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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and biological activity of hydrazide–hydrazones and their corresponding 3-acetyl-2,5-disubstituted-2,3-dihydro-1,3,4-oxadiazoles

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Abstract Various 3-acetyl-2,5-disubstituted-2,3-dihydro-1,3,4-oxadiazoles (**11**–**20**) were prepared by the reaction of aryl substituted hydrazones of 4-fluorobenzoic acid hydrazide (**1**–**10**) with acetic anhydride. The structures of the synthesized compounds **11**–**20**, were confirmed by UV, IR, ^{1}H-NMR and mass spectroscopic methods. Antifungal evaluation of the hydrazide–hydrazones **1**–**10** and corresponding 3-acetyl-2,5-disubstituted-2,3-dihydro-1,3,4-oxadiazoles **11**–**20**, against clinical and standard *Candida* pathogens have been performed by using agar diffusion to indentify the active compounds, which were later subjected to a broth microdilution assay to justify the activity level in terms of minimum inhibitory concentrations (MIC). 4-Fluorobenzoic acid [(5-bromothiophen-2-yl)methylene]hydrazide, showed the highest inhibitory activity against *Candida albicans* (MIC: 125 μg/ml), and when compared with ketoconazole. In addition, bioauthographic antifungal activity against plant pathogenic fungi such as *Colletotrichum, Botrytis*, *Fusarium*, and *Phomopsis* was conducted. 4-Fluorobenzoic acid [(5-bromothiophen-2-yl)methylene]hydrazide was the most active analog against *P. viticola* with 91% inhibition at 30 μM after 144 h. Furthermore, known and the newly synthesized compounds were also screened through a panel of bioassays to determine their anti-inflammatory, cytotoxic, and antioxidant activities in mammalian cells. 3-Acetyl-5-(4-fluorophenyl)-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-1,3,4-oxadiazole, showed a strong inhibition of NF-κB-dependent transcription in SW1353 cells with IC_{50} value of 0.75 μg/ml. 4-Fluorobenzoic acid [(3-hydroxy-4-methoxyphenyl) methylene]hydrazide, and 3-acetyl-5-(4-fluorophenyl)-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-1,3,4-oxadiazole on intracellular ROS generation in PMA induced HL-60 cells demonstrated potent activity with IC_{50} values of 0.9 μg/ml. A strong inhibition of the activity of iNOS activity in LPS induced RAW 264.7 cells was observed for 3-acetyl-5-(4-fluorophenyl)-2-(4-hydroxyphenyl)-2,3-dihydro-1,3,4-oxadiazole with IC_{50} value of 0.3 μg/ml.

Keywords Hydrazide–hydrazones · 1,3,4-Oxadiazoles · Anti-inflammatory · Cytotoxicity · Antioxidant · Antifungal

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Introduction

Looking at the importance of oxadiazole nucleus, it was thought that it would be worthwhile to synthesize new

oxadiazole derivatives and screen them for potential biological activities. Owing to their diverse biological properties, their synthesis has increased noticeably in recent years (Khalil *et al.*, 2003; Jin *et al.*, 2006; Hassan *et al.*, 2008; Shirote and Bhatia 2011).

In this study, known hydrazide-hydrazone and newly synthesized 1,3,4-oxadiazole derivatives were investigated in a panel of bioassays that include, anti-inflammatory, antioxidant, cytotoxic and antimicrobial activities against human pathogenic bacteria or fungi. All the compounds were also tested for growth inhibition of several plant pathogenic fungi from the genera *Colletotrichum*, *Botrytis*, *Fusarium*, and *Phomopsis*. To the best of our knowledge we are reporting for the first time the biological activity of new 1,3,4-oxadiazole derivatives in this present study.

Results and discussion

Chemistry

General procedures for the preparation of target compounds **11–20** are represented in the Scheme 1. Hydrazide–hydrazones derivatives **1–10** were prepared by condensation of 4-fluorobenzoic acid hydrazide with appropriate aldehydes (Koçyigit-Kaymakcıoglu *et al.*, 2006; Kaymakcıoglu *et al.*, 2009). Physicochemical and spectroscopic characterization of the hyrazide-hydrazones **1–10** have been previously described (Koçyigit-Kaymakcıoglu *et al.*, 2006). 3-Acetyl-2,5-disubstituted-2,3-dihydro-1,3,4-oxadiazoles **11–20** were synthesized by reacting compounds (**1–10)** with Ac_2O (Rollas *et al.*, 2002). All 3-acetyl-2,5-disubstituted-2,3-dihydro-1,3,4-oxadiazoles, except compound **11** (Li *et al.*, 2010) are new compounds. Compounds **11–20** were isolated in satisfactory yields (49–90%) and purified by recrystallisation, using ethanol. The purities of the synthesized compounds were checked by reversed phase HPLC (Chromasil C_{18} 3.6 × 150 mm column using acetonitrile and water (50:50 v/v) as the eluent). All compounds showed a single peak with a retention time of 3.219–4.693 min. The structures of **11–20** were supported by elemental analysis and by the spectral data achieved from UV, IR, ^{1}H-NMR, and mass spectroscopy, which were in agreement with the proposed structures.

The IR spectra of **11–20** had different characteristics from those of the hydrazone derivatives as they showed no N–H stretching bands in the 3248–3450 cm^{-1} region and only C=O bands in the 1,673–1,685 cm^{-1} region, which were attributed to the C=O stretching of acetyl group. The ^{1}H NMR data were also consistent with the assigned structures. The spectra of **11–20** displayed the O-CHR-N resonance of the oxadiazoline ring at δ 6.68–7.26 ppm in accordance with the literature (Rollas *et al.*, 2002; Ergenç *et al.*, 1989). All the other aromatic and aliphatic protons were observed at the expected region in their NMR spectrum. In LC–MS, molecular ion peaks were in agreement with proposed molecular weight. All new compounds gave satisfactory elemental analysis results.

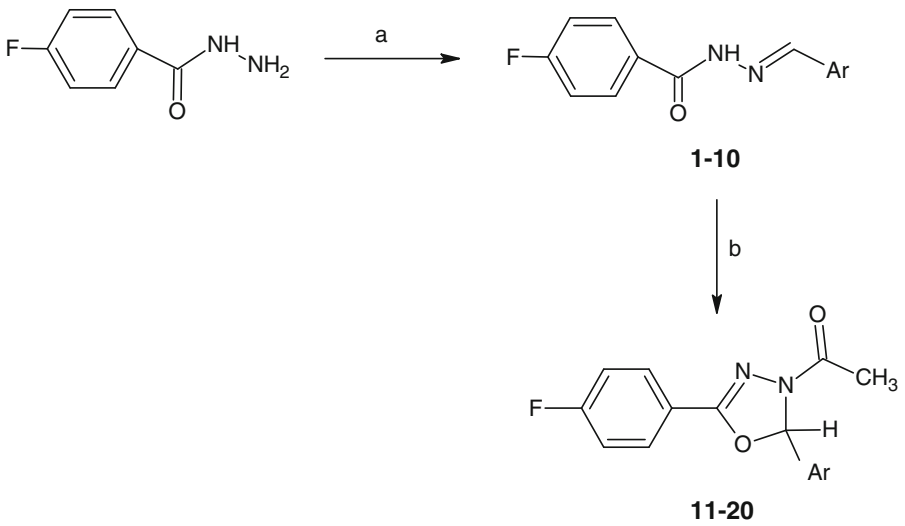
Antifungal activity against human pathogens

The antifungal activity of the compounds was studied with eight pathogenic *Candida* sp. Ketoconazole, was used as reference agents or inhibitory activity against the tested fungi. Minimal inhibitory concentrations (MIC) were recorded as the minimum concentration of a compound that inhibits the growth of tested microorganisms. All of the compounds tested illustrated medium to very good anticandidal inhibitory activity when compared with the reference agents. The MIC values were found within the range of 125–1,000 μg/ml against all evaluated strains. The results are summarized in Table 1. In comparing MIC values with the standard reference agent, compound **9**, 4-fluorobenzoic acid [(5-bromothiophen-2-yl)methylene]hydrazide, showed equal activity (125 μg/ml) against *C. albicans* (NRRL Y-12983). Compound **18**, 3-acetyl-5-(4-fluorophenyl)-2-[2-(4-(dimethylamino)phenyl) ethenyl]-2,3-dihydro-1,3,4-oxadiazole, showed moderate inhibitory activity (125 μg/ml) against *C. tropicalis* (NRRL Y-12968) and *C. krusei* (NRRL Y-7179). Compound **7,** 4-fluorobenzoic acid [(4-dimethylaminocinnamyl)methylene]hydrazide, and compound **14**, 3-acetyl-5-(4-fluorophenyl)-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-1,3,4-oxadiazole, showed moderate inhibition with the 125 μg/mL MIC value towards *C. glabrata* (clinical isolate) and *C. utilis* (NRRL Y-900), respectively. The other compounds were found less active when compared with ketoconazole against the tested microorganisms.

Antifungal activity against plant pathogens

Biological activity of a natural product involves several key characteristics that apply regardless of whether the activity is for pharmaceutical or agrochemical application (Wedge and Camper, 2000). Several 1,3,4-oxadiazole derivatives were chosen for further investigation in a research program at the United States Department of Agriculture aimed at identifying natural fungicides and biopesticides. A total of 20 compounds were subsequently evaluated in a 96-well micro-dilution broth assay for antifungal activity against *Colletrichum acutatum, C. fragariae, C. gloeosporioides*, *Botrytis cinerea*, *Phomopsis obscurans*, *P. viticola* and *Fusarium oxysporum*. This 96-well microtiter assay has been extensively used to determine and compare the sensitivity of fungal plant pathogens to natural and synthetic compounds with known commercial fungicides (Sobolev *et al.*, 2011). Oxadiazole

Scheme 1 Synthetic pathways used for the preparation of **1–20**. Reagents and conditions: (*a*) Ar–CHO, C_2H_5OH, Δ; (*b*) Ac_2O, Δ



Compound	Ar	Compound	Ar
1, 11	C_6H_5 -	**6, 16**	3-OH-4-OCH_3-C_6H_4-
2, 12	4-BrC_6H_4-	**7, 17**	$(CH_3)_2$N-C_6H_4-
3, 13	4-F-C_6H_4-	**8, 18**	$(CH_3)_2$N-C_6H_4CH=CH-
4, 14	4-OH-C_6H_4-	**9, 19**	5-Bromo-thiophen-2-yl-
5, 15	4-OCH_3-C_6H_4-	**10, 20**	Furan-2-yl

Reagents and conditions: (a) Ar-CHO, C_2H_5OH, Δ; (b) Ac_2O, Δ.

derivatives were found to be active only against five species: *C. acutatum, C. gloeosporioides*, *F. oxysporum*, *P. obscurans* and *P. viticola*, (Figs. 1, 2, 3, 4, 5). No antifungal activity was observed against *C. fragariae* and *B. cinerea*. In the microtiter broth bioassays, compound **19** also showed 49% inhibition against *C. acutatum* at 30 μM after 72 h (Fig. 1). The most active compounds **2**, **9**, and **19** inhibited growth of *C. gloeosporioides* 22, 32, and 66% at a dose of 30 μM, after 72 h treatment, respectively (Fig. 2). Any test compound possessing <50% growth inhibition at 30 μM in this bioassay is considered to have weak antifungal activity. The same compounds **2**, **9**, and **19** demonstrated 35, 40, and 67% growth inhibition against *F. oxysporum* at 30 μM after 72 h (Fig. 3). Of the twenty compounds tested, compound **19** demonstrated the greatest level of antifunal activity against *P. obscurans* with 65% inhibition at 30 μM after 144 treatments (Fig. 4). Compound **9** was the most active analog against *P. viticola* with 91% inhibition at 30 μM after 144 h (Fig. 5). The structural modification of hydrazone derivatives, i.e., replacement of bromo at C4 of phenyl ring with C5 of thiophene moiety resulted in an increase in the inhibition of *C. gloeosporioides*. The comparison of inhibition of *C. gloeosporioides* of hydrazone **9** and oxadiazole derivative **19** demonstrated that the cyclization of hydrazones increased the activity profile.

Furthermore, it was thought that the antifungal activity influenced by the aromatic ring substituted by a bromo atom due was to the increase in lipophilicity. This change may allow for great transport across the lipid bilayer of the fungal membrane and hence achieve a higher concentration of antifungal molecules inside the fungal mycelia.

Cytotoxic, anti-inflammatory, and antioxidant activity

All synthesized compounds **1–20** were screened in a panel of bioassays to evaluate their antioxidant, anti-inflammatory and cytotoxic activities. The transcription factor NF-κB plays a key role in regulation of genes involved in immune and inflammatory responses and apoptosis. The activation of NF-κB has been implicated in different types of cancers and in many human chronic inflammatory diseases. Therefore, NF-κB is a central target for a variety of anti-inflammatory agents (Ankisetty *et al.*, 2010). A large

Table 1 Anticandidal evaluation of hydrazide-hydrazones (**1–10**) and 1,3,4-oxadiazole derivatives (**11–20**) as MIC values in μg/ml

Compounds	A*	B	C	D	E	F	G	H*
1	nt	500	500	nt	500	250	500	250
2	500	500	1000	nt	500	1000	500	250
3	250	500	500	500	500	500	500	nt
4	nt	500	500	500	500	500	500	nt
5	nt	500	500	1000	500	500	500	250
6	nt	500	500	250	500	500	500	250
7	nt	500	500	500	500	500	500	125
8	500	500	500	500	500	500	500	500
9	250	250	250	250	250	250	125	250
10	250	500	500	500	500	500	500	250
11	500	1000	1000	1000	1000	1000	500	1000
12	250	1000	500	250	500	500	500	500
13	500	500	500	500	500	500	500	500
14	250	500	125	250	250	250	500	500
15	250	500	250	500	500	500	500	250
16	250	500	250	500	500	500	500	250
17	250	500	500	500	500	1000	500	500
18	500	500	250	125	125	250	500	500
19	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
20	500	1000	500	500	500	1000	500	500
Ketoconazole	16	62	62	62	62	125	125	31

A*, *Candida albicans* (clinical isolate); B, *Candida albicans* (ATCC 90028); C, *Candida utilis* (NRRL Y-900); D, *Candida tropicalis* (NRRL Y-12968); E, *Candida krusei* (NRRL Y-7179); F, *Candida parapsilosis* (NRRL Y-12696); G, *Candida albicans* (NRRL Y-12983); H*, *Candida glabrata* (clinical isolate); nt, not determined

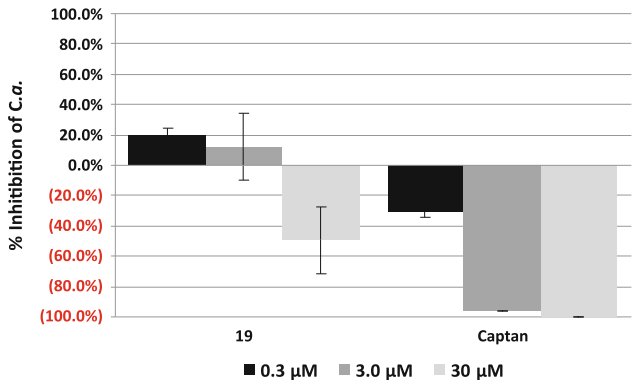


Fig. 1 Growth inhibition of *Colletotrichum acutatum* (Ca) after 72 h using 96-well microdilution broth assay in a dose response and the commercial fungicide standard captan

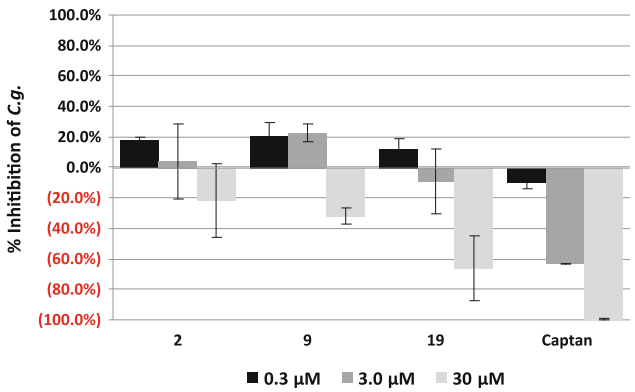


Fig. 2 Growth inhibition of *Colletotrichum gloeosporioides* (Cg) after 72 h using 96-well microdilution broth assay in a dose response and the commercial fungicide standard captan

number of natural and synthetic compounds are currently being investigated for their effect on NF-κB activity. Of the tested compounds, **16** showed a strong inhibition of NF-κB-dependent transcription in SW1353 cells induced by phorbol myristate acetate (PMA) with IC_{50} values of 0.75 μg/ml, which was comparable with the activity of positive control, parthenolide (IC_{50} of 0.68 μg/ml) (Table 2). Compounds **1** and **14** also showed considerable activity with IC_{50} values of 2.8 and 5.5 μg/ml, respectively. Moderate activity was observed for compound **3** with IC_{50} value of 15 μg/ml. A luciferase construct with binding sites for Sp-1 was used as a control because this transcription factor is relatively unresponsive to inflammatory mediators. Measurement of Sp-1-mediated luciferase expression in parallel to NF-kB mediated luciferase expression is employed for detecting agents that non-specifically inhibit

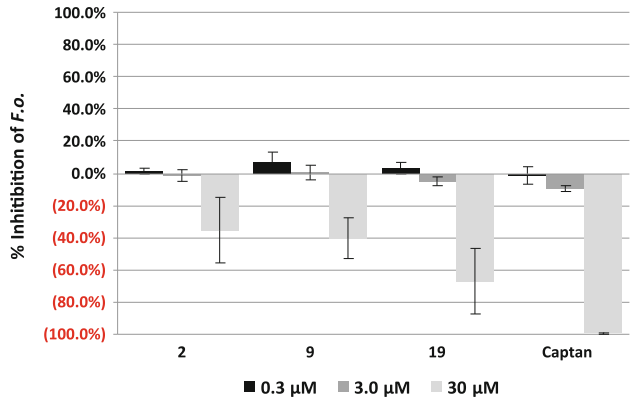


Fig. 3 Growth inhibition of *Fusarium oxysporum* (Fo) after 72 h using 96-well microdilution broth assay in a dose response and the commercial fungicide standard captan

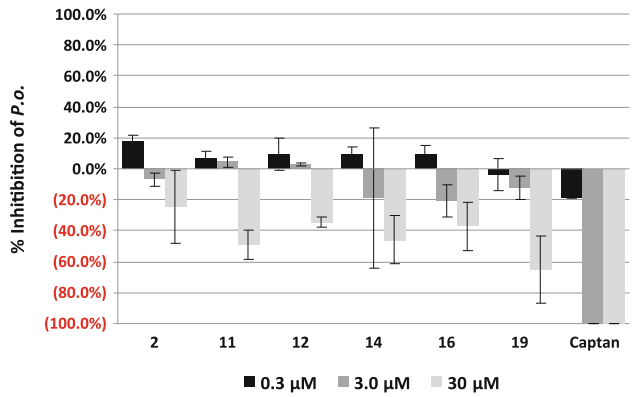


Fig. 4 Growth inhibition of *P. obscurans* (Po) after 144 h using 96-well microdilution broth assay in a dose response and the commercial fungicide standard captan

luciferase expression due to cytotoxicity, inhibition of luciferase enzyme activity or light output (Tabanca *et al.*, 2007a, b). None of the compounds inhibited Sp-1 dependent luciferase expression except **1**. Compound **1** inhibited Sp-1-mediated activity at much higher concentration (IC_{50} = 14 µg/ml) than the concentration responsible for inhibiting NF-κB activity (IC_{50} = 2.8 µg/ml).

The introduction of methoxy group at 4-position of phenyl ring in oxadiazole moiety and also replacement of the hydroxyl group at 4-position to 3-position of phenyl ring (compound **16**) resulted stonger inhibition of NF-κB activity compared to compound **14** which only had a hydroxyl group at 4-position of phenyl ring.

Inducible nitric oxide synthase (iNOS) plays a key role in the development of inflammatory diseases. Thus an iNOS inhibitor could be considered a potential anti-inflammatory agent (Ankisetty *et al.*, 2010) Inhibition of lipopolysaccharide (LPS)-induced nitric oxide synthase activity in RAW 264.7 cells was determined (Table 2) by measuring the decrease in nitrite production in cells treated with compounds **1**–**20** in comparison to untreated vehicle

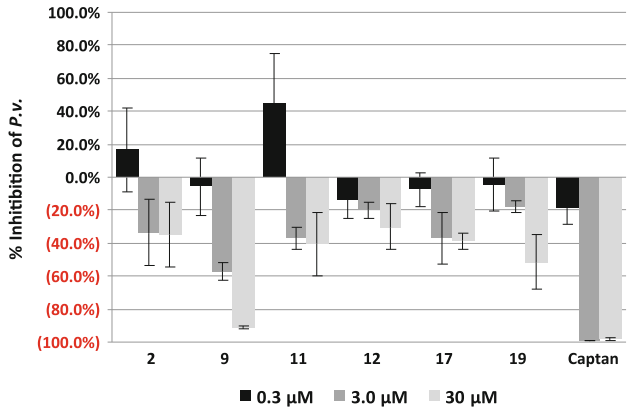


Fig. 5 Growth inhibition of *Phomopsis viticola* (Pv) after 144 h using 96 well microdilution broth assay in a dose response and the commercial fungicide standard captan

controls. A strong inhibition of the activity of iNOS activity in LPS induced RAW 264.7 cells was observed for compound **14** with IC_{50} value of 0.3 µg/ml. The effect was comparable with the positive control parthenolide (IC_{50} = 0.4 µg/ml). Compound **16** also inhibited iNOS activity with IC_{50} value of 4.0 µg/ml. The other compounds, **4**, **6**, **9**–**12**, **17** were moderately active (inhibition of >30% at the highest concentration), while **1**–**3**, **5**, **7**–**8**, **13**, **15**, **18**–**20** were inactive. Cytotoxicity of test samples to macrophages was also determined in parallel to determine if the inhibition of iNOS is due to cytotoxic effects. None of the compounds were cytotoxic to RAW 264.7 cells. The comparison of iNOS activity of hydrazone and oxadiazole derivatives showed that oxadiazoles bearing a phenyl, 4-bromophenyl, 4-hydroxyphenyl, 3-hydroxy-4-methoxy-phenyl, 4-dimethyl aminophenyl were active compounds.

Especially, the substitution of a hydroxyl to a phenyl group in the 4-position (compound **14**) resulted activity comparable to parthenolide. The inhibitory activity of compound **16** which had hydroxyl and methoxyl group at 3- and 4-position of phenyl ring was also significant (Table 2).

Since reactive oxygen species (ROS) and oxidative stress play an important role in major human degenerative diseases such as cancer, inflammation, atherosclerosis, and aging, we investigated the effect of these compounds on intracellular ROS generation in PMA induced HL-60 cells. A strong antioxidant effect was demonstrated by, **6**, **10**, **16** with IC_{50} values in range of 0.7–0.9 µg/ml. Compounds **5** and **20** were also effective with IC_{50} of 2.5 and 5.2 µg/ml, respectively. Compounds **3** and **7** showed mild activity (IC_{50} = 20 and 23 µg/ml, respectively). Moderate cytotoxicity was observed towards HL-60 cells by several of them (see Table 2). Among the most active compounds (**6**, **10**, and **16**) **6** was noncytotoxic while **10** and **16** showed cytotoxicity at much higher concentrations than the effective concentrations for antioxidative activity (Table 2) indicating that the antioxidant effect of these compounds is

Table 2 Anti-inflammatory and antioxidant activities of tested compounds

Compounds	Inhibition of NF-κB activity in SW1353 cells		Antioxidant Activity (inhibition of ROS generation) in HL-60 cells		Inhibition of iNOS activity (NO production) in RAW 264.7 cells	
	IC_{50} (μg/ml)		IC_{50} (μg/ml)		IC_{50} (μg/ml)	
	NF-κB activity	SP-1	Antioxidant activity	Cytotoxicity	iNOS activity	Cytotoxicity
1	2.8	14	NA	NA	NA	NA
2	NA	NA	NA	NA	NA	NA
3	15	NA	20	NA	NA	NA
4	NA	NA	NA	NA	>25	>25
5	NA	NA	2.5	20	NA	NA
6	NA	NA	0.9	NA	22	NA
7	NA	NA	23	>31.25	NA	NA
8	NA	NA	NA	NA	NA	NA
9	NA	NA	NA	16	>25	NA
10	NA	NA	0.7	27	>25	NA
11	NA	NA	NA	31.3	>25	NA
12	NA	NA	NA	NA	>25	NA
13	NA	NA	NA	NA	NA	NA
14	5.5	NA	NA	NA	0.3	NA
15	NA	NA	NA	NA	NA	NA
16	0.75	NA	0.9	20	4	NA
17	NA	NA	NA	NA	20	NA
18	NA	NA	NA	NA	NA	NA
19	NA	NA	NA	5.9	NA	NA
20	NA	NA	5.2	24	NA	NA
Doxorubicin[a]				0.16		
Parthenolidea	0.68	7.5			0.4	13
Trolox[a]			0.3			

[a] Standard compounds with known biological activities. *NA* no activity up to $IC_{50} = 25$ μg/ml

not related to their effect on cell viability. Out of the hydrazone and oxadiazole compounds, the antioxidant activity of compound **6** may be explained due to the presence of hydroxyl and methoxyl groups.

In addition, these **1–20** synthesized compounds were also tested in a panel of mammalian kidney cells (Vero and LLC-PK_{11}) and cancer cells (SK-MEL, KB, BT-549, and SK-OV-3) up to a concentration of 25 μg/ml to see their cytotoxic and anti-cell proliferative effects but none showed any effect (data not shown).

Conclusions

In conclusion, a detailed study concerning synthesis, structure and biological activity of known hydrazide–hydrazones and newly synthesized 1,3,4-oxadiazole derivatives is reported. The in vitro antifungal activity against human pathogenic fungi and plant pathogens were investigated and some of the compounds have showed remarkable activity. Compound **9** showed equal activity with ketoconazole against *Candida albicans*. The same compound also demonstrated potential for further development for control of *Phomopsis viticola*. *Phomopsis* cane and leaf spot, caused by the fungus *P. viticola*, can cause significant losses to the vine grape industry in the worldwide. Evaluation of the biological activity of some of these compounds in the present study indicated their potential as anti-inflammatory agents through their effects on various targets. Some compounds displayed remarkable antioxidant activity. Hydrazide-hydrazone compounds **1**, **6**, **10** and 1,3,4-oxadiazole compounds **14**, **16**, **20** can be interesting source for lead compounds for anti-inflammatory research.

Experimental

Chemistry

All chemicals and solvents were procured from Merck and Aldrich (both Germany). Reactions were monitored by thin

layer chromatography (TLC) and purity of the products was checked by high performance liquid chromatography (HPLC). TLC was performed on Merck 60 F-254 silica gel plates with visualization by UV-light using chloroform and methanol as solvent system. HPLC (Agilent, Palo Alto, CA, USA) was performed using a Chromasil C_{18} 3.9 × 150 mm column. The eluent was acetonitrile and water (50:50 v/v) and the flow rate was 1 ml/min, diode array detection at 254 nm.

Melting points were determined on a SMP II apparatus (Gehrden, Germany). The IR spectra were recorded on a Schimadzu FTIR 8400S spectrometer (Kyoto, Japan). ^{1}H NMR spectra were recorded on Bruker Avance-DPX-400 spectrometer (Brillerica, MA, USA) in d_6-DMSO. Chemical shifts were recorded in parts per million downfield from TMS. The splitting patterns of ^{1}H-NMR were designed as follows: s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet. Mass spectra were recorded on LC–MS-Agilent 1100 (Palo Alto, CA, USA) series in the electrospray mode. Elemental analysis was performed on Leco CHNS-932 analyzer (Michigan, USA). (^{1}H-NMR, mass and elemental analyses were provided by the Scientific and Technical Research Council of Turkey, TUBITAK).

Procedure for the preparation of the 3-acetyl-2,5-disubstituted-2,3-dihydro-1,3,4-oxadiazole derivatives ***11–20***

Physicochemical and spectroscopic characterization of the initial hydrazide–hydrazones, 4-fluorobenzoic acid (substitutedmethylene)hydrazides (**1**–**10**), have been described in our previous study (Koçyigit-Kaymakcıoglu *et al.*, 2006). A mixture of an appropriate hydrazide-hydrazone (1 mmol) and Ac_2O (5 ml) was heated under reflux for 2 h. After the mixture was cooled to room temperature, excess Ac_2O was decomposed by addition of H_2O and the mixture was stirred for 30 min. The separated product was filtered and crystallized from EtOH.

*3-Acetyl-5-(4-fluorophenyl)-2-phenyl-2,3-dihydro-1,3,4-oxadiazole (**11**)* Yield: 76%. M.p. 103–105°C. R_t(acetonitrile and water, 50:50 v/v) 4.555 min. IR (υ_{max}, cm^{-1}): 2950, 2860, 1678, 1580, 1470, 1420, 1220, 1030, 730. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 2.28 (s, 3H, –COC<u>H</u>$_3$); 6.96-8.01 (m, 10H, –OCHR and Ar–H). Anal. calc for $C_{16}H_{13}FN_2O_2$ (284.28): C, 67.60; H, 4.61; N, 9.85. Found: C, 67.65; H, 4.66; N, 9.83. MS-ES (*m*/*z*): 285.28 (MH^+).

*3-Acetyl-5-(4-fluorophenyl)-2-(4-bromophenyl)-2,3-dihydro-1,3,4-oxadiazole (**12**)* Yield: 90%. M.p. 126–128°C. Rt(acetonitrile and water, 50:50 v/v) 4.693 min. IR (υ_{max}, cm^{-1}): 2948, 2850, 1685, 1580, 1465, 1420, 1225, 1040, 735. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 2.26 (s, 3H, –COC<u>H</u>$_3$); 7.20 (s, 1H, -O<u>CH</u>R); 7.28-7.42 (t, 2H, ortho-protons to F), 7.46 (d, 2H, ortho-protons to Br, J: 8.46 Hz); 7.66 (d, 2H, meta-protons to Br, J: 8.43 Hz); 7.84–7.94 (m, 2H, meta-protons to F). Anal. calcd for $C_{16}H_{12}BrFN_2O_2$ (363.18) : C, 52.91; H, 3.33; N, 7.71. Found: C, 52.75; H, 3.36; N, 7.69. MS-ES (*m*/*z*): 364.18 (MH^+).

*3-Acetyl-2,5-bis(4-fluorophenyl)-2,3-dihydro-1,3,4-oxadiazole (**13**)* Yield: 87%. M.p. 126-128°C. Rt(acetonitrile and water, 50:50 v/v) 3.219 min. IR (υ_{max}, cm^{-1}): 2970, 2860, 1675, 1570, 1475, 1420, 1210, 1040, 733. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 2.35 (s, 3H, –COC<u>H</u>$_3$); 7.16–7.52 (m, 5H, –O<u>CH</u>R and ortho-protons to F), 7.84–8.11 (m, 4H, meta-protons to F). Anal. calcd for $C_{16}H_{12}F_2N_2O_2$ (302.27): C, 63.57; H, 4.00; N, 9.27. Found: C, 63.56; H, 4.09; N, 9.35. MS-ES (*m*/*z*): 303.27 (MH^+).

*3-Acetyl-5-(4-fluorophenyl)-2-(4-hydroxyphenyl)-2,3-dihydro-1,3,4-oxadiazole (**14**)* Yield: 49%. M.p. 137–139°C. Rt(acetonitrile and water, 50:50 v/v) 3.970 min. IR (υ_{max}, cm^{-1}): 2960, 2850, 1680, 1565, 1475, 1410, 1230, 1045, 732. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm) 2.28 (s, 3H, –COC<u>H</u>$_3$); 7.20-7.22 (m, 3H, –O<u>CH</u>R and ortho-protons to OH); 7.36–7.41 (t, 2H, ortho-protons to F), 7.53 (d, 2H, meta-protons to OH, J: 8.53 Hz); 7.89–7.93 (m, 2H, meta-protons to F), 10.60 (s, 1H, OH). Anal. calcd for $C_{16}H_{13}FN_2O_3$ (300.28): C, 64.00; H, 4.36; N, 9.33. Found: C, 64.02; H, 4.38; N, 9.29. MS-ES (*m*/*z*): 301.28 (MH^+).

*3-Acetyl-5-(4-fluorophenyl)-2-(4-methoxyphenyl)-2,3-dihydro-1,3,4-oxadiazole (**15**)* Yield: 85%. M.p. 165–167°C. Rt(acetonitrile and water, 50:50 v/v) 4.525 min. IR (υ_{max}, cm^{-1}): 2955, 2880, 1673, 1590, 1480, 1425, 1230, 1035, 735. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm) 2.06 (s, 3H, –COC<u>H</u>$_3$); 3.76 (s, 3H, –OC<u>H</u>$_3$); 6.76-7.10 (m, 7H, –O<u>CH</u>R, ortho-protons to OCH_3, ortho-protons to F and meta-protons to OCH_3); 7.54–7.68 (m, 2H, meta-protons to F). Anal. calcd for $C_{17}H_{15}FN_2O_3$ (314.31): C, 64.96; H, 4.81; N, 8.91. Found: C, 64.84; H, 4.79; N, 8.95. MS-ES (*m*/*z*): 315.31 (MH^+).

*3-Acetyl-5-(4-fluorophenyl)-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-1,3,4-oxadiazole (**16**)* Yield: 82%. M.p. 135–137°C. Rt(acetonitrile and water, 50:50 v/v) 3.913 min. IR (υ_{max}, cm^{-1}): 2990, 2870, 1675, 1590, 1465, 1428, 1225, 1040, 733. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 1.96 (s, 3H, -COC<u>H</u>$_3$); 3.82 (s, 3H, -OC<u>H</u>$_3$); 7.03-7.47 (m, 6H, –O<u>CH</u>R, aromatic protons and ortho-protons to F); 7.90–7.93 (m, 2H, meta-protons to F). Anal. calcd for $C_{17}H_{15}FN_2O_4$ (330.31): C, 61.82; H, 4.58; N, 8.48. Found: C, 61.88; H, 4.59; N, 8.47. MS-ES (*m*/*z*): 331.31 (MH^+).

*3-Acetyl-5-(4-fluorophenyl)-2-(4-(dimethylamino)phenyl)-2,3-dihydro-1,3,4-oxadiazole (**17**)* Yield: 65%. M.p. 119–121°C. Rt(acetonitrile and water, 50:50 v/v) 3.650 min. IR (υ_{max}, cm^{-1}): 2960, 2870, 1674, 1580, 1475, 1410, 1220, 1035, 730. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 1.92 (s, 3H, –COC$\underline{H}_3$); 3.05 (s, 6H, –N(C$\underline{H}_3$)$_2$); 6.72–6.87 (m, 3H, –OC$\underline{H}$R and ortho-protons to ter.amine); 7.25–7.45 (m, 2H, ortho-protons to F), 7.70 (d, 2H, meta-protons to ter. amine J: 8.95 Hz); 7.86–8.09 (m, 2H, meta-protons to F). Anal. calcd for $C_{18}H_{18}FN_3O_2$ (327.35): C, 66.04; H, 5.54; N, 12.84. Found: C, 66.08; H, 5.52; N, 12.85. MS-ES (*m/z*): 328.35 (MH^+).

Yield: 54%. M.p. 153–155°C. Rt(acetonitrile and water, 50:50 v/v) 3.840 min. IR (υ_{max}, cm^{-1}): 2965, 2870, 1678, 1585, 1460, 1430, 1225, 1032, 736. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 1.92 (s, 3H, –COC$\underline{H}_3$); 2.78 (s, 6H, -N(C$\underline{H}_3$)$_2$); 3.00–3.10 (m, 2H, –CH_2–); 6.74-6.77 (m, 3H, –OC$\underline{H}$R, ortho-protons to ter. amine); 7.02–7.06 (m, 2H, –C$\underline{H}$=C$\underline{H}$–); 7.46-7.51 (m, 2H, ortho-protons to F), 7.56–7.73 (m, 3H, meta-protons to ter.amine, –CH=); 8.13-8.16 (m, 2H, meta-protons to F). Anal. calcd for $C_{20}H_{20}FN_3O_2$ (353.39): C, 67.97; H, 5.70; N, 11.89. Found: C, 67.96; H, 5.72; N, 11.85. MS-ES (*m/z*): 354.39 (MH^+).

*3-Acetyl-2-(5-bromothiophen-2-yl)-5-(4-fluorophenyl)-2,3-dihydro-1,3,4-oxadiazole (**19**)* Yield: 65%. M.p. 145–147°C. Rt(acetonitrile and water, 50:50 v/v) 4.150 min. IR (υ_{max}, cm^{-1}): 2955, 2860, 1680, 1580, 1475, 1425, 1220, 1033, 730. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 2.20 (s, 3H, –COC$\underline{H}_3$); 6.82 (s, 1H, C_3-H proton of thiophen); 7.16 (s, 1H, –OCHR); 7.30–7.45 (m, 3H, ortho-protons to F and C_4-H proton of thiophen), 7.75–7.97 (m, 2H, meta-protons to F). Anal. calcd for $C_{14}H_{10}BrFN_2O_2S$ (369.20) : C, 45.54; H, 2.73; N, 7.59; S, 8.68. Found: C, 45.50; H, 2.74; N, 8.66; S, 8.68. MS-ES (*m/z*): 370.20 (MH^+).

*3-Acetyl-5-(4-fluorophenyl)-2-(furan-2-yl)-2,3-dihydro-1,3,4-oxadiazole (**20**)* Yield: 67%. M.p. 130–132°C. Rt(acetonitrile and water, 50:50 v/v) 3.835 min. IR (υ_{max}, cm^{-1}): 2950, 2870, 1685, 1560, 1450, 1430, 1230, 1040, 732. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 2.25 (s, 3H, –COC$\underline{H}_3$); 6.52 (d, 1H, J: 3.25 Hz, C_3-H proton of furan); 6.78 (t, 1H, C_4-H proton of furan); 7.26 (s, 1H, –OCHR); 7.29–7.44 (m, 2H, ortho-protons to F), 7.73 (s, 1H, C_5-H proton of furan), 7.88–8.00 (m, 2H, meta-protons to F). Anal. calcd for $C_{14}H_{11}FN_2O_3$ (274.24): C, 61.61; H, 4.04; N, 10.21. Found: C, 61.60; H, 4.05; N, 10.19. MS-ES (*m/z*): 275.24 (MH^+).

Biological assays

Antifungal activity against human pathogens

The antifungal properties of compounds **1**–**20** were evaluated by the broth microdilution method according to a modified adaptation of the NCCLS reference document M27-A2 (NCCLS, 2002; Özdemir *et al.*, 2010) against *Candida albicans* (clinical isolate), *Candida albicans* (ATCC 90028), *Candida glabrata* (clinical isolate), *Candida utilis* (NRRL Y-900), *Candida tropicalis* (NRRL Y-12968), *Candida parapsilosis* (NRRL Y-12696), *Candida albicans* (NRRL Y-12983). Antifungal evaluation of the hydrazide–hydrazones **1**–**10** and corresponding 3-acetyl-2,5-disubstituted-2,3-dihydro-1,3,4-oxadiazoles **11**–**20** against clinical and standard *Candida* pathogenic strains have been performed by using agar diffusion to the indentify the active compounds which were later subjected to a broth microdilution assay to justify the activity level in terms of MIC (NCCLS, 2002; Özdemir *et al.*, 2010). Ketoconazole was used as positive control.

Anticandidal assay

The activity of the compounds **1**–**20** were first screened using an agar diffusion method for *C. albicans* (clinical isolate) and *C. tropicalis* and all active compounds (inhibition zones >9–11 mm, at 2,000 μg/ml concentration) were further evaluated using the microdilution broth method to identify the MIC against all *Candida* spp.

Broth microdilution assay

The test compounds and the antimicrobial standards were first dissolved in dimethyl sulfoxide (DMSO) which was used to prepare the stock solutions at an initial concentration of 2,000 μg/ml. Serial dilution series were prepared in 100 μl MHB with an equal amount of the test samples. The last row was filled only with water as growth control for the microorganism. Overnight grown microorganism suspensions were first diluted in double strength MHB and standardized to 10^8 CFU/ml (using McFarland No: 0.5) under sterile conditions. Then each microorganism suspension was pippetted into each well and incubated at 37°C for 24 h. Ketoconazole was used as a standard antifungal agent against *Candida* spp. Sterile distilled water and medium served as a positive growth control. The first well without turbidity was assigned as the minimum inhibitory concentration (MIC, in μg/ml).

Antifungal assay against plant pathogens

A standardized 96-well micro-dilution broth assay developed by Wedge and Kuhajek (1998) for the discovery of natural fungicidal agents was used to evaluate the antifungal activity of test compounds (Wedge and Camper 2000). Isolates of *Colletotrichum acutatum* Simmonds, *Colletotrichum fragariae* Brooks, *Colletotrichum gloeosporioides* (Penz.) Penz & Sacc. In Penz, *Botrytis cinerea*

Pers.:Fr, *Fusarium oxysporum* Schlechtend:Fr, *Phomopsis obscurans* (Ellis and Everh.) *B. sutton*, and *P. viticola* Sacc., were used to evaluate the antifungal activity of the test compounds using in vitro micro-dilution broth assay. Each microtiter test well received 80 µl of RPMI 1640 (Roswell Park Memorial Institute mycological broth 1640, Life Technologies, Grand Island, New York) and 3[*N*-morpholino]propanesulfonic acid (MOPS, Sigma Chemical Co., St. Louis, Missouri) buffered broth, 100 µl of conidial suspension at 1.0×10^4 conidia/ml, and 20 µl of test compound solution. The commercial fungicide captan was used as an internal fungicide standard in all assays. Each fungus was challenged in a dose–response format using test compounds, with final treatment concentrations of 0.3, 3.0, and 30.0 µM. Microtiter plates (Nunc MicroWell, untreated; Roskilde, Denmark) were covered with a plastic lid and incubated in a growth chamber at 24 ± 1°C and a 12-h photoperiod under a light intensity of 60 ± 5 µmol/m^2/s. Growth was then evaluated by measuring absorbance (620 nm) of each well using a microplate reader (model SpectraCount; Packard Instrument Company, Meriden, Connecticut).

Using the 96-well plate micro-bioassay format, each chemical was evaluated in duplicate at three concentrations. The experiments were repeated three times over time. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 h and 72 h, except for *P. obscurans* and *P. viticola* the data were recorded at 144 h. Analysis of variance of means for percent inhibition/stimulation of each fungal species at each dose of test compound relative to the untreated positive growth controls was used to evaluate fungal growth. Treatments were arranged as a split-plot design repeated four times. Whole-plots were fungal isolates and sub-plots were chemicals. Each dose level and response time was analyzed separately. The SAS system analysis of variance procedure (Statistical Analysis System, Cary, North Carolina) was used to identify significant factors, and Fisher's protected LSD was used to separate means (Steel and Torrie, 1980).

Anti-inflammatory assays

Inhibition of NF-κB mediated transcription was determined in human chondrosarcoma (SW1353) cells by a reporter gene assay as described earlier (Tabanca *et al.*, 2007a, b). In brief, at about 75% confluency, cells were harvested and transfected with NF-κB reporter luciferase plasmid construct at 160 V and one 70-ms pulse in a BTX Electro Square Porator T 820. Transfected cells were plated in 96-well plates (1×10^5 cells/well) and incubated for 24 h. After 24 h, cells were exposed to test samples for 30 min and then incubated for 8 h with PMA (70 ng/ml) for the activation of NF-κB. After removing medium, cells were lysed by adding 40 µl of a 1:1 mixture of LucLite reagent and PBS containing 1 mM calcium and magnesium. Luciferase activity was measured as light output on a SpectraMax plate reader. IC_{50} values were obtained from dose response curves. Sp-1 was used as a control transcription factor to evaluate the toxicity of tested compounds in the same assay. Parthenolide was used as the positive control.

Inhibition of intracellular NO production as a result of iNOS activity was assayed in mouse macrophages (RAW 264.7 cells) as described (Ankisetty *et al.*, 2010; Quang *et al.*, 2006). Cells were seeded in 96-well plates at a density of 50,000 cells/well and grown for 24 h for a confluency of 75% or more. Test samples were added at various concentrations and after 30 min LPS (5 µg/ml) was added and cells were further incubated for 24 h. The concentration of NO was determined by measuring the level of nitrite in the cell culture supernatant with Griess reagent. The degree of inhibition of nitrite production was calculated in comparison to the vehicle control. IC_{50} values were obtained from dose response curves. Cytotoxicity of test samples to macrophages was also determined in parallel to check if the inhibition of iNOS is due to cytotoxic effects. Parthenolide was included in each assay as the positive control.

Inhibition of intracellular ROS generation (antioxidant activity) was assayed in human promyelocytic leukemia (HL-60) cells. Cells were seeded in 96-well plates (100,000 cells/well) and treated with different concentrations of test samples for 30 min. Cells were then stimulated with PMA (100 ng/ml) for 30 min. ROS generation is determined by using DCFH-DA as described previously (Tabanca *et al.*, 2007a, b). DCFH-DA is a non-fluorescent probe that diffuses into the cells. Cytoplasmic esterases hydrolyze the DCFH-DA to 2′,7′-dichlorofluorescin (DCFH). The reactive oxygen species (ROS) generated within cells oxidize DCFH to 2′,7'-dichlorofluorescin (DCF) that fluoresces. The ability of the test compounds to inhibit production of DCF in PMA treated HL-60 cells was measured in comparison to the vehicle control. The IC_{50} values were calculated from dose curves of the % DCF production versus test concentrations. Trolox was used as positive control.

Cytotoxicity assay

Cytotoxicity was determined against a panel of four human tumor cell lines [SK-MEL (malignant melanoma); KB (oral epidermal carcinoma); BT-549 (breast ductal carcinoma); SK-OV-3 (ovary carcinoma)] and two noncancerous cell lines [Vero (African green monkey kidney fibroblasts) and LLC-PK_{11} (pig kidney epithelial cells)] as described earlier (Tabanca *et al.*, 2003). Cells were seeded at a density of 25,000 cells/well in 96-well plates and

grown for 24 h. Samples were added and plates were incubated for 48 h. Cell viability was determined by using Neutral Red. Doxorubicin was used as a positive control.

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