenic Candida species (Mycopathologia 2002;172:235-239).
- *C. albicans* morphology mainly divided into three types: yeast form, pseudohyphae type, and hyphae and the first theory proposes that *C.albicans* must undergo morphogenic conversion from yeast to hyphal forms within the circulation which makes it more capable of invading the tissues (Infect. Immun 2000;68:3485-3490; Infect. Immun 2000;72:598-601).
- Through observing urine, blood, sputum and other common human body fluids, isolated Candida strains was mostly collected from urine, and up to 45.2% of Candida use urine as a source of nutrition (Diagnostic Microbiology and Infectious Disease 2013;76(2):182–186). The mortality rates after candidiasis infection is as high as 30-60% (Infectious Disease Society of America 1995;20:1530–4).

C. albicans-induced Proinflammatory Response in Epithelial Cells

- Mucosal epithelia possess distinct mechanisms that enable discrimination between harmless commensal organisms and disease-causing pathogenic organisms. This process results in either nonresponsiveness or homeostasis (commensals) or activation of an immune response (pathogens) (Cell Host & Microbe 2010;8:225-235).
- Under suitable predisposing conditions, *C. albicans* is able to cause a variety of mucosal diseases with significant morbidity and potentially fatal disseminated infections in immunocompromised individuals and patients on immunosuppressive regimens (Candida and Candidiasis. Philadelphia: Mosby Elsevier, 2008 ; Crit. Rev. Microbiol 2010;36:1-53).
- Glycosylation of *C. albicans* cell wall proteins is critical for induction of innate immune responses and IL-6, IL-8 or other proinflammatory cytokines activation and apoptosis of epithelial. (Infect. Immun. Dec 2001;4902-4910; PLOS ONE 2012;7(11): e50918; Cell Host Microbe 2010;8:225-235).

Aim

This study aimed to identify the proinflammatory mediators in *C. albicans*–infected human bladder epithelial cells. We want to investigate proinflammatory mechanism of bladder epithelial cell infected by *C. albicans*. We hope to supply some alternative methods that can help patients with urinary tract infected by *C. albicans*.

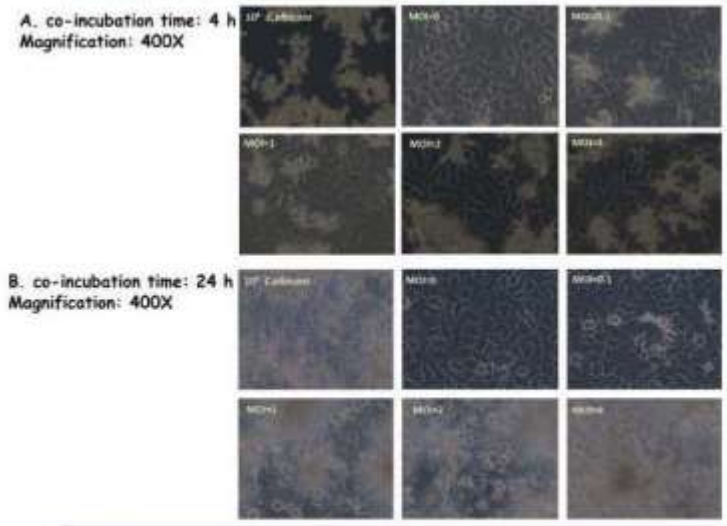
Materials and methods

Urinary epithelial cells co-incubated with *C. albicans* → TSGH 8301 cells →

- Microscopy
- LDH assay
- RT-PCR
- Western blot
- PGE_2 assay

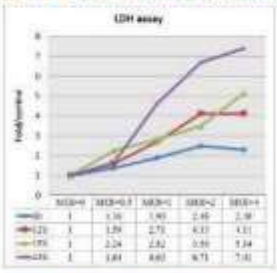
Results

Figure 1 Morphology of human bladder epithelial cells TSGH 8301 co-incubated with *C. albicans* for fungal burden



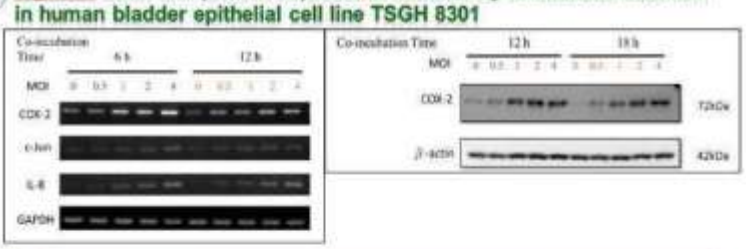
1. After infection for 4 h, major form of *C. albicans* are germ tube and yeast type, and at 24 h, the major form shifts to hyphae and pseudohypha.
2. Compare to multiplicity of infection (MOI) of 0, the bladder epithelial cells morphology had change and damage after of *C. albicans* infection for 24 h.

Figure 2 Induction of epithelial cell damage by *C. albicans* infection



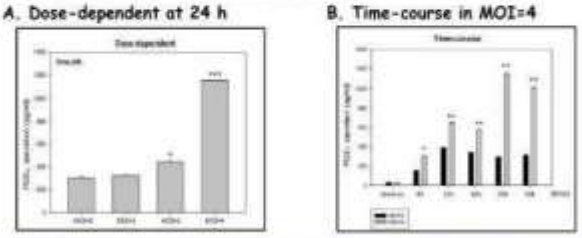
1. At different infection time, the higher the number of *C. albicans*, the more damage of TSGH8301 cells.
2. In MOI of 4, TSGH8301 cells are significant damage after infection for 24 h.

Figure 3 Gene and protein expression induced by *C. albicans* infection in human bladder epithelial cell line TSGH 8301



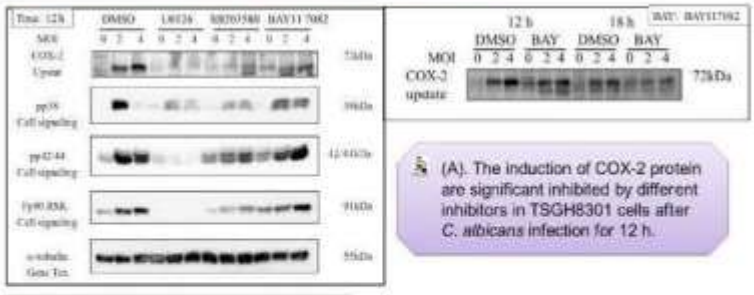
The induction of IL-8 and COX-2 gene or protein are significant expression in TSGH8301 cells after *C. albicans* infection at 6 h, 12 h and 18 h.

Figure 4 Culture medium prostaglandin E_2 (PGE_2) accumulation after TSGH 8301 cells infected by *C. albicans*



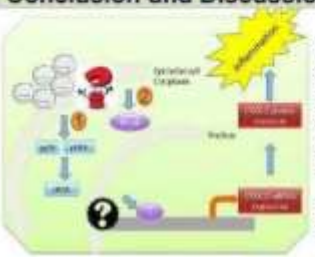
(A). After infection for 24 h, culture medium PGE_2 accumulation are evident in MOI of 2 and MOI of 4.
(B). In MOI of 4, culture medium PGE_2 accumulation are evident after TSGH 8301 cells infected by *C. albicans* for 8-24 h.
*P<0.05, **P<0.01, ***P<0.001 (MOI=0 versus MOI=4).

Figure 5 *C. albicans*-induced COX-2 expression is suppressed by inhibitors of MAPK and NF-κB signal pathways



(A). The induction of COX-2 protein are significant inhibited by different inhibitors in TSGH8301 cells after *C. albicans* infection for 12 h.

Conclusion and Discussion



In this study, we found that *C. albicans* caused the bladder epithelial cells morphology change, cell damage and inflammatory response including cyclooxygenase-2 (COX-2) and interleulin-8 (IL-8) expression and PGE_2 accumulation. The COX-2 gene was induced majorly though MAPK and partially NF-κB pathway.

In the future, we will identify the transcription factor(s) and promoter binding site(s) of *C. albicans*-induced COX-2 gene in bladder epithelial cells.

Synthesis of New Lipophilic Substituent Baicalein Derivatives and Biological Evaluation as Potent Antitumor Agents

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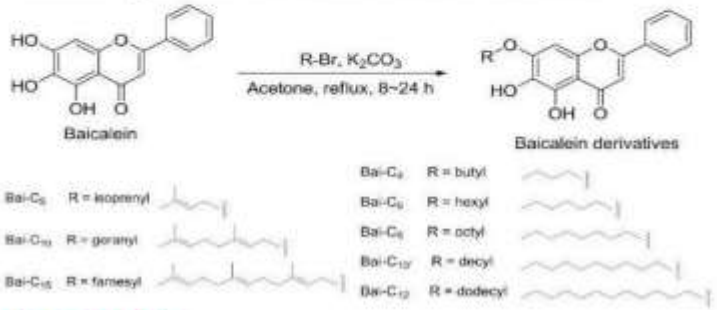
INTRODUCTION

Colon cancer is one of the most common malignant diseases worldwide. It always accompany poor prognosis and the more person are diagnosed in recent years in Taiwan. Baicalein, a flavonoid from *Scutellariae baicalensis* Georgi (*S. baicalensis*), has many pharmacological effect and demonstrates cytotoxicity in a variety of tumor cell line. But, baicalein is become over 90% converted to baicalein-7-*O*-glucuronide (baicalin) in the intestinal. In hepatic metabolic study, baicalein is more rapid glucuronidation and sulfation in 7th position of hydroxy group that in the intestinal. In the study, we modified baicalein by lipophilic substitution at the 7th position hydroxy group of A ring to terpenyl derivatives, including isoprenyl, geranyl, farnesyl groups and alkyl derivatives, including butyl, hexyl, octyl, decyl, dodecyl groups. The cytotoxic effects treated with baicalein or 7-*O*-farnesylbaicalein (**Bai-C_{15}**) in human colon cancer cell lines, human hepatocellular carcinoma and normal embryonic murine BNL CL.2 cell lines, cell growth inhibition was evaluated by MTT assay. Using flow cytometry examined cell cycle distribution by PI staining and apoptosis by annexin V-FITC/PI dual staining methods on human colon cancer SW480 cell lines. There from among those small-molecule compounds, 7-*O*-farnesylbaicalein (**Bai-C_{15}**) is significant potential anticancer activity in human colon cancer cell lines (HT-29, SW480 and DLD-1). **Bai-C_{15}** significantly inhibits the growth of human colon cancer cell lines, a little or no cytotoxicity in human hepatocellular carcinoma HepG2 cell lines and normal embryonic murine BNL CL.2 cell lines. In cell cycle analysis, **Bai-C_{15}** induces SW480 that arrested cell cycle at S phase and induced apoptosis. In wound-healing assay, **Bai-C_{15}** had a strong effect in inhibition cell migration. In summary, these preliminary results showed that **Bai-C_{15}** has a variety of function to inhibition in human colon cancer cells. That could to develop a new potential drug in human colon cancer therapy.

MATERIALS AND METHODS

Chemistry

Scheme 1: Synthesis of 7-*O*-substituted baicalein derivatives.



RESULTS

Table 1. Cytotoxicity of baicalein and its derivatives on human cancer cell line for 48 h

	R	IC_{50} (μM)				
		SW480 Colon	HT-29 Colon	DLD－1 Colon	HepG2 Liver	BNL CL.2 Normal cells
Baicalein	H	18.18 ± 0.89	30.61 ± 0.46	27.88 ± 0.31	28.09 ± 0.51	> 40
Bai-C_1	Methyl	29.41 ± 0.46	> 40	30.93 ± 0.65	> 40	> 40
Bai-C_5	Isoprenyl	8.60 ± 0.37	> 20	> 20	> 20	> 20
Bai-C_{10}	Geranyl	2.84 ± 0.43	17.02 ± 0.25	9.77 ± 0.93	> 20	> 20
Bai-C_{15}	Farnesyl	**1.15 ± 0.15**	14. 95 ± 0.63	6.97 ± 0.15	> 20	> 20
Bai-C_4	Butyl	> 20	19.48 ± 0.35	> 20	> 20	> 20
Bai-C_6	Hexyl	9.48 ± 0.47	17.65 ± 0.16	19.01 ± 0.52	> 20	> 20
Bai-C_8	Octyl	3.03 ± 0.46	16.25 ± 0.62	15.41 ± 0.76	> 20	> 20
Bai-C_{10}	Decyl	1.99 ± 0.38	> 20	15.32 ± 0.47	> 20	> 20
Bai-C_{12}	Dodecyl	**1.57 ± 0.29**	> 20	9.26 ± 0.10	> 20	> 20
Wogonin	H	35.06 ± 3.84	39.33 ± 0.23	36.87 ± 0.65	37.89 ± 2.26	> 40
Wo-C_1	Methyl	> 20	> 20	> 20	2.72 ± 0.84	> 20
Wo-C_5	Isoprenyl	> 20	> 20	> 20	> 20	> 20
Wo-C_{10}	Geranyl	> 20	> 20	> 20	> 20	> 20
Wo-C_{15}	Farnesyl	> 20	> 20	> 20	> 20	> 20
Chrysin	H	31.08 ± 2.96	19.49 ± 0.38	18.62 ± 0.81	16.50 ± 0.36	> 40
C-C_1	Methyl	> 20	> 20	> 20	> 20	> 20
C-C_5	Isoprenyl	> 20	> 20	> 20	> 20	> 20
C-C_{10}	Geranyl	> 20	> 20	> 20	> 20	> 20
C-C_{15}	Farnesyl	> 20	> 20	> 20	> 20	> 20

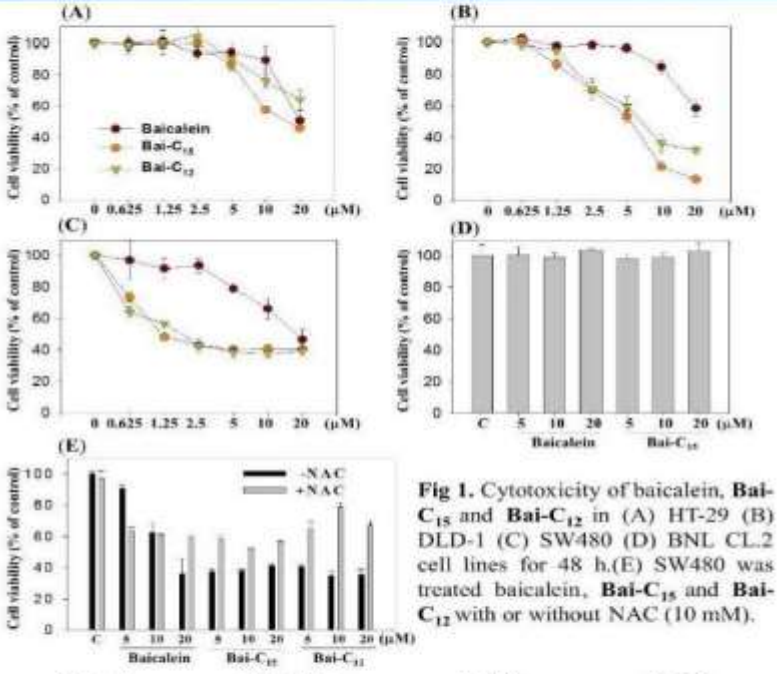


Fig 1. Cytotoxicity of baicalein, **Bai-C_{15}** and **Bai-C_{12}** in (A) HT-29 (B) DLD-1 (C) SW480 (D) BNL CL.2 cell lines for 48 h.(E) SW480 was treated baicalein, **Bai-C_{15}** and **Bai-C_{12}** with or without NAC (10 mM).

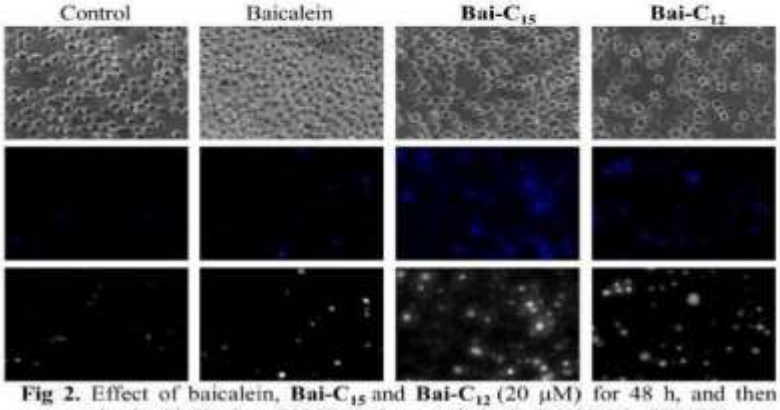


Fig 2. Effect of baicalein, **Bai-C_{15}** and **Bai-C_{12}** (20 μM) for 48 h, and then were stained with Hochest 33258 on the morphological (400×) in SW480.

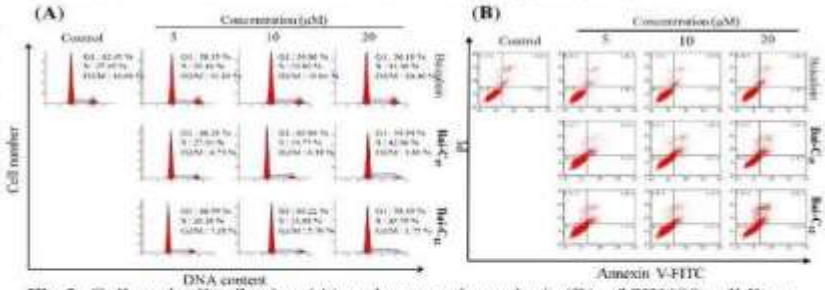


Fig 3. Cell cycle distribution (A) and apoptotic analysis (B) of SW480 cell lines treated with baicalein, **Bai-C_{15}** and **Bai-C_{12}** (5–20 μM) by flow cytometer.

CONCLUSIONS

In this study, we synthesized a series of lipophilic substitution flavonoid derivatives on 7th position hydroxyl group. In addition, **Bai-C_{15}** and **Bai-C_{12}** were better activity in human colon cancer cells. **Bai-C_{15}** compare with baicalein and it's derivatives that has the lowest IC_{50} value in SW480 cells and no cytotoxicity in BNL CL.2 (normal murine cell lines). However, NAC partly reduced the accumulation of ROS induced by **Bai-C_{15}**. Treatment with **Bai-C_{15}** increased cell shrinkage and DNA fragment in SW480 cells. The flow cytometric analysis, **Bai-C_{15}** mainly caused S phase arrest and increased apoptotic rate in SW480 cells. In conclusion, **Bai-C_{15}** can as a potential antitumor agent on human colon cancer cell lines in the further.

Molecular Epidemiology Studies of Invasive Infections Caused by *C. albicans* in a Hospital in North Taiwan from 2003 to 2011

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Background: *Candida albicans* is a commensal of humans but is also the main fungal pathogen of humans. As the changing of medical environment, candidemia is an increasing common cause of blood infection in hospitalized patients. Investigation of genetic evolution of *C. albicans* demonstrated the geographic clustering, and also hinted some association between antifungal resistance and genetic traits. **Methods:** Multilocus sequence typing (MLST), which based on sequencing 7 selected house-keeping genes and identifying polymorphic nucleotide sites, has been introduced to the characterization of *C. albicans* isolates for epidemiological purposes. **Results:** After analysis of allelic sequences of 330 *C. albicans* isolates from Chang-Gung Memorial Hospital Linkou (CGMHL) from 2003 to 2011, 125 isolates were assigned diploid sequence types (DSTs) but more than hundred isolates matched no DST in MLST database. Furthermore, by a phylogenetics algorithm eBURST analysis, we found that DST659 is possibly the most common clonal founder in CGMHL isolates during 2003 to 2011. With four much variable allele sequences of seven, UPGMA and neighbor joining methods were applied to create phenograms. Three cluster groups, DST659-like, DST669-like and DST69-like, were clearly separated. Interestingly, a subset of DST659-like group showed significantly higher biofilm formation than other groups. **Conclusions:** The association between biofilm formation and the cluster expansion of DST659 is worth further investigation.

Materials & Methods

Candida albicans isolates

All *C. albicans* isolates were collected from Chang-Gung Memorial Hospital Linkou (CGMHL) from 2003 to 2011. 288 isolates (87.3%) were collected from blood cultures obtained from invasive candidiasis patients, and 42 (12.7%) were isolated from candiuria patients without candidedemia. All of these isolates were identified by conventional methods, and the results were further confirmed with the API ID32C system (bioMerieux) or CHROMagar Candida (BD).

Multilocus sequence typing (MLST) analysis

The *C. albicans* MLST is based on the method developed by Bougnoux et al., *C. albicans* MLST typing is based on variations of 7 housekeeping gene loci, including AAT1a - ACC1 - VPS13 - MPIb - ADP1 - ZWF1b and SYA1. The internal regions of these genes were PCR amplified and sequenced. Each sequence result was assigned with an allele number (http://calbicans.mlst.net/), and then the combination of the 7 allele numbers defined a unique diploid strain types (DSTs) representing its genotype. The numbered clades of *C. albicans* were described as previously. A dendrogram was constructed with the use of un-weighted pair group method with arithmetic Mean (UPGMA). Numbers at nodal points indicate bootstrap values for 1,000 replications.

eBURST analysis

Putative relationships between isolates were determined by eBURST (http://eburst.mlst.net/). The eBURST algorithm was used for not only placing all related isolates into clonal complexes but also predicting the ancestral DST of each complex. The results of eBURST displayed the most parsimonious patterns of each descent from the ancestral DST type.

Antifungal susceptibility testing

A commercially prepared dried colorimetric microdilution panel (Sensititre YeastOne, TREK Diagnostic Systems) was used for susceptibility testing of *Candida* to 5-flucytosine, amphotericin B, anidulafungin, caspofungin, fluconazole, itraconazole, micafungin, posaconazole, and voriconazole, in accordance with the manual provided. In brief, 24-h incubated yeast isolates were suspended into distilled water and the density was adjusted to 0.5 McFarland, followed by 1/550 dilution into 11 ml YeastOne medium (1.5-8 x 10^3 cfu/ml at first). Diluted solution and antifungal agents were added into 96-microtiter wells, which were then incubated at 35 ℃ without CO_2 for 24 h. The minimal inhibition concentration (MIC) was finally determined according to the guideline provided. MIC_{50} and MIC_{90} values of *C. albicans* isolates against each antifungal agent were also calculated.

In vitro biofilm weighting analysis

Sterile MF-Millipore membrane (0.8 μm pore size) (Millipore) were placed into 12-well culture wells, and incubated with 1-ml fetal bovine serum for 16 hr. The membranes were PBS washed and wet with Spider medium (10 g/L mannitol, 20 g/L Bacto peptone, 10 g/L Nutrient broth, 10 g/L yeast extract, 4 g/L potassium phosphate dibasic, pH7.2), following inoculation with *C. albicans* for 90 min at 37 ℃. Unbound *C. albicans* yeasts were removed by PBS washing, and then incubated with sterile Spider medium for 60 hours. Finally, the PBS washed filter membranes were air dried and weighted. Blank was performed with non-inoculation membrane in the same procedure, while a biofilm defective strain ΔTEC1 was used for negative control and its congenic parental wild type *C. albicans* SC5314 was used for positive control. The mean with 2 standard deviations of ΔTEC1 membrane weights were used for the threshold of biofilm formation. *C. albicans* SC5314 (a/a) and ΔTEC1 were performed with 9 different replicates independently.

Results

Table 1. The list of *C. albicans* isolates collected from different years in CGMHL

year	Numbers of *C. albicans*	ICU		Pediatrics	Candiduria
		Candidemia	Candidemia Candiduria		
2011	40	29	0	6	5
2010	38	18	6	7	7
2009	44	14	13	7	10
2008	45	20	11	5	9
2007	48	19	13	10	6
2006	34	21	3	9	1
2005	31	14	5	9	3
2004	30	21	0	8	1
2003	26	14	0	12	0
Total	330	164	51	73	42

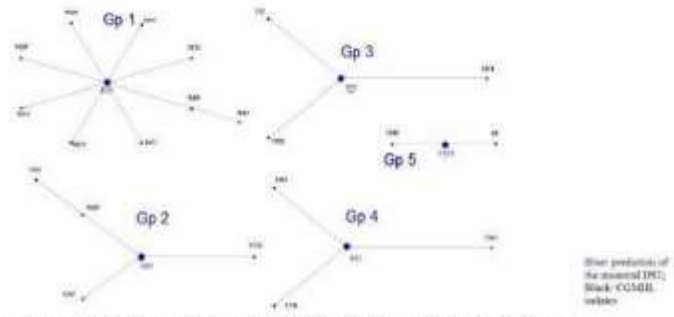

Figure 1. The diagram of eBURST analysis for *C. albicans* in CGMHL

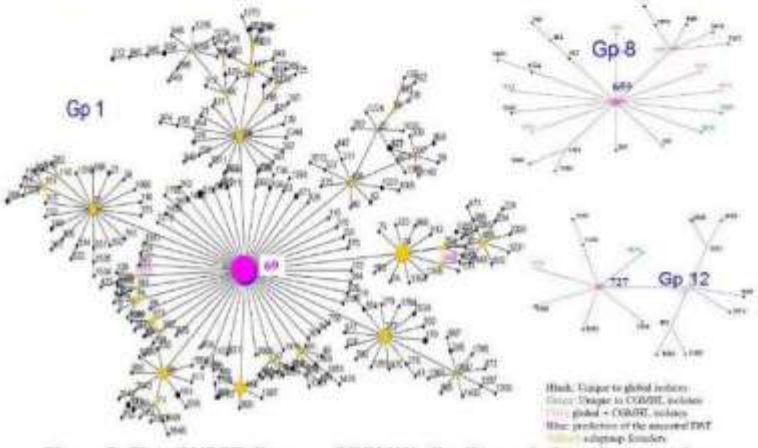

Figure 2. The eBURST diagram of CGMHL *C. albicans* isolates in comparison with global isolates

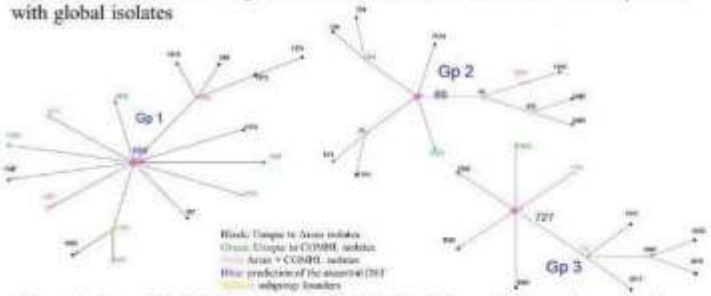

Figure 3. The eBURST diagram of CGMHL *C. albicans* isolates in comparison with Asian isolates

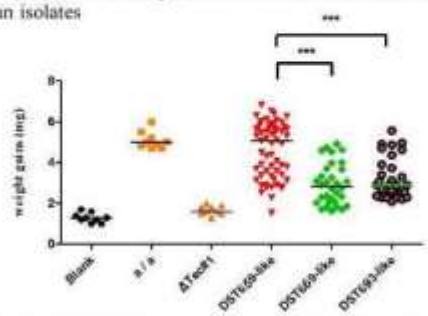

Figure 4. *Candida* biofilm analysis by weighting membrane. Note: a/a stands for positive control using *C. albicans* SC5314 strain; Tec#1 stands for negative control using *C. albicans* SC5314 *ΔTEC1*.

Conclusions

1. DST659 is the main cluster founder of *C. albicans* isolates in CGMHL by eBURST analysis.
2. DST659-like group showed significantly higher biofilm formation than other groups.