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Short communication

Liquid chromatography–tandem mass spectrometric method for the analysis of fluconazole and evaluation of the impact of phenolic compounds on the concentration of fluconazole in *Candida albicans*

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Abstract

A bioanalytical method using liquid chromatography–tandem mass spectrometry was developed for the analysis of fluconazole in *Candida albicans* after incubation with phenolic compounds, which have been proved possessing antifungal properties and have synergetic activity against *C. albicans* when in combination with fluconazole. Samples of *C. albicans* thallus obtained by centrifuging the mixed culture after 24 h incubation were saponified and centrifuged. The supernatant was evaporated to dryness, reconstituted, and injected on a C18 column using an organic-aqueous mobile phase. The chromatographic run time was 3.5 min per injection, with retention times of 2.4 min for fluconazole. The detection was by monitoring fluconazole at *m/z* 305 → 191. The standard curve range was 1.0–100.0 ng ml^{-1} with a mean correlation coefficient 0.9992. The precision and accuracy of the quality control (QC) samples were R.S.D. < 5.5%, R.E. < 3% for intra-day and R.S.D. < 6.2%, R.E. < 4% for inter-day. The concentration of fluconazole in *C. albicans* was found to be increased with the increment of the tested compounds concentration when they were in combination.

Keywords: Fluconazole; Phenolic compounds; HPLC–MS/MS; *Candida albicans*

1. Introduction

Fluconazole, a highly selective inhibitor of fungal cytochrome P-450 sterol C-14 alpha-demethylation, is an active agent against yeasts, yeast-like fungi and dimorphic fungi [1,2]. Clinical pharmacokinetics of fluconazole indicates that fluconazole is effective at a wide range of body sites because of its large distribution volume and the long half-life, combined with a rapid absorption after oral administration [3,4]. It has been widely used for the prevention and treatment of a broad range of fungal infections as a first-line

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antifungal drug [5–8]. But with its wide use, the resistance of fluconazole to the most common pathogen *Candida albicans* was frequently reported these years [9–11]. Extensive biochemical studies highlighted the significant diversity in the mechanisms conferring resistance to azoles, which include alterations in sterol biosynthesis, target site, uptake and efflux. But the prime mechanism is reduced intracellular accumulation of azoles because of decreased amount of uptake and the increased levels of active efflux of drugs [12,13]. The increasing clinical difficulty for the treatment of drug-resistance fungal infection has lead to intensive research on the mechanisms of fungal resistance, the way how to avoid and reverse the resistance to the available antifungals as well as potent antifungal compounds screening and new antifungals development.

In our continuous program focusing on the research of antifungal activities of phenolic compounds from liverworts [14] and other plants, we have tried to determine their synergetic antifungal effects to *C. albicnas* and to elucidate their action mechanism. In present work, two natural phenolic compounds and one derivative with fluorine, which have been proved possessing antifungal activities and having synergetic effects with fluconazole against *C. albicans* according to the National Committee for Clinical Laboratory Standards (NCCLS) microdilution method M27-A protocol [15], were studied for their impact on concentration of fluconazole in *C. albicans* after 24 h incubation together. A high performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) was employed for the quantitation analysis of fluconazole in *C. albicans*. The result will account for one of the possible mechanisms of the synergy effects we have observed [16].

2. Experimental

2.1. Strains and media

A strain of *C. albicans* isolated from a patient hospitalised in a tertiary care hospital (800 beds) in 2002 was identified by standard mycological methods [17–19]. Chromogenic medium BBL® CHROMagar™ Candida (BBL®, Becton Dickinson, USA) and biochemical test ID 32 C (bioMerieux, France) were used and all media and biochemical tests were incubated at 30 °C for 48 h. Its susceptibility (MIC) to fluconazole was 64 μg ml^{-1} determined according to NCCLS microdilution method M27-A protocol. The isolate was kept at −70 °C and was passaged at least twice on Sabouraud dextrose agar at 35 °C prior to being tested.

RPMI 1640 medium, pH was 6.3, with L-glutamine but without sodium bicarbonate (Dulbecco's Modified Eagle Medium, lot no. 1095479, 24 h Chemical Hazard Emergency No. USA (301) 431–8585), was supplemented with dextrose to a final concentration of 2% and 0.165 M morpholine-propanesulfonic acid (MOPS), and H was adjusted to 7.0.

2.2. Chemicals and reagents

Two natural phenolic compounds and one synthetic phenolic derivative with fluorine (Fig. 1) were obtained from our department and their structures have been identified unequivocally by spectral methods. Fluconazole was kindly provided by Pfizer Pharmaceutical Ltd., China. Sodium hydroxide, monopotassium phosphate, hydrochloric acid, methanol, ammonium hydroxide, dimethyl sulfoxide and water were of LC grade and were purchased from Huamei medical reagent Ltd., Shanghai, China.

2.3. Calibration standards and quality control samples

Standards and quality controls (QCs) were made from one stock solutions (1 mg ml^{-1} in ethanol–water 1:1, v/v) of fluconazole. The stock solutions were stable for at least 1 month when kept in a refrigerator at 2–8 °C. Calibration standards at concentrations of 1.0, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0, and 100.0 ng ml^{-1} were prepared in ethanol. QCs at levels of 1.0, 60.0, and 100.0 ng ml^{-1} were prepared for the determination of intra-day and inter-day accuracy and precision. All standards and QCs were aliquoted into pre-labeled 1.5 ml vials and stored at −20 °C.

2.4. HPLC–MS/MS

The HPLC–MS/MS system consisted of an Agilent HPLC system (1100 series, USA) and a AB Sciex API 4000 tandem mass spectrometer (Applied Biosystems,

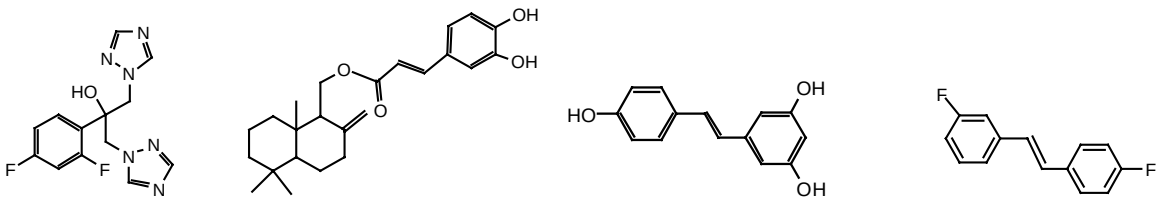

Fig. 1. Chemical structures of fluconazole and tested compounds.

USA) with (−) ESI. The analytical column C18 (HP ODS Hypersil 3 μm, 125 mm × 3 mm) was used. The final mobile phase was methanol–water–ammonium hydroxide (80:20:0.001, v/v/v). The injection volume was 10 μl; run time was 3.5 min; flow rate was 0.3 ml min^{-1}. Between two consecutive pipetting, the Multiprobe needles were washed with water and ethanol. Auto-sampler carry-over was determined by injecting the highest calibration standard then a blank sample. No carry-over was observed, as indicated by the lack of fluconazole peak in the blank sample. The background noise in the blank sample was also not elevated. The column performance throughout the study was monitored by measuring the retention time (t_R), peak symmetry factor (B/A) at the 10% peak height where B and A were the distances after and before the peak center respectively, and the plate count (N) as 5.54 $(t_R/W_{0.5})^2$ where $W_{0.5}$ was the peak width at the half of the peak height.

The sensitivity of the multiple reactions monitoring (MRM) was optimized by testing with an infusion of 100 ng ml^{-1} fluconazole solution. The ionspray needle was maintained at −4.5 kV. The turbo gas temperature was 350 °C and the auxiliary gas flow was 20 l min^{-1}. Ion source gas, curtain gas, and collision gas flows were at instrument settings of 25, 10, and 15, respectively. The declustering potential and focusing potential were at 46 and 200 V, respectively. The mass spectrometer was operated under MRM mode with collision energy of 24 eV. The transitions (precursor to product) monitored were m/z 305 → 191 for fluconazole. The dwell time was 150 ms for each transition. Both quadrupoles were maintained at unit resolution. Chromatograms were integrated using the ANALYST version 1.3.1 software. A weighted 1/concentration^2 linear regression was used to generate calibration curves from standards and calculate the concentrations of QC samples.

2.5. Sample preparation

The test *C. albicans* was prepared from Sabouraud glucose agar subcultures incubated at 35 °C for 24 h and the resulting suspension was adjusted to a density equivalent to 10^8 cfu ml^{-1} by comparing with the isolates density standard (Drug and Biological products Identification Unit, China). Stock solutions of tested compounds were prepared in dimethylsulfoxide (DMSO). To achieve the desired concentrations of the tested compounds, appropriate amounts of the stock solutions were added to 50 ml polypropylene tubes and diluted with medium. The final concentrations were 2, 8 and 32 μg ml^{-1} for albicanyl caffeate and 8, 32 and 128 for resveratrol and 3,4′-difluorostilbene, which were equivalent to the concentration levels of 0.5 MIC, 2 MIC and 8 MIC we determined in the former experiment. The final concentration of DMSO in the assay did not exceed 2% which was proved no effects at this concentration against *C. albicans*. To each tube 1 ml of the isolate suspension and 1 ml of fluconazole stock solution (12.5 μg ml^{-1}) were added, respectively. The final isolates size and fluconazole concentration were 2.1×10^7 cfu ml^{-1} and 0.25 μg ml^{-1}, respectively. The mixture was incubated at 35 °C in ambient air with agitation for 24 h. *C. albicans* thallus was obtained by centrifuging the culture for 20 min at 3000 rpm after 24 h of incubation. Added 0.2 ml phosphate buffer (pH = 7.4, monopotassium phosphate 1.36 g, added 0.1 mol l^{-1} sodium hydroxide solution 79 ml and water to 200 ml) and 0.4 ml 15% alcoholic sodium hydroxide (15 g NaOH and 10 ml sterile distilled water, brought to 100 ml with ethanol) to 5 mg of frozen dried thallus, vortex-mixed the mixture briefly and

saponified over 60 min at 80 °C. After centrifugation of the mixture at 3000 rpm for 5 min at room temperature, the supernatant were transferred into polypropylene eppendorf cups and incorporated with 0.4 ml residual washing solution of ethanol. Added 0.2 ml hydrochloric ethanol solution (ethanol–hydrochloric solution 1:1 v/v) into the collection, and centrifuged again for 5 min at 10 000 rpm. The supernatant was transferred into another polypropylene eppendorf cups, and evaporated under a gentle stream of nitrogen, then frozen to dryness and reconstituted with 0.1 ml ethanol. All tests were carried out in triplicate.

2.6. Validation of the HPLC–MS/MS method

Validation was performed according to the current recommendations for analytical method validation, that require a standard curve with five to eight points with reproducible linear or non-linear responses and statistical fits [20]. This method was validated by three consecutive analytical curves on three separate days. Each calibration curve contained a single set of eight calibration standards and three QCs. Each curve also contained other test samples such as processing and storage stability samples. Calibration standards and QCs were randomized through the curve. An extracted blank sample was always placed after the upper limit of quantization standard (ULOQ) to determine carry-over of the HPLC–MS/MS system.

The method specificity was evaluated by screening six lots of blank sample. These lots were spiked with fluconazole at 0.00, 5.0, and 60.0 ng ml^{-1}. The spiked samples were processed and analyzed to confirm lack of interference and absence of lot-to-lot variation. The matrix suppression was determined by comparing the peak areas of analyte spiked post processing with those dissolved in ethanol at corresponding concentrations.

Analyte stability was tested by subjecting QCs through multiple freeze/thaw cycles, and on the bench at room temperature. Post-processed analyte stability was also determined.

The recovery of sample processing was determined by comparing the quantity increment of fluconazole measured in the sample spiked with fluconazole and the quantity spiked. The volumes of the spiking solutions were always kept below 1% of sample solution volumes.

3. Results and discussion

3.1. HPLC–MS/MS

The selection of C18 column and organic-aqueous mobile phase for quantitative analysis of fluconazole was based on the reported method [21,22]. Ammonium hydroxide can facilitate the ionization of analyte by changing pH and enhanced the signal intensity. The C18 column demonstrated excellent stability indicated by very stable t_R and unchanged peak shape for fluconazole.

Compounds ketoconazole and phenacetin were evaluated for potential use as the internal standard (IS), it was soon discovered that neither of these two compounds tracked fluconazole well in the sample processing or chromatographic elution. Because of a too large variation of extraction recovery of these two compounds, the use of an internal standard was omitted in the analysis of fluconazole. The described method has been demonstrated to be accurate and reliable without the use of an internal standard.

The described process technique, combined with measurement on a C18 analytical column, preceded by a guard column, provides a good resolution of fluconazole. Typical chromatograms of samples were shown in Figs. 2 and 3. And the MS spectra of fluconazole were shown in Fig. 4 determined in negative

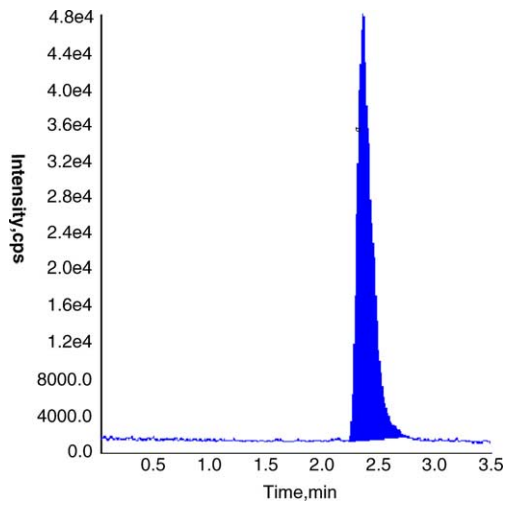

Fig. 2. Selected ion current traces of fluconazole *C. albicans* thallus spiked with 100 ng ml^{-1} fluconazole.

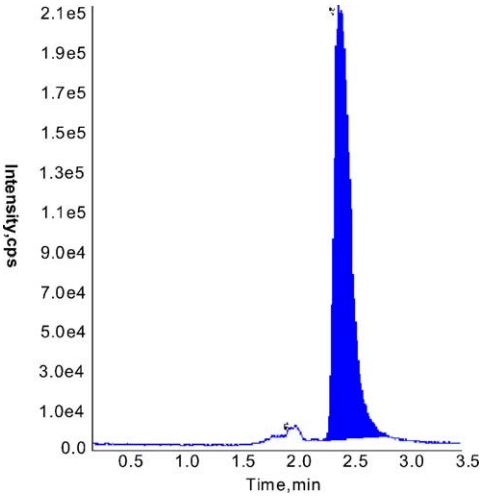

Fig. 3. Selected ion current traces of fluconazole in samples.

mode. The possible fragmentation way was shown in Fig. 5.

3.2. Validation results

Validation characteristics of the assay in terms of linearity, accuracy and precision at different concentrations of fluconazole have been tabulated.

Six lots of blank control sample were tested for matrix interference. Matrix effects from co-eluting endogenous components in *C. albicans* have been well studied in order to compromise the reproducibility and accuracy of the analysis. The region of the analyte peaks was free from interference. When the samples were spiked with fluconazole at low QC concentration, which is 5.0 ng ml^{-1}, the R.S.D. and R.E. were 3.28 and −2.4%, respectively. For the samples spiked with fluconazole at higher QC concentration

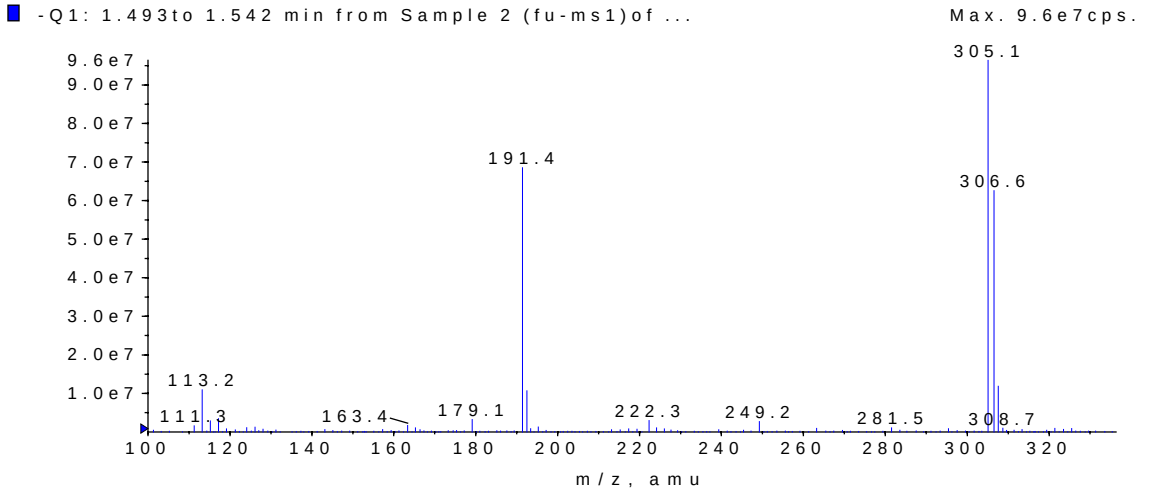

Fig. 4. MS spectra showing the parent ions and the corresponding *m*/*z* of fluconazole.

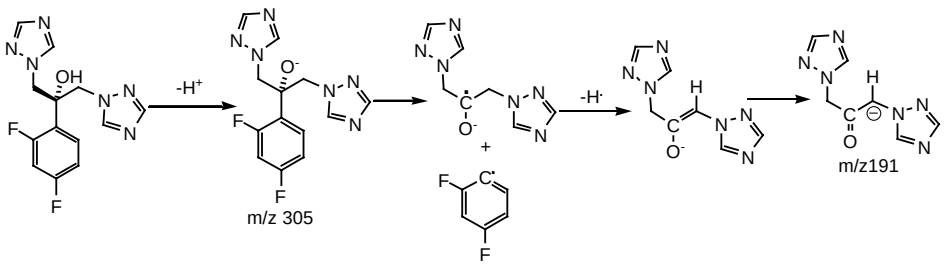

Fig. 5. The possible pattern of fragmentation of fluconazole in the negative mode.

Table 1
Precision and accuracy of calibration standards ($n = 3$)

C (ng ml^{-1})	1.00	5.00	10.00	20.00	40.00	60.00	80.00	100.00	r
Batch 1	0.965	4.97	9.12	19.8	36.2	59.3	82.5	98.1	0.9988
Batch 2	1.05	5.25	9.87	20.3	40.0	56.1	78.7	100	0.9994
Batch 3	1.02	4.86	10.3	19.1	41.2	55.9	83.5	93.5	0.9968
Mean	1.01	5.02	9.80	19.8	39.4	57.8	81.2	97.9	0.9992
R.S.D. (%)	4.1	4.0	6.1	3.06	6.6	3.3	3.0	3.4	N/A
R.E. (%)	+0.95	+0.4	−1.78	−1	−1.6	−3.6	+1.5	−2.1	N/A

R.S.D.: relative standard deviation calculated as (standard deviation of measured value/measured mean value × 100)%. R.E.: relative error calculated as (measured value/nominal value × 100 − 100)%; N/A: not applicable.

that is 60.0 ng ml^{-1}, the R.S.D. and R.E. were 2.9 and −3.3%, respectively. These tight R.S.D. and R.E. values indicate no significant lot-to-lot variation in matrix effects. The absolute magnitude of matrix suppression was determined by comparing peak areas of post-processed spiked samples with those from unprocessed analyte at the same concentration. For fluconazole, the matrix suppression is <7% (<7% of the analyte signal was suppressed) for both 5.0 and 60.0 ng ml^{-1} samples. This suppression is insignificant and could be well within the measurement errors.

The recovery of sample processing was determined by comparing the quantity increment of fluconazole measured in the sample spiked with fluconazole and the quantity spiked. The fluconazole was spiked with 1.0, 40.0 and 80.0 ng mg^{-1}. The recoveries of fluconazole were 72% at 1.0 ng mg^{-1}, 78% at 40.0 ng mg^{-1} and 81% at 80.0 ng mg^{-1}. Acceptable and consistent recoveries were obtained for fluconazole.

Calibration curve parameters and data were listed in Table 1. The correlation coefficients of the three validation curves were all >0.996. The standards showed a linear range of 1.0–100 ng ml^{-1}, using weighted (1/concentration^2) least-square linear regression.

The accuracy and precision of the method were determined on three different occasions by replicate analyses of three known concentrations over the calibration curve. The precision and accuracy data for QC samples were summarized in Table 2. The data showed that this method was consistent and reliable with low R.S.D.s and R.E.s values. For the LLOQ (lower limit of quantization) QCs, the R.S.D. ($n = 6$)

Table 2
Precision and accuracy of QC samples

	Intra-day, $n = 6$ (ng ml^{-1})			Inter-day, $n = 10$ (ng ml^{-1})		
	1.0	60.0	100.0	1.0	60.0	100.0
Mean	0.965	60.8	103	0.957	57.7	104
R.S.D. (%)	5.5	4.9	3.7	6.2	2.5	3.3
R.E. (%)	−3.5	+1.3	+3	−4.3	−3.8	+4

Table 3
Stability of the samples ($n = 6$)

	Five freeze/thaw cycle concentration		24 h bench-top concentration		−20 °C for 90 days concentration		72 h processed concentration	
	1.0	60.0	1.0	60.0	1.0	60.0	5.0	60.0
Mean	0.962	61.5	0.958	58.7	1.05	57.6	4.83	61.9
R.S.D. (%)	6.1	4.8	7.3	5.1	9.2	4.9	3.5	2.9
R.E. (%)	−3.8	+2.5	−4.2	−2.17	+5.0	−4.0	−3.4	+3.2

Concentration is in ng ml^{-1}; 72 h processed, post-processed blank sample spiked with fluconazole.

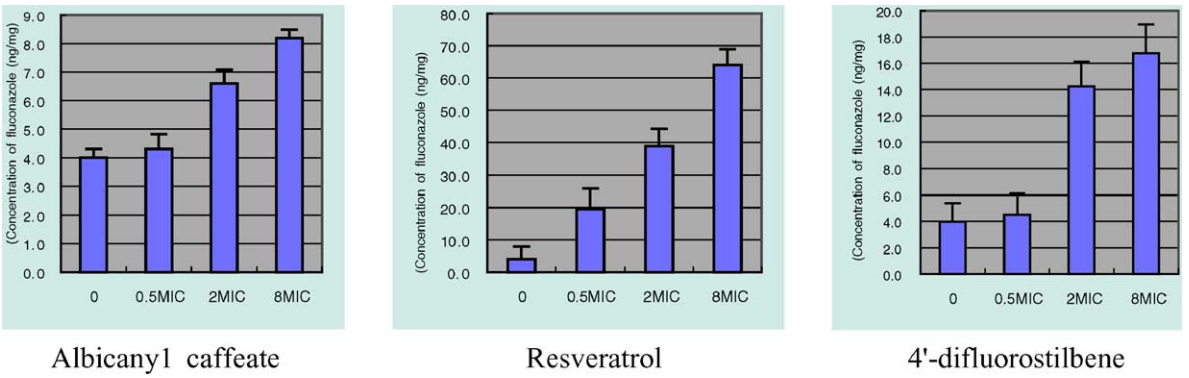

Fig. 6. The impact of phenolic compounds on the concentration of fluconazole in *C. albicans*. MIC, minimum inhibitory concentration of the tested compounds against *C. albicans*.

of the measured concentration was 5.5%. The R.E. of the mean of the measured concentrations was −3.5%.

The stability tests were designed to cover the anticipated conditions that the test samples may experience. Stability of sample processing (freeze/thaw, bench-top and storage) and analyte chromatography were tested and established. The results were summarized in Table 3. Five freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to analysis appeared to have little effect on the quantization. QC samples stored in a freezer at −20 °C remained stable for at least 90 days. Extracted calibration standards and QC samples were allowed to stand at 2–8 °C for 72 h prior to injection. No effect on quantization of the calibration standards or QC samples was observed.

3.3. The concentrations of fluconazole in C. albicans

The concentrations of fluconazole in *C. albicans* were determined after incubation *C. albicans* with fluconazole alone at the concentration of 0.25 μg ml^{-1} and in combination with different tested phenolic compounds at three concentration levels of 0.5 MIC, 2 MIC and 8 MIC. The results were illustrated in Fig. 6.

The results indicated that the quantity of fluconazole accumulated in *C. albicans* increased with the increment of phenolic compounds concentration. This may be one of the mechanisms accounting for the synergetic effects we observed in the antifungal activity evaluation test. In addition, the test results may also be attributed to the result we got in another experiment that the phenolic compounds can decrease the synthesis of ergosterol, the main component of *C. albicans* membrane. Other mechanism is still under researching.

4. Conclusion

A sensitive, reliable and highly reproducible HPLC–MS/MS method for the measurement of fluconazole in *C. albicans* has been developed and validated. A C18 column and an organic- aqueous mobile phase were used to improve the sensitivity. The LLOQ was 1.0 ng ml^{-1} for fluconazole. The concentration of fluconazole in *C. albicans* was determined successfully using the method developed, and the results showed that the tested compounds can increase the quantity of fluconazole accessed into *C. albicans* cells when they were in combination.

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