ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2013, Vol. 39, No. 3, pp. 318–328. © Pleiades Publishing, Ltd., 2013.

Synthesis and Synergistic, Additive Inhibitory Effects of Novel Spiro Derivatives against Ringworm Infections[1]

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Received May 14, 2012; in final form, November 12, 2012

Abstract—An environmentally benign solvent free synthesis of various spiro-1,4-dihydropyridines (1,4-DHPs) incorporating 2-oxindole/piperidines is performed in 5–8 min with reasonable purity in 80–90% yield under microwave irradiation using montmorillonite KSF as an inorganic solid support. The reaction is found to be general with respect to various cyclic carbonyl compounds, e.g. cyclohexanone, substituted indole-2,3-dione, and piperidinone derivatives. In our study, these compounds were also found effective against dermatophytes and other fungal organisms. Our results suggest that novel spiro derivatives can be used for the treatment of dermatophytosis or ringworm infections.

Keywords: montmorillonite KSF, microwave irradiation, MCRs, spiro-1,4-DHPs

DOI: 10.1134/S106816201303014X

The rapid assembly of molecular diversity is an important goal of synthetic organic chemistry and one of key paradigms of modern drug discovery. One approach to address this challenge involves the use of multicomponent reactions (MCRs), in which three or more reactants are combined together in a single reaction flask to generate a product incorporating most of the atoms contained in the starting material. Due to their intrinsic atom economy, selectivity, simple procedures and equipment, time and energy saving, as well as environmental friendliness, MCRs are gaining much importance in both academia and industry [1–4].

Amlodipine [5, 8] is comparable—from a dynamic point of view—to and nifedipine [6] and is in late stage clinical evaluation for the once-daily treatment of angina and hypertension [7]. Recent structure-activity assessment of a new generation of 1,4-DHPs indicates that the presence of oxypropanolamine moiety on phenyl ring at position-4 of the DHP nucleus imparts the agents with β- or α-/β-adrenoceptor blocking activities [9, 10]. In addition, *Barnidipine* and *Furnidipine* are well tolerated, as are other 1,4-DHP calcium channel anatagonists and vasodilators; headache, flushing, and peripheral oedema account for most of the adverse events reported [11]. Oedema is less frequent than with amlodipine and nitredipine. Also its use is not associated with reflex tachychardia. 1,4-Dihydropyridine derivatives possess a variety of biological activities such as HIV protease inhibition [12, 13], MDR reversal [14–16], radioprotection [17], vasodilaton [18], antitumor, bronchodilator, and hepatoprotective activities [19]. A large number of publications is available on the synthesis of these biodynamic scaffolds [20].

Spirooxindole derivatives are also becoming key building blocks for drug discovery as these templates have been shown to exhibit a variety of interesting biological activities, such as antioneoplastic [21], antibiotic [22], cytostatic [23], monoamine transporter inhibiting [24], bradykinin antagonist [25], and cell cycle inhibiting activities [26]. Due to their structure, they interact with a wide range of receptors and this activity has resulted in significant interest in developing efficient methods to prepare spirocompounds. On the other hand, spiropiperidines have also received great attention because of their promising therapeutic applications [27–30]. The spiro(indoline-3,4'-piperidine) scaffold is a key structural feature in MK-0677, a potent peptidomimetic growth hormone secretagogue (GHS) [31], a serine-derived NK1 antagonist, and a potent and selective melanocortin subtype-4 (MC-4) receptor agonist [32]. It is also found in oxytocin, somatostatin, tachykinines, anaphylatoxin chemotactic receptor ligands, and is considered as a privileged structure for general G-protein coupled receptor (GPCR) ligands [33].

To the best of our knowledge, no work has been reported on the multicomponent synthesis of spiro-1,4-DHPs incorporating N-substituted piperidine or

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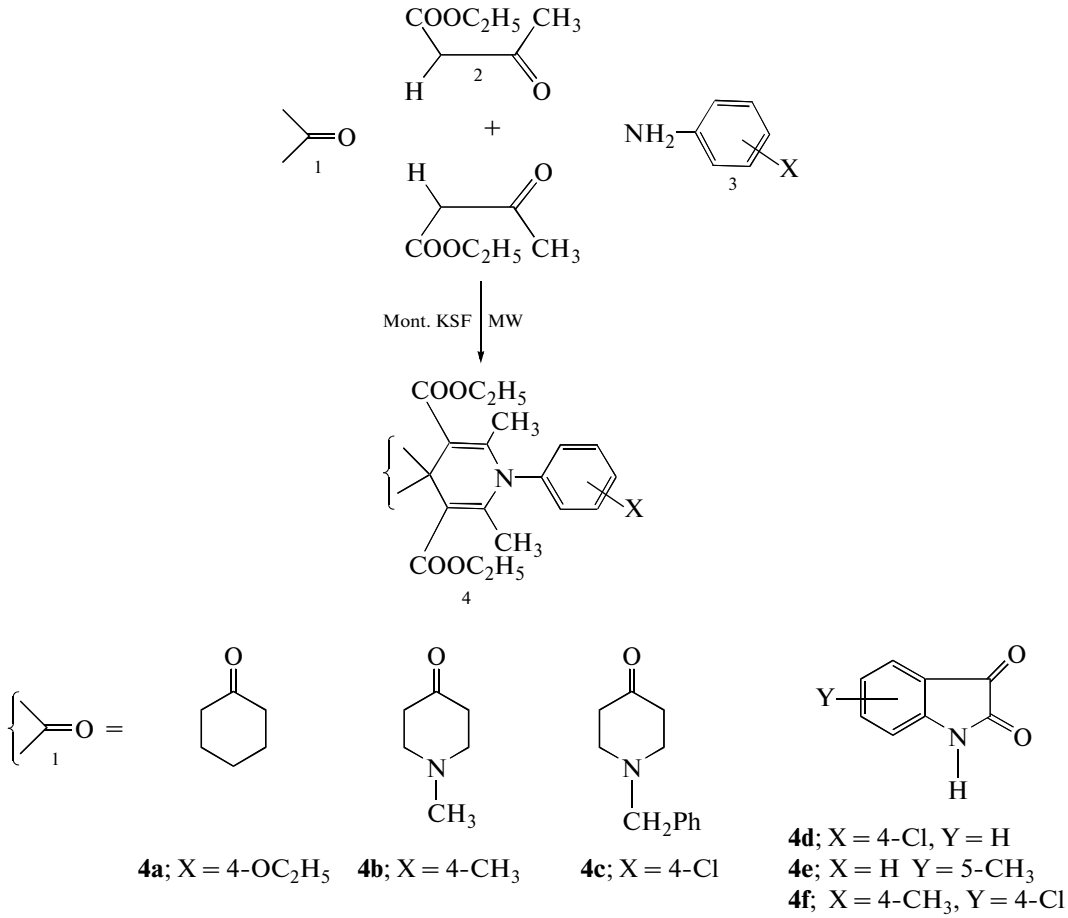
2-oxindoline motif in a single molecular framework. Hence, promoted by these observations and in continuation of our earlier interest on the green chemical synthesis of biodynamic spiro and annulated derivatives [34, 35] using non-traditional approach, an attempt has been made to synthesize various spiro-1,4-DHP derivatives with the assumption that the incorporation of more than one bioactive heterocyclic moiety into a single framework may result in the production of novel heterocycles with enhanced/altered bioactivity. Therefore, we herein report novel spiro derivatives combining heterocyclic moieties with 1,4-DHPs by the multicomponent reaction of cyclic carbonyl compounds (**1**), β-ketoester (**2**), and substituted anilines (**3**), as depicted in Scheme, using environmentally benign methodologies.



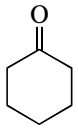
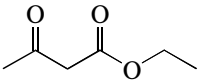
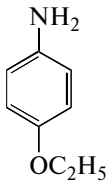
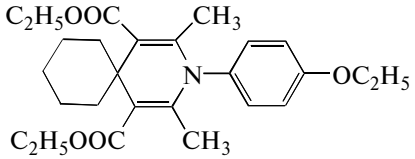
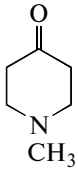
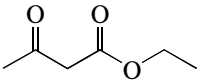
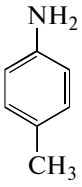
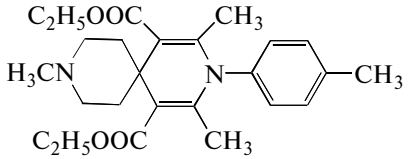
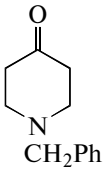
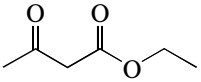
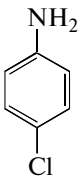
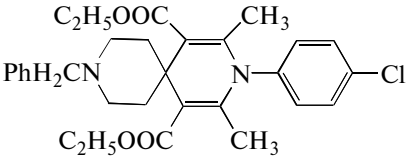
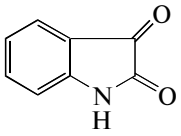
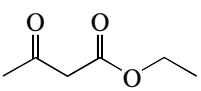
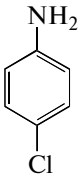
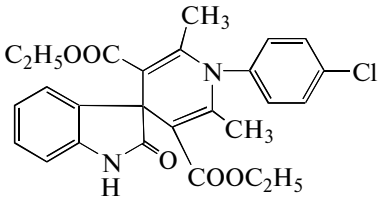
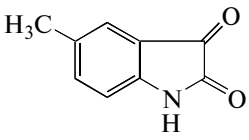
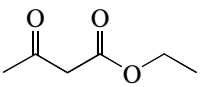
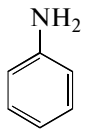
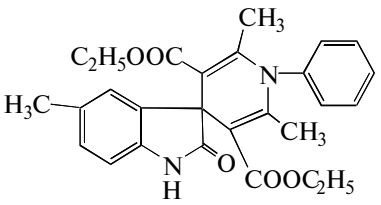
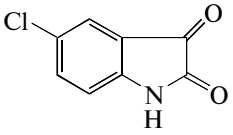
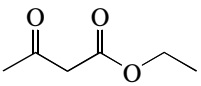
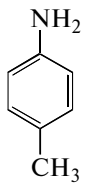
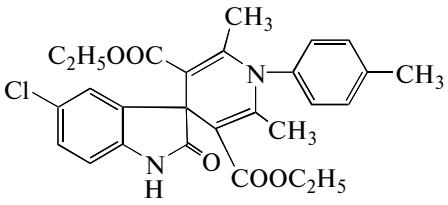
Scheme.

A series of novel N-substituted spiro-1,4-dihydropyridines (**4a**–**4f**) have been synthesized in one pot under microwave irradiation using solvent-free conditions (Table 1). The reaction was performed under different conditions to find out the best method giving product in higher yield with operational simplicity. In the present investigation, we study the synthesis of 3-(4-ethoxyphenyl)-2,4-dimethyl-3-azaspiro[5.5]undeca-1,4-diene-1,5-dicarboxylic acid diethyl ester (**4a**) via multicomponent reaction of cyclohexanone (**1**), β-ketoester (**2**), and *p*-phenatidine (**3**) varying the reaction parameters as shown in Table 2.

Results reported in Table 2 evidence that the multicomponent reaction of (**1**), (**2**), and (**3a**) occurred successfully in ethanol without using any catalyst both under microwave irradiation and conventionally. However, the yield of the product was low when the reaction was carried out conventionally in ethanol. The reaction was also performed under neat conditions without adding any solvent or support, which could be expected to be the most economical method. The reaction proceeded efficiently (~100% conversion, indicated by TLC) but the yield of isolated crystalline product decreased to 72% due to the tedious work-up procedure which requires trituration with petroleum ether followed by crystallization from methanol.

To further improve the procedure, reaction was also studied using different types of inorganic solid supports, e. g., montmorillonite KSF, neutral alumina,

Table 1. Novel spiro-1,4-DHP derivatives

Entry	Reactants			Product
	(1)	**(2)**	**(3)**	
(4a)				
(4b)				
(4c)				
(4d)				
(4e)				
(4f)				

and silica gel. The montmorillonite KSF was found to be the best solid support giving the maximum (90%) yield of the required product reasonable purity (TLC) with shortest time and easier work-up (Table 2). However, for spectral studies and elemental analyses, compounds **(4a–4f)** were recrystallized from methanol.

The three-component condensation of cyclic ketones **(1)**, β-ketoester **(2)**, and substituted anilines **(3)** gave corresponding spiro-1,4-dihydropyridine derivatives incorporating indoline/piperiding ring.

The structure assigned for the reaction product is established from analytical and spectral data. The IR

Table 2. Comparative results obtained in the synthesis of (**4a**) using classical method (Δ) and microwave assistance (MW).

Exp.	Medium	Mode of activation	Time, min	Temp.[a], °C	Yield[b], %
1	EtOH	MW	15	78	85
2	Neat	MW	5	135	72
3	Neutral alumina	MW	7	120	70
4	Acidic alumina	MW	8	122	78
5	Silica gel	MW	10	125	70
6	**Mont. KSF**	**MW**	**6**	**138**	**90**
7	EtOH + AcONa	Δ	600–840	Reflux	50

Note: a—Final temperature is measured at the end of MW irradiation by introducing a glass thermometer in the reaction mixture. b—Isolated yield.

Table 3. Antifungal activity of compounds (the newly synthesized spiro-1,4-DHPs derivatives (**4a**–**4f**)) against *Trichophyton rubrum*

Compound	Concentration of compounds, %	IZ of sample, mm	IZ of a standard, ketoconazole, mm	AI	IZ of a standard, clotrimazole, mm	AI
(**4a**)	100	68	60	1.1	36	1.8
(**4b**)	100	72	60	1.2	36	2.0
(**4c**)	100	82	60	1.3	36	2.2
(**4d**)	100	80	60	1.3	36	2.2
(**4e**)	100	79	60	1.3	36	2.1
(**4f**)	100	78	60	1.3	36	2.1

spectrum of spiro compounds displayed characteristic absorption band in the region of 1694–1724 cm^{-1} due to C = O vibrations. Aromatic and aliphatic C–H absorption bands were observed at 3060–3080 and 2960–2985 cm^{-1}, respectively. The aromatic skeletal vibrations of C–C appeared at 1600, 1580, and 1450 cm^{-1} and the broad and intense absorption, occurred at 1595–1610 cm^{-1} in all spectra, was assigned to C=C stretching. ^1H NMR spectra of compounds (**4a**–**4f**) exhibited one sharp singlet at 8.05 ppm due to NH proton along with a multiplet at 7.09–7.26 ppm for the aromatic protons. A triplet and a quartet appeared at 1.15–1.28 ppm and 4.03–4.10 ppm due to the presence of methyl and methylene protons of carbethoxy group, respectively. A singlet was found at 2.32 ppm due to the methyl protons. The presence and position of NH was confirmed with deuteration.

Further, the structure of spiro compounds was also supported by ^{13}C NMR and mass spectra. In the ^{13}C NMR spectrum of (**4a**) the quaternary C-6 spiro carbon appeared at 84.67 ppm. The aromatic and carbonyl carbon resonated at 126.73–159.98 and 170.45 ppm, respectively. Other signals were observed at 14.04–63.592 due to methyl and methylene carbon of carbethoxy group. The signal of olefinic carbon of tetrahydropyridine ring was observed at 114.71 ppm. The mass spectrum of (**4a**) showed molecular ion peak at m/z 441 $[M]^+$ (12.3%), along with base peak observed at m/z 269 (100%).

Antidermatophytic activity of the newly synthesized spiro-1,4-DHPs derivatives (**4a**–**4f**) was screened using disc diffusion and MIC (minimum inhibitory concentration) method on *T. rubrum* and *M. gypseum* (etiological agent of dermatophytoses). The results are presented in Tables 3 through 7 and Fig. 1 through 7; they demonstrate the important antifungal activity of the compounds alone and in combinations. The diameters of the inhibition zones obtained upon treatment with compounds and mixture of compounds at concentration of 100% was 68, 72, 82, 80, 79, and 78 mm against *T. rubrum* and 56, 69, 79, 78, 76, and 79 mm, against *M. gypseum*. Standard reference drugs, i. e. clotrimazole and ketoconazole, showed inhibition zones of 36 and 60 mm against *T. rubrum* and 41 and 26 mm, against *M. gypseum*, respectively. Inhibition zones of the mixture of compounds were found to be higher than those of single compounds and reference antibiotics. The results show that the compounds exhibited inhibitory effect against *T. rubrum* at concentration of 1.6 to 2 μg/mL. MIC of compounds against *M. gypseum* was found to be within the range of 0.04 to 1.2 μg/mL. Even after 4, 8, and 12 days interval, no growth was observed at that low concentrations and control taken without compounds showed 100% growth of *T. rubrum* and *M. gyp-*

Table 4. Antifungal activity of compounds (the newly synthesized spiro-1,4-DHPs derivatives (**4a**–**4f**)) against *Microsporum gypseum*

Compound	Concentration of compounds, %	IZ of sample, mm	IZ of a standard, ketoconazole, mm	AI	IZ of a standard, clotrimazole, mm	AI
(**4a**)	100	56	26	2.1	41	1.3
(**4b**)	100	69	26	2.6	41	1.6
(**4c**)	100	79	26	3.0	41	1.9
(**4d**)	100	78	26	3.0	41	1.9
(**4e**)	100	76	26	2.9	41	1.8
(**4f**)	100	79	26	3.0	41	1.9

Table 5. Antifungal activity of mixture of compounds (the newly synthesized spiro-1,4-DHPs derivatives, (**4a**–**4f**)) against *T. rubrum* and *M. gypseum*

Compound	Test strain	Concentration of compounds, %	IZ of sample, mm	IZ of a standard, ketoconazole, mm	AI	IZ of a standard, clotrimazole, mm	AI
Mixture of compounds	*T. rubrum*	100	82	26	3.1	41	2.0
Mixture of compounds	*M. gypseum*	100	79	26	3.0	41	1.9

Note: IZ, inhibition zone, in mm, including the diameter of disc, 6 mm; AI, activity index.

Table 6. MIC of compound (the newly synthesized spiro-1,4-DHPs derivatives, (**4a**–**4f**)) against *T. rubrum*

Concentration of compounds, μg/mL	Growth visually inspected at different concentrations of compounds	
1	+4	+4
1.2	+3	+3
1.4	+2	+2
1.6	0	0
1.8	0	0
2	0	0
Control without oil	1100% growth	100% growth

Table 7. MIC of the compounds (the newly synthesized spiro-1,4-DHPs derivatives, (**4a**–**4f**) against *M. gypseum*

Concentration of compounds, μg/mL	Growth visually inspected at different concentrations of compounds	
0.02	+1	+1
0.04	0	+2
0.06	0	0
0.08	0	0
1.0	0	0
1.2	0	0
Control without oil	100% growth	100% growth

seum. We conclude that both, mixture of compounds and single compounds can be used as antifungal agents against *T. rubrum* and *M. gypseum*, the causal organisms of dermatophytic infections.

CONCLUSION

Here, we describe an efficient, clean, and green methodology for montmorillonite KSF-mediated one-pot synthesis of spiro-1,4-dihydropyridines. The advantages—(i) no need for additional reagent/catalyst, (ii) non-inflammable and nontoxic reaction medium, (iii) high yields, (iv) virtually no waste generation, and (v) ease of product isolation/purification—fulfill the triple bottom-line philosophy of green chemistry and make the present methodology environmentally benign.

EXPERIMENTAL

Melting points were determined on a Toshniwal apparatus and are reported uncorrected. The purity of compounds was checked on thin layers of silica gel in

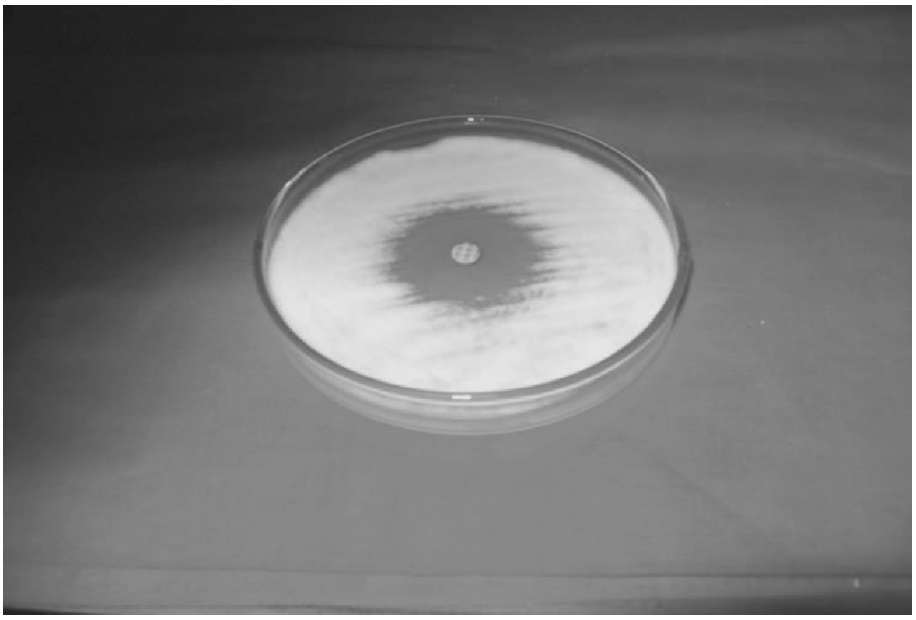


Fig. 1. Inhibition zone of clotrimazole against *Trichophyton rubrum*.

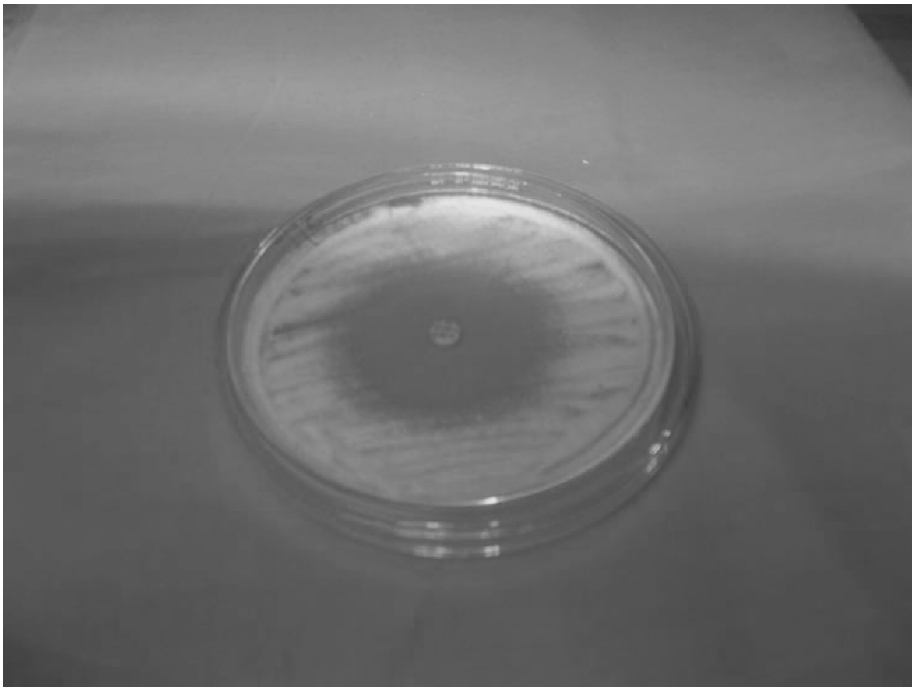


Fig. 2. Inhibition zone of ketoconazole against *M. gypseum*.

various non-aqueous solvent systems, e.g. benzene : ethylacetate, 9 : 1, benzene : dichloromethane, 8 : 2. IR spectra (KBr) were recorded on a Shimadzu FT IR-8400S spectrophotometer and ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX-300 equipment using $CDCl_3$ at 300.15 and 75.47 MHz respectively. TMS was used as an internal reference. Mass spectra of representative compounds were recorded on a Kratos 50 mass spectrometer at 70 eV. The microwave-assisted reactions were carried out in a modified multimode MW oven (Panasonic-NN-781JF) equipped with inverter technology

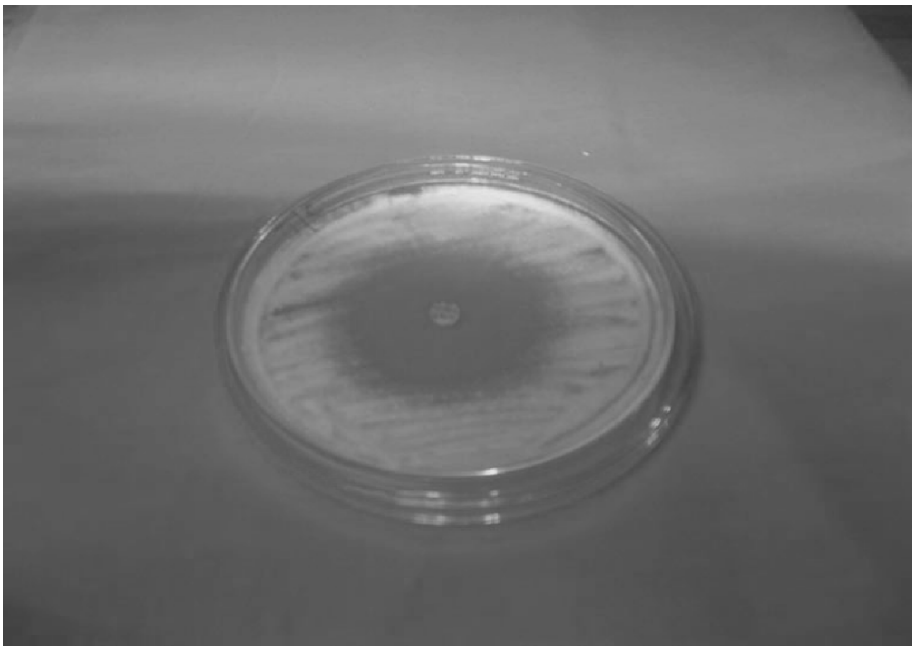


Fig. 3. Inhibition zone of ketoconazole against *Trichophyton rubrum*.

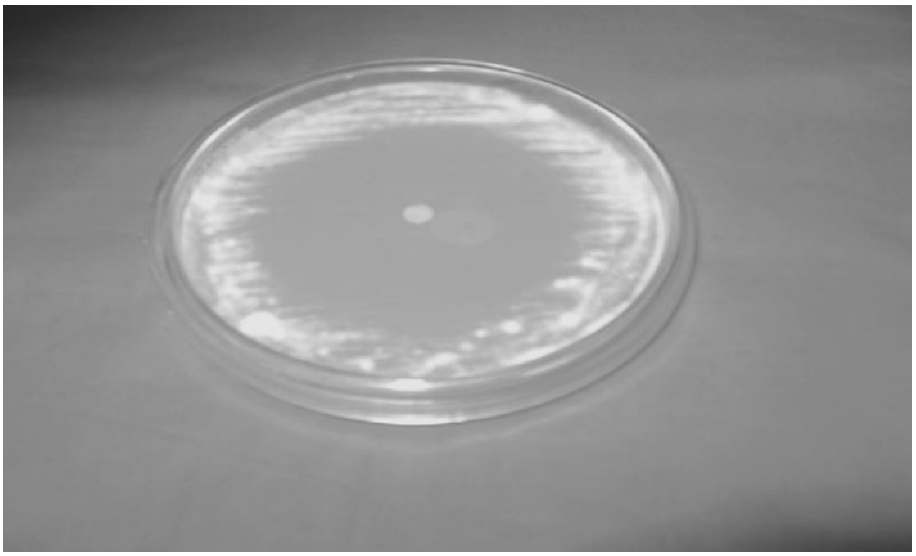


Fig. 4. Inhibition zone of the novel synthesized spiro-1, 4-DHPs derivative (**4c**) against *Trichophyton rubrum*.

(generating fixed frequency throughout the required time) operating at 1000 W generating 2450 MHz frequency.

The synthesis of spiro compound (**4a**) was carried out using different methods: (a) classical method, and (b) microwave-mediated synthesis.

(a) Classical Method

A mixture of 0.01 mol cyclohexanone (**1**), 0.02 mol ethyl acetoacetate (**2**), and 0.01 mol *p*-phenatidine (**3a**), containing 1–2 g of fused sodium acetate, was heated (without solvent) on steam bath for 10–12 h. After elimination of water, ethanol (25 mL) was added directly to the reaction mixture and refluxed for 15 h. Then the reaction mixture was poured into ice water, the separated solid mass was extracted with diethyl ether (50 mL), dried over magnesium sulfate, and then concentrated. The solid product was treated with methanol. The product was filtered and recrystallized from ethyl acetate.

(b) Microwave-Mediated Synthesis

(i) Using polar solvent. A mixture of 0.01 mol (**1**), 0.02 mol (**2**), and 0.01 mol (**3a**) was put into a borosil

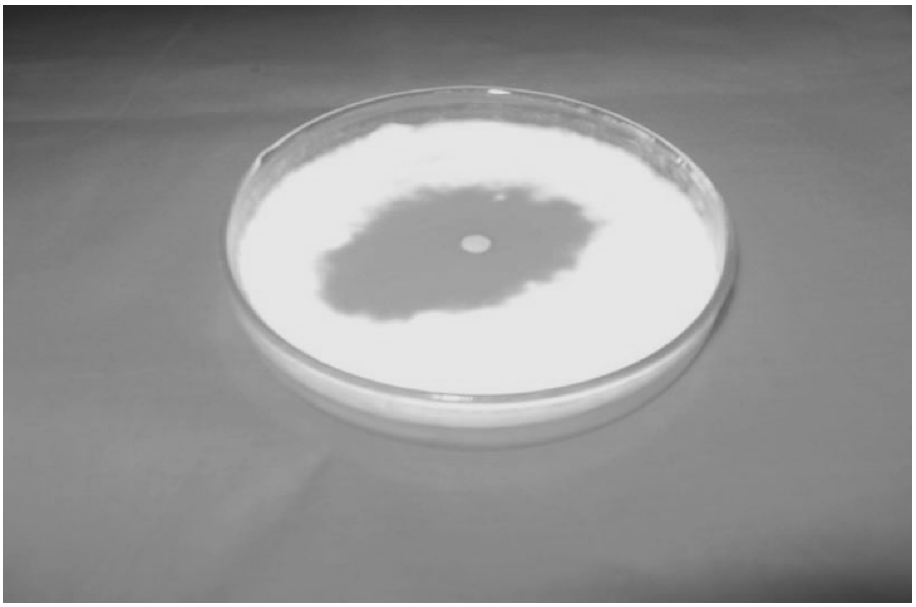


Fig. 5. Inhibition zone of the novel synthesized spiro-1,4-DHPs derivative (**4f**) against *M. gypseum*.

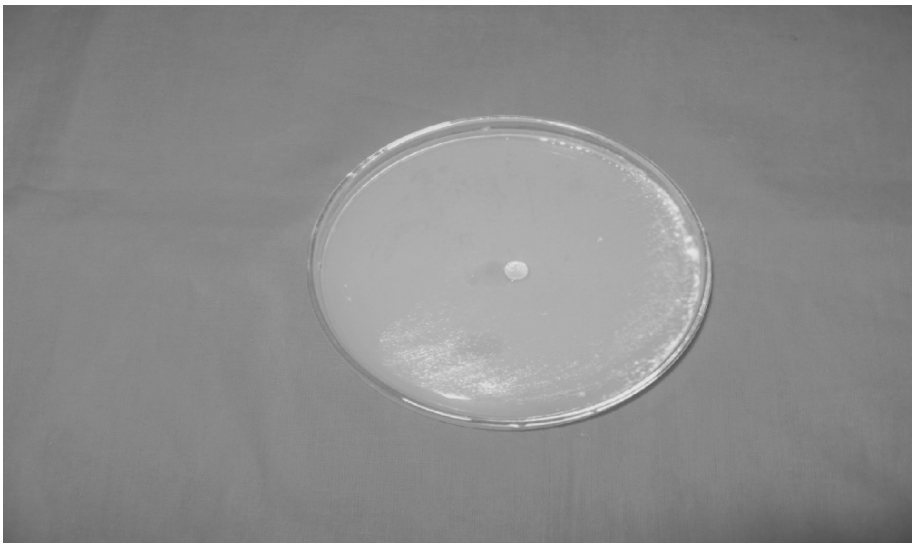


Fig. 6. Inhibition zone of the mixture of compounds: spiro-1,4-DHPs derivatives (**4a–4f**) against *T. rubrum*.

glass vessel containing 5 mL ethanol and irradiated inside a microwave oven at 340 W. After every 2 min, an interval of 1 min without irradiation was allowed, to avoid excessive evaporation of solvent, until the completion of the reaction (15 min, TLC). The reaction mixture was cooled and the product was filtered, dried, and recrystallized from methanol.

(ii) Neat reaction. A mixture of 0.01 mol (**1**), 0.02 mol (**2**), and 0.01 mol (**3a**) was irradiated under neat conditions at 640 W under microwave irradiation until the completion of the reaction (TLC). After trituration with pet-ether, 5 mL methanol was added to the dark brown-colored solid mass. After washing with petroleum ether, shiny crystals were isolated by filtration and found to be pure by TLC.

(iii) Using inorganic solid support. A mixture of 0.01 mol (**1**), 0.02 mol (**2**), and 0.01 mol (**3a**) was adsorbed on inorganic solid supports, such as montmorillonite KSF (4 g), silica gel (4 g), or neutral alumina (4 g), via methanolic solution, and swirled for a while followed by removal of solvent under gentle vacuum. The dry powder thus obtained was placed into a pyrex-glass vessel and irradiated in a microwave oven at power output of (640 W) for appropriate time

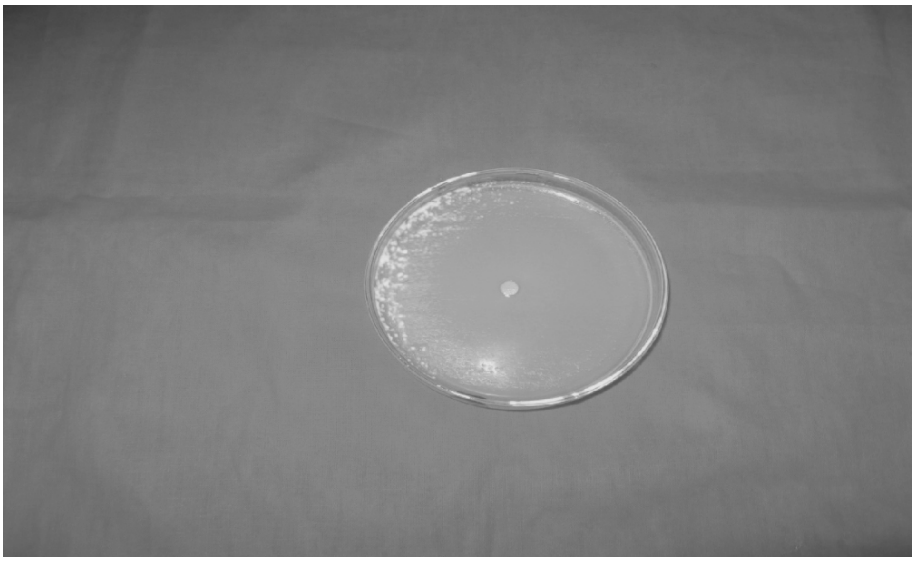


Fig. 7. Inhibition zone of the mixture of compounds: spiro-1,4-DHPs derivatives (**4a**–**4f**) against *M. gypseum*.

(TLC). The product was extracted with methanol and found to pure by TLC.

The compounds synthesized by various methods were identified by their mixed melting point and spectral studies. The results presented in Table 2 show that the montmorillonite KSF was found to be the best solid support for the synthesis of spiro compound (**4a**), hence all other compounds listed in Table 1 were also synthesized by microwave method using montmorillointe clay as inorganic solid support providing the required product in pure form (TLC) with no need for further purification. However, for spectral studies and elemental analyses, compounds (**4a**–**4f**) were further recrystallized from methanol.

(**4a**) Yield 92%; mp 48–50°C; reaction time, 7 min. Anal. Found: C, 70.50; H, 7.98; N, 3.013. Calcd. $C_{26}H_{35}NO_5$: C, 70.72; H, 7.99; N, 3.17. IR (KBr) ν_{max} 3080 (aromatic C–H), 2985 (aliphatic C–H), 1694 (C=O), 1595 (C=C). ^{1}H NMR ($CDCl_3$) (δ, ppm): 0.75–2.08 (m, 10H $(CH_2)_5$ and 9H, $(CH_3)_3$), 2.32 (s, 6H, $(CH_3)_2$), 3.91 (q, 2H, OCH), 4.03 (q, 4H, $O(CH_2)_2$), 6.90–7.12 (m, 4H, ArH).

(**4b**) Yield 89%; mp 138–140°C; reaction time, 5 min. Anal. Found: C, 70.69; H, 8.05; N, 6.60. Calcd. $C_{25}H_{34}N_2O_4$: C, 70.39; H, 8.03; N, 6.57. IR (KBr) ν_{max} 3086 (aromatic C–H), 2996 (aliphatic C–H), 1705 (C=O), 1585 (C=C). ^{1}H NMR ($CDCl_3$) (δ, ppm): 0.89–2.05 (m, 8H, $(CH_2)_4$ and $(-OC\underline{H}_2CH_3)_2$, 2.24 (s, 6H, $(CH_3)_2$), 2.31 (s, 3H, CH_3), 3.1 (s, 3H, N–CH_3), 4.01 (q, 4H, $(-OC\underline{H}_2CH_3)_2$), 6.78–7.04 (m, 4H, ArH).

(**4c**) Yield 87%; mp 220–221°C; reaction time, 6 min. Anal. Found: C, 70.02; H, 6.75; N, 5.33. Calcd. $C_{30}H_{35}ClN_2O_4$: C, 68.89; H, 6.74; N, 5.36. IR (KBr) ν_{max} 3078 (aromatic C–H), 2985 (aliphatic C–H), 1694 (C=O), 1595 (C=C). ^{1}H NMR ($CDCl_3$) (δ, ppm): 0.93–2.35 (m, 8H, $(CH_2)_4$, and 6H, $(-OCH_2C\underline{H}_3)_2$, 2.21 (s, 6H, $(CH_3)_2$), 3.5 (s, 2H, $C\underline{H}_2Ph$), 4.21 (q, 4H, $(-OC\underline{H}_2CH_3)_2$), 6.69–7.59 (m, 9H, ArH).

(**4d**) Yield 88%; mp 180–181°C; reaction time, 6 min. Anal. Found: C, 65.02; H, 5.22; N, 5.32. Calcd. $C_{26}H_{25}ClN_2$: C, 64.93; H, 5.24; N, 5.28. IR (KBr) ν_{max} 3298 (NH), 3018 (aromatic C–H), 2998 (aliphatic C–H), 1705 (NHC=O), 1685 (C=O), 1610 (C=C). ^{1}H NMR ($CDCl_3$) (δ, ppm): 1.22 (t, 6H, $(CH_2C\underline{H}_3)_2$), 2.32(s, 6H, $(CH_3)_2$), 4.08 (q, 4H, $(C\underline{H}_2CH_3)_2$), 7.09–7.26 (m, 8H, ArH), 8.05 (s, 1H, NH).

(**4e**) Yield 91%; mp 163–164°C; reaction time, 7 min. Anal. Found: C, 70.61; H, 6.12; N, 6.10. Calcd. $C_{27}H_{28}N_2O_5$: C, 70.42; H, 6.13; N, 6.08. IR (KBr) ν_{max} 3310 (NH), 3010 (aromatic C–H), 2994 (aliphatic C–H), 1700 (NHC=O), 1689 (C=O), 1627 (C=C); ^{1}H NMR ($CDCl_3$) (δ, ppm): 1.21 (s, 3H, CH_3), 1.28 (t, 6H, $(CH_2C\underline{H}_3)_2$), 2.23 (s, 6H, $(CH_3)_2$, 3.91 (q, 4H, $(C\underline{H}_2CH_3)_2$, 6.91–7.20 (m, 8H, ArH), 8.01 (s, 1H, NH).

(**4f**) Yield 94%; mp 140–144°C; reaction time, 7 min. Anal. Found: C, 65.63; H, 5.49; N, 5.70. Calcd. $C_{27}H_{27}ClN_2O_5$: C, 65.52; H, 5.50; N, 5.66. IR (KBr) ν_{max} 3310 (NH), 3288 (NH), 3012 (aromatic C–H), 2987 (aliphatic C–H), 1702 (NHC=O), 1691 (C=O), 1617 (C=C). ^{1}H NMR ($CDCl_3$) (δ, ppm): 0.91 (s, 3H, CH_3), 1.28 (t, 6H, $(CH_2C\underline{H}_3)_2$, 2.35 (s, 6H, $(CH_3)_2$, 3.89 (q, 4H, $(C\underline{H}_2CH_3)_2$, 6.86–7.32 (m, 7H, ArH), 8.01(s, 1H, NH).

Biological Experiment

Novel spiro compounds were screened for their antifungal activity against molds by disc diffusion method [36]. Standard size Whatman No.1 filter paper discs, 6.0 mm in diameter, sterilized by dry heat at 140°C in an oven for one hour were used to determine antifungal activity. SDA medium for disc diffusion test was prepared. After sterilization, it was poured into sterilized petri plates and allowed to solidify. In case of molds, spore suspension of each of the fungi was prepared from 8 to 10 days old cultures separately. The suspension was vortexed and 0.1 mL aliquots were spread over the respective agar medium plates. Sterilized filter paper discs were soaked in neat, undiluted (100%) concentration of novel synthesized compounds and mixtures of compounds. Using an ethanol dipped and flamed forceps, saturated discs of 100% concentration of pure compounds and mixtures of compounds were aseptically placed over Sabouraud dextrose agar plates seeded with the respective test microorganism. The antibiotic discs of gentamycin (30 μg/disc), streptomycin (10 μg/disc), clotrimazole and ketoconazole (10 μg/disc) concentration were also aseptically placed over the seeded Sabouraud dextrose agar plates as a standard drugs for comparison of antifungal activity to different compounds. The plates were incubated for molds at 30°C for 48–72 hours. The diameter of the inhibition zones was measured in mm. Three replicates were kept in each case and average values were calculated. After the screening of different compounds (**4a**–**4f**) modified microdilution method was followed to determine MIC [37].

Activity Index = Inhibition zone of sample/Inhibition zone of standard.

Media used for MIC was semisolid agar media (Brain Heart Infusion Agar).

ACKNOWLEDGMENTS

The authors are thankful to Chairperson and Chief Mentor, Suresh Gyan Vihar University for providing technical and laboratory facilities.

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