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Diversity-oriented synthesis of pyrazoles derivatives from flavones and isoflavones leads to the discovery of promising reversal agents of fluconazole resistance in *Candida albicans*

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ABSTRACT

Diversity-oriented synthesis of derivatives of natural products is an important approach for the discovery of novel drugs. In this paper, a series of novel 3,4-diaryl-1*H*-pyrazoles and 3,5-diaryl-1*H*-pyrazoles derivatives were synthesized through the one-pot reaction of flavones and isoflavones with the hydrazine hydrate and substituted hydrazine hydrate. Some of these novel compounds exhibited antifungal effects against *Candida albicans* SC5314, and displayed more potent inhibitory activities against the efflux-pump-deficient strain DSY654. In addition, compounds **25**, **28** and **32a** displayed outstanding reversal activity of azole resistance against clinical azole-resistant *Candida albicans* in combination with fluconazole (FLC), with FICI values ranging from 0.012 to 0.141. The preliminary structure-activity relationship (SAR) of these compounds was also discussed. In conclusion, this study provides several novel agents that displayed potent antifungal activities alone or together with fluconazole, which makes progress for development of antifungal drugs.

Candida albicans, isolated from human oral, gastrointestinal, vaginal, cutaneous and mucosal surfaces, is one of the most common human opportunistic fungal pathogen, causing high mortality in nosocomial bloodstream infections. Currently, azole antifungal agents are widely used as first-line antifungal therapy by inhibiting fungal lanosterol 14α-demethylase, and fluconazole (FLC) is the most commonly used azole drug to treat *C. albicans* in prophylaxis and therapy. Although contemporary antifungal medications are still effective, the usefulness of these drugs is compromised by the frequent emergence of high-grade resistance.[1–6] This acquired drug resistance has been rapidly increasing worldwide and posed a grave threat to human health. Therefore, the development of new and more potent antifungal drugs becomes even more urgent.

Natural products are considered as great sources for the development of novel pharmaceuticals and their scaffolds are also recognized as "privileged structures" for further modifications. Recently, it has been reported that flavones and isoflavones, such as flavone (**1**) and formononetin (**2**) (Fig. 1) exhibited moderate antifungal activity, which could be used as the precursor for the discovery of novel antifungal agents.[7–10] In addition, Furlan and Gu have revealed a strategy to generate chemically engineered extracts through chemical diversification of natural product mixtures.[11,12] Specifically, the extract of flavones was reacted with hydrazine monohydrate, and the following bioactivity-guided fractionation of the semisynthetic mixture led to the isolation of 3,5-diaryl-1*H*-pyrazole **3**, which displayed excellent antifungal activity (Fig. 2).[11] Moreover, it was reported that the isoflavones could also react with hydrazine to generate 3,4-diaryl-1*H*-pyrazoles in one step with good-to-excellent yields.[13,14] Furthermore, pyrazole was considered as an important antifungal pharmacophore, and many pyrazole derivatives have been reported to exhibit effective antifungal activity.[15,16,3] All these findings motivated us to prepare a series of 3,4-diaryl- and 3,5-diaryl-pyrazole derivatives by the reaction of flavones and isoflavones with hydrazine hydrate and substituted hydrazine hydrate, and these novel pyrazole derivatives were then evaluated for their antifungal activity.

Firstly, each of the natural isolated flavones **1**, **10**, **11**, **13**, **14** and isoflavones **2**, **4**, **5**, **12** was reacted with hydrazine monohydrate, respectively, under reflux in ethanol. Interestingly, only four pyrazoles (compounds **6**–**9**) were obtained (Scheme 1), and the starting material bearing 5-hydroxyl or 8-hydroxyl group

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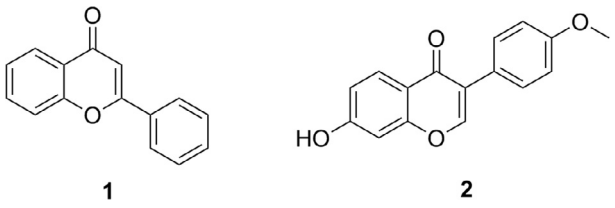


Fig. 1. The chemical structures of flavone (**1**) and formononetin (**2**).

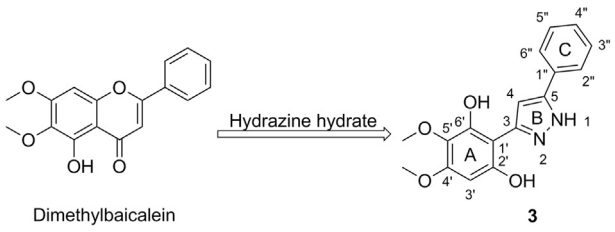
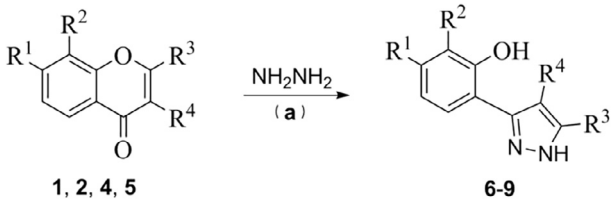


Fig. 2. Synthesis of diarylpyrazole **3**.



1,6: $R^1=R^2=R^4=H$, $R^3=Ph$
2,7: $R^2=R^3=H$, $R^1=OH$, $R^4=4\text{-}OCH_3\text{-}Ph$
4,8: $R^2=R^3=H$, $R^1=OH$, $R^4=4\text{-}OH\text{-}Ph$
5,9: $R^3=H$, $R^1=OH$, $R^2=Glc$, $R^4=Ph$

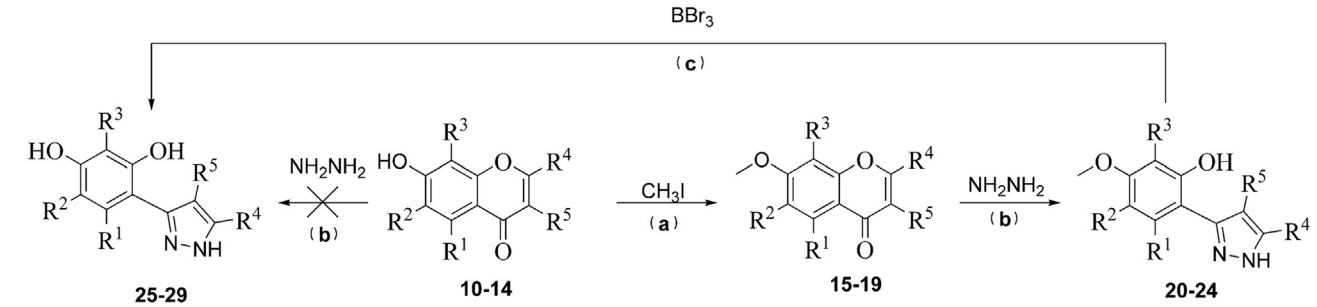
Scheme 1. Synthesis of pyrazoles derivatives **6**–**9**. Reagents and conditions: (a) EtOH, 90 °C, reflux for 8–12 h.

(compounds **10**–**14**) could not react with hydrazine under this condition, which might because of the intramolecular hydrogen bond impacting the ring-open process. Compounds **10**–**14** were then methylated by CH_3I, and the resulting intermediates **15**–**19** reacted with hydrazine hydrate to provide the pyrazoles **20**–**24** as we expected. The following demethylation by boron tribromide resulted in the formation of pyrazoles derivatives **25**–**29** in satisfied yield (Scheme 2).[17] The proposed reaction mechanism of flavones and isoflavones with hydrazine is illustrated in Fig. 3. Nucleophilic attack of hydrazine at C-2 of flavone or isoflavone followed by ring-opening afforded the intermediate VI. The further nucleophilic attack of the second nitrogen atom at the carbonyl carbon of VI and subsequent dehydration could lead to the formation of the pyrazole ring. The obtained pyrazoles may exist as mixtures of OH—N and NH—O tautomers.

Secondly, to prepare more structurally diversed pyrazoles derivatives and define their antifungal structure-activity relationship (SAR), the flavones and isoflavones **1**, **2**, **4** and **5** as well as the methylated intermediates **15**–**17** and **19** were then reacted with substituted hydrazines containing electron-donating groups, such as ethyl, phenyl, *p*-methoxyphenyl, 2-hydroxy ethyl etc, and twenty-five more novel pyrazoles derivatives were obtained (Scheme 3). However, when the substituted hydrazines bearing electron-withdrawing groups, the reaction did not work, and these observations indicated that the nucleophilic potency of hydrazine derivatives was essential for this reaction.

In addition, as shown in Scheme 4, we also studied the reaction of flavones with ethylenediamine. The compounds **1**, **14**, **15**, **18**, and **19** were treated with ethylenediamine under reflux in ethanol, and the novel 5,7-diaryl-2,3-dihydro-1,4-diazepine derivatives **47**–**51** were then obtained in satisfied yield.[18,19]

All the synthetic pyrazoles and diazepines derivatives were evaluated for antifungal activities against *C. albicans* SC5314 (the wild-type strain) and DSY654 (the Cdr1, Cdr2 efflux pumps deficient strain), and fluconazole served as the positive antifungal agent.[20] The minimum inhibitory concentrations (MICs) of active compounds are summarized in Table 1. Compounds **6**, **20** and **29** exhibited antifungal activity against the SC5314 with MIC_{80} values ranging from 8 to 16 μg/mL. Surprisingly, compounds **20**, **21**, **40b** and **43a** displayed very strong inhibitory activity against DSY654 with MIC_{80} values ranging from 1 to 4 μg/mL, and compound **20** was found to be the most potent antifungal agent (MIC_{80} = 1 μg/mL). However, the growth inhibitory activity of all the synthesized compounds against *C. albicans* SC5314 and DSY654 was less potent than the reference drug FLC, with MIC_{80} values of 2 and 0.25 μg/mL, respectively. Among all the derivatives, compound **6** displayed moderate antifungal activity against SC5314 and DSY654, with MIC_{80} values of 16 and 8 μg/mL, respectively. Introduction of two methoxyl groups at C-4′ and C-6′ on arene *A* led to the most potent compound **20** (MIC_{80} = 8 and 1 μg/mL for SC5314 and DSY654,



10: $R^1=OH$, $R^2=R^3=R^5=H$, $R^4=Ph$
11: $R^1=R^2=OH$, $R^3=R^5=H$, $R^4=Ph$
12: $R^1=OH$, $R^2=R^3=R^4=H$, $R^5=4\text{-}OH\text{-}Ph$
13: $R^1=OH$, $R^2=R^3=R^5=H$, $R^4=4\text{-}OH\text{-}Ph$
14: $R^3=OH$, $R^1=R^2=R^5=H$, $R^4=Ph$

15, 20: $R^1=OCH_3$, $R^2=R^3=R^5=H$, $R^4=Ph$
16, 21: $R^1=R^2=OCH_3$, $R^3=R^5=H$, $R^4=Ph$
17, 22: $R^1=OCH_3$, $R^2=R^3=R^4=H$, $R^5=4\text{-}OCH_3\text{-}Ph$
18, 23: $R^1=OCH_3$, $R^2=R^3=R^5=H$, $R^4=4\text{-}OCH_3\text{-}Ph$
19, 24: $R^3=OCH_3$, $R^1=R^2=R^5=H$, $R^4=Ph$

25: $R^1=OH$, $R^2=R^3=R^5=H$, $R^4=Ph$
26: $R^1=OH$, $R^2=R^3=R^4=H$, $R^5=4\text{-}OH\text{-}Ph$
27: $R^1=OCH_3$, $R^2=R^3=R^4=H$, $R^5=4\text{-}OH\text{-}Ph$
28: $R^1=OH$, $R^2=R^3=R^5=H$, $R^4=4\text{-}OH\text{-}Ph$
29: $R^3=OH$, $R^1=R^2=R^5=H$, $R^4=Ph$

Scheme 2. Synthesis of pyrazoles derivatives **20**–**29**. Reagents and conditions: (a) acetone, 60 °C, reflux for overnight; (b) EtOH, 90 °C, reflux for 8–12 h; (c) DCM, -78 °C, reflux for 1 h, then reflux for overnight at rt.

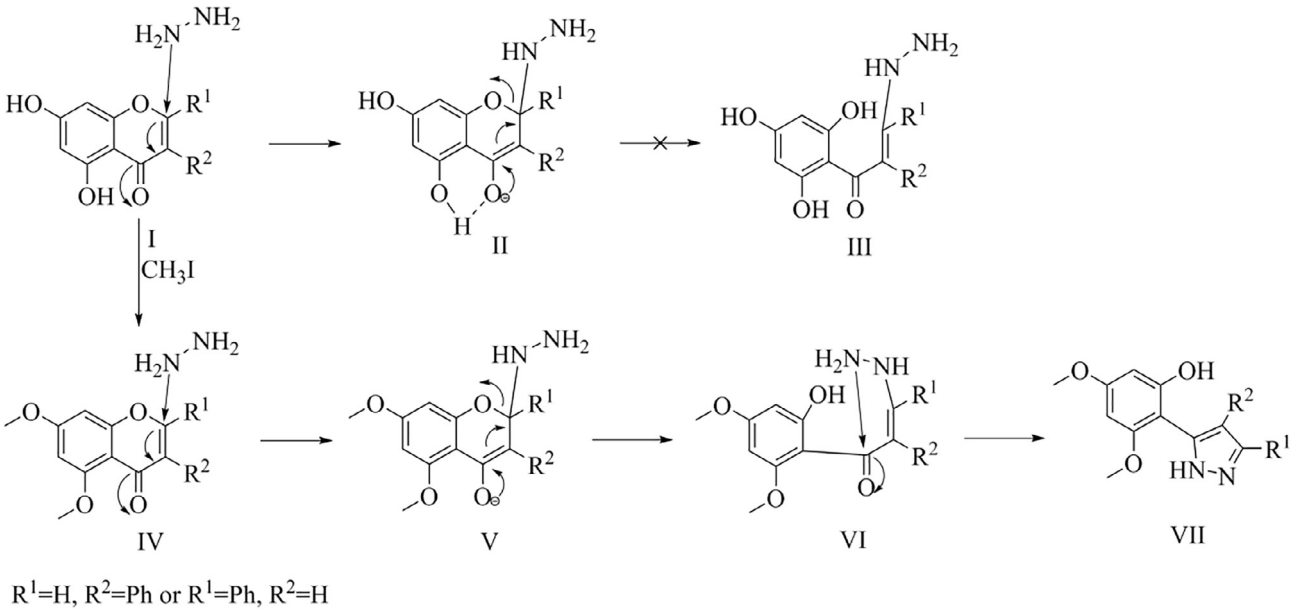
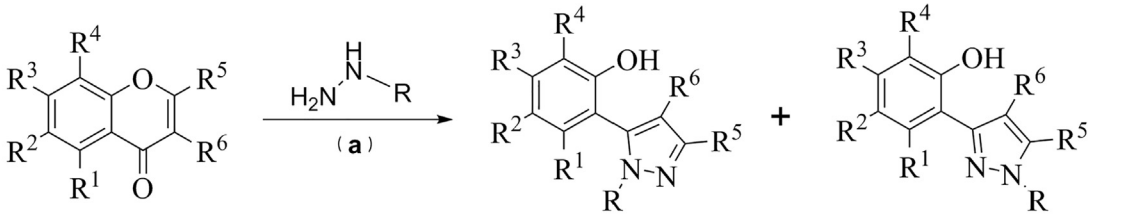


Fig. 3. Proposal of mechanism for the reaction of methylated 5, 7-dihydroxyl flavones and 5, 7-dihydroxyl isoflavones with hydrazine.



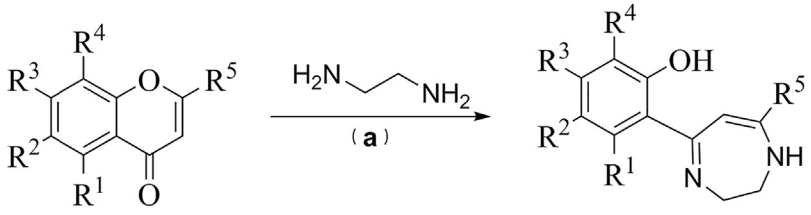
1: R^1=R^2=R^3=R^4=R^6=H, R^5=Ph
2: R^1=R^2=R^4=R^5=H, R^3=OH, R^6=4-OCH_3-Ph
4: R^1=R^2=R^4=R^5=H, R^3=OH, R^6=4-OH-Ph
5: R^1=R^2=R^5=H, R^3=OH, R^4=Glc, R^6=Ph
15: R^1=R^3=OCH_3, R^2=R^4=R^6=H, R^5=Ph
16: R^1=R^2=R^3=OCH_3, R^4=R^6=H, R^5=Ph
17: R^1=R^3=OCH_3, R^2=R^4=R^5=H, R^6=4-OCH_3-Ph
19: R^3=R^4=OCH_3, R^1=R^2=R^6=H, R^5=Ph

30a: R^1=R^2=R^3=R^4=R^6=H, R^5=Ph, R=2-OH-Et
31a: R^1=R^2=R^3=R^4=R^6=H, R^5=Ph, R=Ph
32a: R^1=R^2=R^4=R^5=H, R^3=OH, R^6=4-OCH_3-Ph, R=Ph
33a: R^1=R^2=R^4=R^5=H, R^3=OH.R^6=4-OCH_3-Ph, R=4-OCH_3Ph
34a,34b: R^1=R^2=R^4=R^5=H, R^3=OH, R^6=4-OCH_3-Ph, R=Et
35a,35b: R^1=R^2=R^4=R^5=H, R^3=OH, R^6=4-OCH_3-Ph, R=2-OH-Et
36a: R^1=R^2=R^4=R^5=H, R^3=OH, R^6=4-OH-Ph, R=Ph
37a,37b: R^1=R^2=R^4=R^5=H, R^3=OH, R^6=4-OH-Ph, R=2-OH-Et
38a,38b: R^1=R^2=R^5=H, R^3=OH, R^4=Glc, R^6=Ph,R=Et
39a,39b: R^1=R^2=R^5=H, R^3=OH, R^4=Glc, R^6=Ph, R=2-OH-Et
40b: R^1=R^3=OCH_3, R^2=R^4=R^6=H, R^5=Ph, R=2-OH-Et
41a: R^1=R^3=OCH_3, R^2=R^4=R^6=H, R^5=Ph, R=Ph
42a,42b: R^1=R^2=R^3=OCH_3, R^4=R^6=H, R^5=Ph, R=Ph
43a,43b: R^1=R^2=R^3=OCH_3, R^4=R^6=H, R^5=Ph, R=4-OCH_3-Ph
44a,44b: R^1=R^3=OCH_3, R^2=R^4=R^5=H, R^6=4-OCH_3-Ph, R=Ph
45a: R^3=R^4=OCH_3, R^1=R^2=R^6=H, R^5=Ph, R=2-OH-Et
46a: R^3=R^4=OCH_3, R^1=R^2=R^6=H, R^5=Ph, R=4-OCH_3-Ph

Scheme 3. Synthesis of pyrazoles derivatives **30a–39a**, **41a–46a**, **34b**, **35b**, **37b–40b**, **42b–44b**. Reagents and conditions: (a) EtOH, 90 °C, reflux for 48–60 h.

respectively). This indicated that the methoxyl groups on arene *A* were very important for the antifungal activity. Interestingly, when the substitutive groups were introduced to the ring *B*, the inhibitory activity against SC5314 was diminished and even lost, while the antifungal potency against DSY654 was maintained or improved (**30a**, **31a**, **40b**, **43a** and **46a**). In addition, the replacement of pyrazole ring by the dihydrodiazepine moiety resulted in an obviously decreased potency (**20** vs. **48** and **29** vs. **51**). Furthermore, the derivatives bearing the 3,5-diaryl-pyrazole skeleton displayed more potent antifungal activity than the 3,4-diaryl-pyrazole compounds.

The *in vitro* synergistic antifungal activities of the most potent compound **20** were then tested using the broth microdilution checkboard assay, and the MIC_{80} of **20** and FLC alone or in combination against three clinically isolated FLC-resistant *C. albicans* strains (28A, 28I, and 28J) were summarized in Table 2. The FICI



1, 8, 9,14 ,19 **47-51**

1: $R^1=R^2=R^3=R^4=H$, $R^5=Ph$
14: $R^3=R^4=OH$, $R^1=R^2=H$, $R^5=Ph$
15: $R^1=R^3=OCH_3$, $R^2=R^4=H$, $R^5=Ph$
18: $R^1=R^3=OCH_3$, $R^2=R^4=H$, $R^5=4\text{-}OCH_3\text{-}Ph$
19: $R^3=R^4=OCH_3$, $R^1=R^2=H$, $R^5=Ph$

47: $R^1=R^2=R^3=R^4=H$, $R^5=Ph$
48: $R^1=R^3=OCH_3$, $R^2=R^4=H$, $R^5=Ph$
49: $R^1=R^3=OCH_3$, $R^2=R^4=H$, $R^5=4\text{-}OCH_3\text{-}Ph$
50: $R^3=R^4=OCH_3$, $R^1=R^2=H$, $R^5=Ph$
51: $R^3=R^4=OH$, $R^1=R^2=H$, $R^5=Ph$

Scheme 4. Synthesis of diarylbenzodiazepines **47**–**51**. Reagents and conditions: (a) ethylenediamine, EtOH, 90 °C, reflux for 2–12 h.

Table 1
In vitro antifungal effects of thirteen active compounds against *Candida albicans* SC5314 and DSY654.[a]

Compounds	SC5314	DSY654	Compounds	SC5314	DSY654
6	16	8	**40b**	>128	4
9	>128	16	**43a**	>128	4
20	8	1	**46a**	>128	8
21	64	2	**48**	128	8
29	8	8	**49**	>128	16
30a	>128	8	**51**	>128	32
31a	128	8	FLC	2	0.25

[a] Mean values based on three independent experiments; all data are minimum inhibitory concentrations (MIC_{80}), given as μg/mL.

Table 2
The susceptibility test of compound **20** alone and in combination with FLC against three FLC-resistant *Candida albicans* strains.

Strains	MIC80 (μg/mL)					
	Alone		In combination		FICI	Interpretation[a]
	20	FLC	**20**	FLC		
28A	32	>128	8	0.25	0.252	SYN
28I	32	>128	8	0.25	0.252	SYN
28J	32	>128	8	0.5	0.254	SYN

MIC, minimum inhibitory concentration; FICI, fraction inhibited concentration index.
[a] SYN, synergism. SYN was defined as a FICI of ≤0.5, antagonism was defined as a FICI of >4.0, and Indifference was defined as a FICI of >0.5–4.

values of **20** for strains 28A, 28I, and 28J are 0.252, 0.252 and 0.254, respectively, which indicated that pyrazole derivative **20** displayed potent synergistic activity with FLC. In addition, the data of the synergistic effect of **20** against FLC-resistant *C. albicans* strain 28A was displayed as a heat map using HemI 1.0.3.7 (Fig. 4). These encouraging results motivated us to evaluate all the synthetic derivatives for their synergistic antifungal activity with FLC to discover more potent reversal agents of drug resistance. Compounds **6**, **20**, **21**, **25**, **28**, **32a**, **34b**, **40b** and **48** displayed excellent synergistic activity, with FICI values ranging from 0.012 to 0.266, and compound **28** was the most potent agent with FICI value of 0.012 (Table 3). Compound **6**, bearing the 3,5-diaryl-1*H*-pyrazole skeleton, exhibited excellent synergistic antifungal activity, with a FICI value of 0.266. Introduction of methoxyl groups to the arene *A* led to compounds **20**, **21** and **40b** with FICI values ranging from 0.252 to 0.266, which are comparable to that of compound **6**. This suggests that the methoxyl groups on arene *A* do not provide any obvious effect on the activity. Compound **25**, with three hydroxyl groups at C-2′, C-4′ and C-6′ of arene *A*, exhibited very potent synergistic antifungal activity with FICI value of 0.075. Surprisingly,

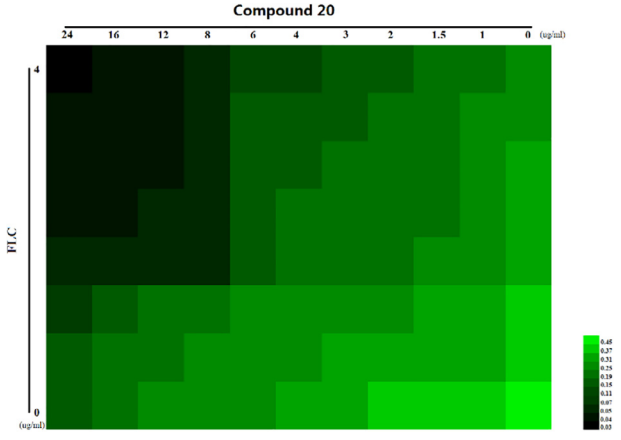


Fig. 4. Compound **20** enhances the efficacy of fluconazole against FLC-resistant *Candida albicans* strain 28A. A dose response matrix was performed in RPIM 1640 medium with gradients of FLC and **20**. Date was analyzed after 48 h at 35 °C and displayed as a heat map using HemI 1.0.3.7, see color bar.

Table 3
In vitro susceptibilities of pyrazoles derivatives and FLC acting alone and in combination against clinical FLC-resistant *Candida albicans* 28A.

Compounds	MIC_{80} (μg/mL)					
	Alone		In combination		FICI	Interpretation[a]
	Pyrazoles	FLC	Pyrazoles	FLC		
6	64	>128	16	2	0.266	SYN
9	>128	>128	>128	>128	2	IND
20	32	>128	8	0.25	0.252	SYN
21	64	>128	16	2	0.266	SYN
25	>128	>128	8	2	0.078	SYN
28	>128	>128	1	0.5	0.012	SYN
30a	>128	>128	>128	>128	2	IND
31a	128	>128	128	2	1.016	IND
32a	>128	>128	16	2	0.141	SYN
34b	>128	>128	32	2	0.266	SYN
40b	>128	>128	32	2	0.266	SYN
43a	>128	>128	>128	>128	2	IND
46a	>128	>128	>128	>128	2	IND
48	128	>128	32	2	0.266	SYN
49	>128	>128	>128	>128	2	IND
51	>128	>128	>128	>128	2	IND

MIC, minimum inhibitory concentration; FICI, fraction inhibited concentration index.
[a] SYN, synergism; IND, indifference. SYN was defined as a FICI of ≤0.5, antagonism was defined as a FICI of >4.0, and IND was defined as a FICI of >0.5–4.

the further introduction of phenolic group at C-4″ of arene *C* led to the most potent compound **28**. These findings indicated that the hydroxyl groups on arene *A* and *C* is critical for the improved synergistic activity.

In summary, we have described an efficient synthesis of a series of pyrazole and dihydrodiazepine derivatives in one-pot by the reaction of flavones and isoflavones with substituted hydrazines and ethylenediamine. Some of these novel compounds exhibited antifungal effects against *Candida albicans* SC5314, and displayed more potent inhibitory activities against the efflux-pump-deficient strain DSY654. Moreover, compounds **25**, **28** and **32a** displayed outstanding reversal activity of drug resistance against clinical drug-resistant *Candida albicans* in combination with FLC, and **28** was proved to be the most potent agent with FICI value of 0.012. The preliminary SAR study indicated that the 3,5-diaryl-pyrazole skeleton was preferable for antifungal activity, and the methoxyl and hydroxyl groups on arene *A* also played critical role in promoting the potency. Further studies on structural optimization are in progress in our group.

Acknowledgments

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A. Supplementary data

Supplementary data (general methods for key intermediates, typical reaction procedure, and experimental data for the synthesized compounds) associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.03.066.

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