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## ***In vitro* Studies of Antimicrobial Activity of Synthetic Ovaliflavone**

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**Abstract:** In the present study 8- $\gamma,\gamma$ -dimethylallyl-5, 7-dimethoxyflavone [ovaliflavone, 6] has been synthesized from phloroacetophenone via chalcone precursor and by its treatment with DMSO/I<sub>2</sub> or diphenyl sulphide. The antibacterial and antifungal activities of ovaliflavone and its corresponding chalcone were found *in vitro* by the filter paper disc diffusion method and poisoned food technique, respectively. Compound 6 showed good antibacterial and antifungal activities to the some selected bacterial and fungal strain.

**Key words:** *Milletia ovalifolia*, ovaliflavone, antibacterial activity, antifungal activity, inhibition zone

### INTRODUCTION

Gupta *et al.*<sup>[1,2]</sup> reported the isolation of ovalichalcone (3'- $\gamma,\gamma$ -dimethylallyl-2'-hydroxy-4', 6-dimethoxychalcone, 5) from the seeds of *Milletia ovalifolia* and also reported its synthesis. Islam *et al.*<sup>[3]</sup> also synthesized this chalcone 5 by following the method of Gupta *et al.*<sup>[2]</sup> and converted this chalcone into its corresponding flavone 6 by using DDQ. Now, in the present study a synthesis of ovaliflavone (6) from its corresponding ovalichalcone by using DMSO/I<sub>2</sub> and diphenyl sulphide as oxidizing agent. The required ovalichalcone was prepared by the alkaline condensation of 3- $\gamma,\gamma$ -dimethylallyl-2-hydroxy-4, 6-dimethoxyacetophenone (2) and benzaldehyde (4). Both the ovaliflavone and its corresponding ovalichalcone were screened *in vitro* for their antibacterial and antifungal activity against four human pathogenic bacteria, viz., *Sarcina lutea* (G<sup>+</sup>), *Bacillus subtilis* (G<sup>+</sup>), *Shigella dysenteriae* (G<sup>-</sup>), *Pseudomonas aeruginosa* (G<sup>-</sup>) and five plant as well as molds fungi, viz., *Colletorichum gloeosporioides* Penz., *Candida albicans*, *Aspergillus nigar*, *Aspergillus flavus* and *Penicillium* sp.

### MATERIALS AND METHODS

**General:** Melting points were recorded on Gallenkamp apparatus and were uncorrected. IR spectra (KBr) were measured using a Shimadzu, DR-8001 spectrophotometer, <sup>1</sup>H NMR spectra (CDCl<sub>3</sub>) on a Bruker WH 200 MHZ instrument with TMS as an internal standard and UV spectra (MeOH) on a LKB 4053 spectrophotometer. Purity of the compounds was checked by TLC.

### **Prenylation of 2-hydroxy-4, 6-dimethoxyacetophenone, 1:**

2-hydroxy-4, 6-dimethoxyphloroacetophenone (1, 20 mmol, 3.92 g) was added to a well cooled solution of KOH (2.5 g) in absolute methanol (50 mL) and the whole solution was cooled to 0°C. Prenyl bromide (20 mmol, 2.98 g) was added to it and the reaction mixture was kept at room temperature for 24 h with constant stirring. Then the reaction mixture diluted with water, acidified with cold dil. HCl and was extracted with ethyl acetate. The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated and was subjected to column chromatography over silica gel and eluted successively with Petroleum spirit-benzene (20: 1), Petroleum spirit-benzene (15:3). It gave a mixture of two products labeled as fraction-I (2) and fraction-II (3).

### **Fraction-I:** 3- $\gamma,\gamma$ -dimethylallyl-2-hydroxy-4, 6-dimethoxyacetophenone, 2

It crystallized from ethanol as white needles (3.30 g), yield 48%, m.p. 64°C, R<sub>f</sub> 0.64 (benzene). It gave positive alcoholic ferric chloride test.

**Anal. found:** C, 68.58; H, 7.85% ; Calc. for C<sub>15</sub>H<sub>20</sub>O<sub>5</sub> ; C, 68.18; H, 7.57%

UV  $\lambda_{\max}^{\text{MeOH}}$ : 243 and 285 nm

IR  $\nu_{\max}^{\text{KBr}}$ : 3420, 1645, 1610, 1585, 1470, 1420, 1390, 1375, 1365, 1205, 1145, 1105, 1040, 980, 910, 835 cm<sup>-1</sup>.

**<sup>1</sup>H NMR (CDCl<sub>3</sub>):**  $\delta$  1.75 [s, 6H, >C(CH<sub>3</sub>)<sub>2</sub>], 2.25 (s, 3H, -COCH<sub>3</sub>), 3.55 (d, 2H, J= 7 Hz, -CH<sub>2</sub>-CH=), 3.75 (s, 3H, -OCH<sub>3</sub>), 3.78 (s, 3H, -OCH<sub>3</sub>), 5.25 (t, 1H, J= 7 Hz, -CH<sub>2</sub>-CH=), 6.01 (s, 1H, C<sub>5</sub>-H), 12.75 (s, 1H, C<sub>2</sub>-OH).

**Fraction II:** 5- $\gamma,\gamma$ -dimethylallyl-2-hydroxy-4, 6-dimethoxyacetophenone, 3

It was obtained as viscous oil (2.1 g) and was characterized as 5- $\gamma,\gamma$ -dimethylallyl-2-hydroxy-4,6-dimethoxyacetophenone (3) from its spectral data and elemental analysis.

**Anal. found:** C, 68.45; H, 7.73%; Calc. for  $C_{15}H_{20}O_4$ ; C, 68.18; H, 7.57%

UV  $\lambda_{max}^{MeOH}$ : 245 and 280 nm.

IR  $\nu_{max}^{KBr}$ : 3410, 1625, 1600, 1590, 1420, 1375, 1365, 1325, 1240, 1205, 1100, 1050, 985, 910, 835, 725  $cm^{-1}$ .

$^1H$  NMR ( $CDCl_3$ ):  $\delta$  1.73 [s, 6H,  $>C(CH_3)_2$ ], 2.28 (s, 3H,  $-COCH_3$ ), 3.50 (d, 2H,  $J=7$  Hz,  $-CH_2-CH=$ ), 3.95 (s, 3H,  $-OCH_3$ ), 3.99 (s, 3H,  $-OCH_3$ ), 5.22 (t, 1H,  $J=7$  Hz,  $-CH_2-CH=$ ), 5.41 (s, 1H,  $C_3-H$ ), 12.01 (s, 1H,  $C_2-OH$ ).

**Synthesis of 3'- $\gamma,\gamma$ -dimethylallyl-2'-hydroxy-4', 6'-dimethoxychalcone (Ovalichalcone, 5):** A mixture of 3- $\gamma,\gamma$ -dimethylallyl-2-hydroxy-4, 6-dimethoxyacetophenone (2, 10 mmol, 2.80 g) and benzaldehyde (4, 1.1 eqv., 11 mmol, 1.17 g) in ethanolic solution of KOH (5%, 15 mL) was kept at room temperature for about 75 h. The reaction mixture was then diluted with ice cold water, acidified with cold dil. HCl and extracted with ether. The ether layer was washed with water, dried over anhydrous  $Na_2SO_4$  and evaporated to dryness. It was purified by preparative TLC over silica gel (60G) using benzene as developing solvent and was crystallized from alcohol as yellow needles (850 mg), yield 48.29%, m.p. 122-123°C (Lit.<sup>1</sup> m. p. 122-123°C),  $R_f$  0.64 (benzene). It gave brown color with alcoholic ferric chloride solution.

**Anal. found:** C, 75.32; H, 6.56%; Calc. for  $C_{22}H_{24}O_4$ ; C, 75.00; H, 6.81%

UV  $\lambda_{max}^{MeOH}$ : 220 and 345 nm.

IR  $\nu_{max}^{KBr}$ : 3470, 1625, 1590, 1575, 1470, 1410, 1375, 1365, 1345, 1220, 180, 1145, 1100, 1050, 1005, 980, 735, 645  $cm^{-1}$ .  $^1HNMR$  ( $CDCl_3$ ):  $\delta$  1.7 [s, 6H,  $>C(CH_3)_2$ ], 3.50 (d, 2H,  $J=7$  Hz,  $-CH_2-CH=$ ), 3.95 (s, 3H,  $-OCH_3$ ), 3.98 (s, 3H,  $-OCH_3$ ), 5.21 (t, 1H,  $J=7$  Hz,  $-CH_2-CH=$ ), 6.75 (s, 1H,  $C_5'-H$ ), 6.98 (s, 5H,  $C_2'-H$ ,  $C_3'-H$ ,  $C_4'-H$ ,  $C_5'-H$  and  $C_6'-H$ ), 7.58 (d, 1H,  $J=16$  Hz,  $C_6-H$ ), 8.18 (d, 1H,  $J=16$  Hz,  $C_6-H$ ), 12.75 (s, 1H,  $C_2'-OH$ ).

**Synthesis of ovaliflavone (6, 8- $\gamma,\gamma$ -dimethylallyl-5,7-dimethoxyflavone) using DMSO/ $I_2$  method:** The chalcone

(5, 3 mmol, 1.15 g) was suspended in dimethyl sulphoxide (DMSO, 7.5 mL) and a crystal of iodine<sup>[4]</sup> was added to it. The mixture was refluxed for 15 min in a silicon oil bath and diluted with water. The solid obtained was filtered off, washed with 20% aq. sodium thiosulphate. The product (6) was purified by preparative TLC over silica gel (60G) using benzene-acetone (15:1) as developing solvent. It crystallized from benzene-Petroleum spirit as yellow needles (0.72 g), yield 62.5%, mp. 142°C (Lit.<sup>3</sup> m. p. 142°C),  $R_f$  0.52 (benzene-acetone; 10:1). It gave positive Mg/HCl and Labat tests and a blue fluorescence in UV light.

**Anal. found:** C, 75.73; H, 6.50%; Calc. for  $C_{22}H_{22}O_4$ ; C, 75.42; H, 6.28%.

UV  $\lambda_{max}^{EtOH}$ : 220, 270 and 360 nm.

IR  $\nu_{max}^{KBr}$ : 1645, 1590, 1560, 1420, 1375, 1365, 1330, 1290, 1145, 1110, 980, 930, 908, 872, 8825, 775, 720, 626  $cm^{-1}$ .

$^1H$  NMR ( $CDCl_3$ ):  $\delta$  1.70 [s, 6H,  $>C(CH_3)_2$ ], 3.52 (d, 2H,  $J=7$  Hz,  $-CH_2-CH=$ ), 3.97 (s, 3H,  $-OCH_3$ ), 3.99 (s, 3H,  $-OCH_3$ ), 5.32 (t, 1H,  $J=7$  Hz,  $-CH_2-CH=$ ), 5.61 (s, 1H,  $C_3-H$ ), 7.25 (s, 1H,  $C_6-H$ ), 7.52 (s, 5H,  $C_2'-H$ ,  $C_3'-H$ ,  $C_4'-H$ ,  $C_5'-H$  and  $C_6'-H$ ).

**Synthesis of ovaliflavone (6, 8- $\gamma,\gamma$ -dimethylallyl-5, 7-dimethoxyflavone) using Ph-S-S-Ph:** The chalcone (5, 3 mmol, 1.15 g) was pasted with diphenyl sulphide<sup>[5]</sup> (125 mg) in a mortar and the mixture was transferred to a 100 mL three necked round bottom flask equipped with nitrogen inlet and outlet tubes. The central neck was closed by a glass stopper. The flask was then dipped into a silicon oil bath and heated at 265°C under nitrogen atmosphere until the distilling of the thiols formed through the other outlet tube ceased (2.5 h). The reaction mixture was then cooled at room temperature and 20 mL chloroform was added. The organic layer was washed with water several times, dried over anhydrous sodium sulphate and the solvent was removed by distillation. The product crystallized from benzene -Petroleum spirit as yellow needles (0.78 g), yield 68%, m. p. 142°C,  $R_f$  0.52 (benzene-acetone; 10:1). It gave positive Mg/HCl and Labat tests and a blue fluorescence in UV light. Spectral data of this flavone (6) was also similar to that prepared by DMSO/ $I_2$  method.

**Antibacterial screening:** The antibacterial activities of synthesized compounds 5 and 6 were studied against four human pathogenic bacteria, viz., *Shigella dysenteriae* ( $G^-$ ), *Pseudomonas aeruginosa* ( $G^-$ ), *Sarcina lutea* ( $G^+$ ) and *Bacillus subtilis* ( $G^+$ ). For the detection of antibacterial activities the filter paper disc diffusion

method<sup>[6,7]</sup> was performed. Kanamycin was used as standard antibiotics for the antibacterial activities. Nutrient Agar (NA) was used as basal medium for test bacteria. These agar media were inoculated with 0.5 mL of the 24 h liquid cultures containing  $10^7$  microorganisms/mL. The diffusion time was 24 h at 5°C for bacteria. The incubation time was 12 h at 37°C for bacteria. Discs with only DMSO were used as control. Inhibitory activity was measured (in mm) as the diameter of the observed inhibition zones.

#### Determination of the minimum inhibitory concentration

**(MIC):** Minimal inhibitory concentration is defined as the lowest concentration that inhibits bacterial growth. To determine of the minimum inhibitory concentration (MIC) the serial dilution technique<sup>[8]</sup> was followed using nutrient broth medium. The MIC value of the compound 5 and 6 were determined against *Pseudomonas aeruginosa* (G<sup>-</sup>) and *Bacillus subtilis* (G<sup>+</sup>). Bacterial culture in Nutrient broth that had been incubated for 24 h was diluted 1000-fold with the same broth. An aliquot (0.9 mL) of the dilute was mixed with 0.1 mL of sample solution that had been dissolved or suspended in 10% aqueous dimethyl sulfoxide (DMSO) in a sterilized culture tube. After incubating overnight at 37°C, the growth of the test bacteria was determined by measuring turbidity.

**Antifungal screening:** The antifungal activities of compound 5 and 6 were studied towards five plants pathogenic and mold fungi, viz., *Colletorichum gloeosporioides* Penz. (Plant pathogen), *Candida albicans* (Human pathogen), *Aspergillus nigar* (Molds), *Aspergillus flavus* (Molds) and *Penicillium* sp. (Blue molds). The antifungal activity was assessed by poisoned food technique<sup>[9]</sup>, in some modified condition<sup>[10]</sup>. Fluconazole (200 µg disc<sup>-1</sup>) was used as standard fungicide for the antifungal activity. Potato Dextrose Agar (PDA) was used as basal medium for test fungi. Glass petridishes were sterilized and sterilized melted PDA medium (~ 45°C) was poured at the rate of 15 mL in each petridish (90 mm). After solidification of the medium the small portions of mycelium of each fungus were spreaded carefully over the center of each PDA plate with the help of sterilized needles. Thus, each fungus was transferred to a number of PDA plates. The PDA plates were then incubated at (25±2)°C and after five days of incubation they were ready for use. The prepared discs of samples were placed gently on the solidified agar plates, freshly seeded with the test organisms with sterile forceps. Control disc was also placed on the test plates to compare the effect of the test samples and to nullify the effect of solvents, respectively. The plates were then kept in a refrigerator at 4°C for 24 h in order that the materials had

sufficient time to diffuse to a considerable area of the plates. After this, the plates were incubated at 37.5°C for 72 h. Dimethyl sulphoxide (DMSO) was used as a solvent to prepare desired solution (10 mg mL<sup>-1</sup>) of the compounds initially. Proper control was maintained with Dimethyl sulphoxide (DMSO).

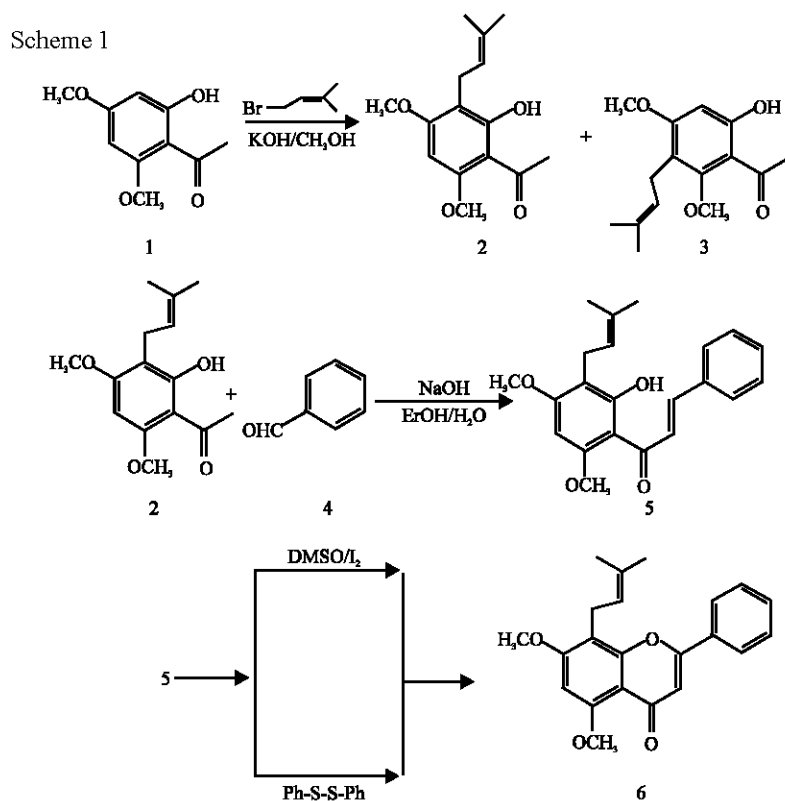
## RESULTS AND DISCUSSION

The synthesis of 8-γ,γ-dimethylallyl-5, 7-dimethoxyflavone [ovaliflavone, 6] was accomplished starting from 2-hydroxy-4, 6-dimethoxyphloroacetophenone<sup>[11]</sup> (1) (scheme-1).

3-γ,γ-dimethylallyl-2-hydroxy-4, 6-dimethoxyacetophenone (2) and 5-γ,γ-dimethylallyl-2-hydroxy-4, 6-dimethoxyacetophenone (3) were obtained from 2-hydroxy-4, 6-dimethoxyacetophenone (1) by the nuclear prenylation using prenyl bromide<sup>[12]</sup>. Compound 2 was obtained as white needles, m.p. 64°C. It showed IR absorption frequency at  $\nu$  3420 and 1645 cm<sup>-1</sup> indicating the presence of a hydroxy (-OH) group and a carbonyl group (>C=O), respectively. The <sup>1</sup>H NMR spectrum of compound 2 showed the presence of a γ,γ-dimethylallyl units by a singlet at  $\delta$  1.75 (6H) and doublet at  $\delta$  3.55 (d, 2H, J=7 Hz, Ar-CH<sub>2</sub>-CH<) indicating the presence of two methyl groups and two methylene protons, respectively. The olefinic proton appeared as a triplet centered at  $\delta$  5.25 (t, 1H, J=7 Hz, Ar-CH<sub>2</sub>-CH<). The compound 2 also showed two singlets at  $\delta$  3.75 and 3.781 for two methoxy groups. The three methyl protons of acetyl group (-COCH<sub>3</sub>) appeared at  $\delta$  2.25 integrating for three protons. The C<sub>5</sub>-H proton and a chelated phenolic proton at C-2' position appeared as two singlets at  $\delta$  6.01 and 12.75 respectively, integrating for one proton each. The above spectral data suggested that the compound 2 would be 3-γ,γ-dimethylallyl-2-hydroxy-4,6-dimethoxyacetophenone. Similarly, the structure of the compound 3 (5-γ,γ-dimethylallyl-2-hydroxy-4, 6-dimethoxyacetophenone) was established by its spectral data and elemental analysis.

Alkaline condensation of 3-γ,γ-dimethylallyl-2-hydroxy-4, 6-dimethoxyacetophenone (2) and benzaldehyde (4) afforded 3'-γ,γ-dimethylallyl-2'-hydroxy-4', 6'-dimethoxy chalcone (5) as yellow needles, mp. 122°C. The mp., spectral data (UV, IR and <sup>1</sup>H NMR) and elemental analysis of this chalcone 5 were very much similar to that of the natural sample of ovalichalcone<sup>[1]</sup>.

Cyclization of chalcone 5 into the corresponding flavone 6 was done by using two different oxidizing reagents DMSO/I<sub>2</sub> or diphenyl sulphide. The flavone 6 was obtained as yellow needles, mp. 142°C and its formation has been supported by spectral data and elemental analysis. The UV spectrum of this flavone 6 ( $\lambda_{max}$  220, 270 and 360 nm) suggested the presence of a



flavone nucleus. The IR absorption frequency at  $\nu$  1645  $\text{cm}^{-1}$  showed the presence of a carbonyl group ( $>\text{C}=\text{O}$ ) and the absence of a hydroxyl group band, confirmed the oxidation of chalcone 5 into flavone 6. In  $^1\text{H}$  NMR spectrum, the protons of  $\gamma$ ,  $\gamma$ -dimethylallyl units of flavone 6 appeared in the usual way<sup>[12]</sup>. The  $\text{C}_3$ -H proton of the flavone nucleus appeared at  $\delta$  5.61 as singlet integrating for one proton. The aromatic protons of the B-ring of the compound 6 ( $\text{C}_2'$ -H,  $\text{C}_3'$ -H,  $\text{C}_4'$ -H,  $\text{C}_5'$ -H and  $\text{C}_6'$ -H) were shown at  $\delta$  7.52 as a singlet integrating for five protons. The two methoxyl groups of flavone 6

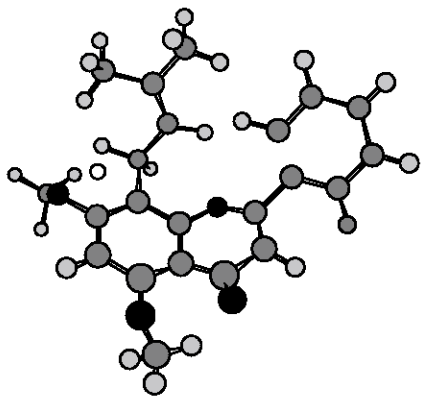


Fig. 1: A computer generated perspective drawing of compound 6. ●, ○ and ○ circles are denoted for oxygen, carbon and hydrogen, respectively

were indicated as two singlets at  $\delta$  3.97 and 3.99 integrating for three protons each. The  $\text{C}_6$ -H proton of the A ring appeared as a singlet at  $\delta$  7.25 integrating for one proton.

**Antibacterial activities:** The antibacterial activities of compounds 5 and 6 have been assayed at the concentration of 100, 200 and 300  $\mu\text{g disc}^{-1}$  against four human pathogenic bacteria. Among them, two were gram-positive and the rest two were gram-negative. The inhibitory effects of compounds 5 and 6 against these organisms (Table 1).

The screening results indicated that compound 5 did not show any antibacterial activities except against *S. dysenteriae* at the concentration 300  $\mu\text{g disc}^{-1}$ , on the other hand compound 6 showed good antibacterial activities to every bacteria. From this result it may be concluded that the flavone skeleton is active against bacteria than its corresponding chalcone skeleton.

**Minimum inhibitory activity:** The minimum inhibitory concentration of the compound 6 were determined against *B. subtilis* and *P. aeruginosa* by serial dilution method. The MIC level of the compound 6 was found 64  $\mu\text{g mL}^{-1}$  against *B. subtilis* and *P. aeruginosa*, respectively.

**Antifungal activities:** The antifungal activities of compounds 5 and 6 have been assayed at the

Table 1: Antibacterial screening for the compound 5 and 6

Comp.	Concentration ( $\mu\text{g disc}^{-1}$ )	<i>S. dysenteriae</i>	<i>P. aeruginosa</i>	<i>S. lntea</i>	<i>B. subtilis</i>
Comp. 5	100	-	-	-	-
	200	-	-	-	-
	300	8	-	-	-
Comp. 6	100	-	10	-	8
	200	10	14	10	12
	300	13	17	11	18
K-30*	30	26	28	34	30

\*Kanamycin-30

Table 2: Antifungal screening for the compound 5

Organisms	Diameter of the zone of inhibition (mm)			
	100 ( $\mu\text{g disc}^{-1}$ )	200 ( $\mu\text{g disc}^{-1}$ )	300 ( $\mu\text{g disc}^{-1}$ )	Fluconazole 200 ( $\mu\text{g disc}^{-1}$ )
<i>Penicillium</i> sp	-	5	8	12
<i>Aspergillus niger</i>	-	-	-	11
<i>Aspergillus flavus</i>	-	-	-	15
<i>Candida albicans</i>	-	-	-	13
<i>Colletorichum gloeosporioides</i> Penz.	-	-	-	10

Table 3: Antifungal screening for the compound 6

Organisms	Diameter of the zone of inhibition (mm)			
	100 ( $\mu\text{g disc}^{-1}$ )	200 ( $\mu\text{g disc}^{-1}$ )	300 ( $\mu\text{g disc}^{-1}$ )	Fluconazole ( $\mu\text{g disc}^{-1}$ )
<i>Penicillium</i> sp	4	5	7	12
<i>Aspergillus niger</i>	3	4	6	11
<i>Aspergillus flavus</i>	3	4	5	15
<i>Candida albicans</i>	4	6	7	13
<i>Colletorichum gloeosporioides</i> Penz.	2	3	5	10

concentration of 100, 200 and 300  $\mu\text{g disc}^{-1}$  against five plant pathogenic and molds fungi. The inhibitory effects of compounds 5 and 6 against these organisms are given in Table 2 and 3, respectively.

The screening results indicated that the compound 5 did not show any antifungal activities to the tested fungi except to the *Penicillium* sp. at the concentration of 200 and 300  $\mu\text{g disc}^{-1}$ . On the other hand, compound 6 showed good antifungal activity to the all tested fungal strain.

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