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# Quinolines: Microwave-assisted Synthesis and their Antifungal, Anticancer and Radical Scavenger Properties

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#### **Graphical Abstract**



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#### ABSTRACT

An efficient method for the synthesis of quinolines using microwave irradiation was developed providing 28 quinolines with good yields. The reaction procedures are environmentally friendly, convenient, mild and of easy work-up. Quinolines were evaluated for their antifungal, anticancer and antioxidant properties and exhibited high activities in all tests performed.

#### 1. Introduction

The quinolic core is repeatedly found in various natural and synthetic products, including several important clinically used drugs and pharmaceutical candidates.<sup>1</sup> Some 2,4-disubstituted

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quinolines have been reported as anti-tuberculosis<sup>2</sup> and anthelmintics<sup>3</sup> agents and also effective for the treatment of leishmaniasis, a protozoan disease widespread in tropical areas of South America.<sup>4</sup> Functionalized aminoquinolines<sup>5</sup> and 2-heteroarylquinolines<sup>6</sup> have been reported as potential antifungal agents. Synthesized amido and ureido quinoline derivatives substituted with 2-*N*-methylamido-pyridin-4-yloxy group effectively inhibited the proliferation of renal carcinoma, ovarian and lung cancer cells with potency higher than that of sorafenib, a reference drug.<sup>7</sup> Diarylureas containing a 4-aryl-8-amino(acetamido)quinoline moiety are notable for their activities against melanoma cells as they exhibited concentration necessary to inhibit cell proliferation by 50% (IC<sub>50</sub>) in the order of nanomolar.<sup>8</sup> Some quinoline hybrids with chalcogenide<sup>9</sup>, ferrocene<sup>10</sup> or coumarins<sup>11</sup> were shown to be good antioxidants.

Due to the broad range of applications in medicinal, industrial and bioorganic processes and in the synthetic organic chemistry area, there has been an increasing interest in developing efficient methods for the synthesis of quinolines. A variety of synthetic methods have been developed for obtaining 2,4-disubstituted quinolines<sup>12</sup>, in which the three-component Povarov reaction<sup>13</sup> has been proved to be the most empowering and versatile approach. Indeed, the three-component Povarov reaction is as convenient as the classical synthesis for this purpose in terms of efficacy, speed and atom economy. Then, the Povarov reaction, based on an inverse electron-demand aza-Diels-Alder reaction between an aniline, aldehyde and an electron-rich alkene to afford a tetrahydroquinoline, quinoline or julolidine, has long attracted the interest of synthetic chemists. This three-component reaction is reported to be catalyzed by BF<sub>3</sub>/Et<sub>2</sub>O<sup>14</sup>, lanthanide(III) triflates<sup>15</sup>, molecular iodine<sup>16</sup>, SnCl<sub>2</sub><sup>17</sup>, TMSCl<sup>18</sup>, TEMPO salt (T<sup>+</sup>BF<sub>4</sub>)<sup>19</sup>, fluorinated alcohols<sup>20</sup>, AG®50W-X2 resin<sup>21</sup>, cellulose sulphuric acid<sup>22</sup> and triphenylmethylium cation<sup>23</sup>. Our group and others have used calix[n]arenes as organocatalysts in multicomponent reactions (MRCs)<sup>24</sup>, including a Povarov reaction designed for the synthesis of julolidines.<sup>25</sup> Recently, we have reported the direct synthesis of 2,4-disubstituted quinolines using the Povarov reaction among anilines, benzaldehyde and styrene followed by *in situ* oxidation assisted by the catalyst *p*-sulfonic acid calix[4]arene (CX4SO<sub>3</sub>H).<sup>13a</sup> Despite the good yields obtained from a single step reaction (38-71%), the formation of 2,4disubstituted quinolines occurred after long periods of reaction incubation (12 h) in the presence of acetonitrile as the solvent.

Microwave-assisted organic synthesis has become an interesting tool in organic synthesis for obtaining desired products from environmentally-friendly reactions based on the use of catalysts and free of solvents. Atom-economical processes have collectively contributed to promote the school of modern synthesis.<sup>26</sup> To the best of our knowledge, the use of microwave irradiation to promote the Povarov reaction is still underexplored.<sup>27</sup> Additionally, the design of an efficient and green approach based on the application of a reusable catalyst for the synthesis of 2,4-disubstituted quinolines is of great interest.

In this work, we investigated a simple and efficient protocol for the microwave-assisted synthesis of a series of 2,4disubstituted quinolines under solvent-free conditions, with potential to inhibit the growth of fungal of clinical interest and proliferation of cancer cells and to scavenge free radicals.

#### 2. Results and Discussion

#### Synthesis of quinolines

A series of solvents as well as solvent-free conditions (Table 1) were tested to determine the best condition for obtaining quinolones in good yields. For this purpose, a model reaction constituted of 4-bromoaniline (1a), benzaldehyde (2a) and styrene (3) was chosen and carried out in the presence of *p*-sulfonic acid calix[4]arene (CX4SO<sub>3</sub>H) as catalyst (1 mol%) under microwave irradiation. The use of ethanol or water as protic solvents yielded the quinoline Q1 in only 8% and 5%, respectively, in which the imine I1 was isolated as the major product (up to 27% yield) and the amine A1 in up to 15% yield, respectively (Table 1, entries 1 and 2).

#### Table 1 Optimization of the reaction conditions<sup>a</sup>



Entry	Solvent	CX4SO <sub>3</sub> H	Q1 yield	I1 yield	A1 yield
Entry	Solvent	(mol%)	$(\%)^d$	$(\%)^d$	$(\%)^d$
	ethanol <sup>b</sup>	1.0	8	20	15
2	water <sup>b</sup>	1.0	5	27	11
3	acetonitrile <sup>b</sup>	1.0	15	13	12
4	$DCM^b$	1.0	15	47	-
5	none <sup>c</sup>	1.0	64	20	-
6	none <sup>c</sup>	0.5	43	15	-
7	none <sup>c</sup>	2.0	65	16	-
8	none <sup>c</sup>	5.0	58	17	-
9	none <sup>c</sup>	0.0	0.0	30	20

<sup>*a*</sup>Reagents and conditions: 4-bromoaniline **1a** (1.0 mmol), benzaldehyde **2a** (1.2 mmol) and styrene **3** (1.5 mmol) for 20 min under power of 50 W in a sealed tube. <sup>b</sup>Under reflux. <sup>c</sup>Reactions at 200 °C. <sup>*d*</sup>Isolated yield. DCM, dichloromethane.

The Q1 was also obtained in low yield when aprotic solvents, such as acetonitrile or dichloromethane were used, conditions that yielded I1 in almost 50% and A1 in 12% in reactions performed with the former solvent (Table 1, entries 3 and 4). Under solvent-free conditions, the quinoline Q1 was isolated in 64% yield along with imine I1 in 20% yield. Once determined that a solvent-free reaction was the best condition to obtain Q1, we further investigated the minimum amount of catalyst (CX4SO<sub>3</sub>H) required to achieve the maximum reaction yield. The Q1 yield decreased to 43% when the amount of CX4SO<sub>3</sub>H was diminished from 1.0 mol% to 0.5 mol% (Table 1, entry 6). No further yield increment was achieved when the concentration of CX4SO<sub>3</sub>H was increased from 1 to 2 mol% (Table 1, entry 7). The use of CX4SO<sub>3</sub>H at 5 mol% provided Q1 at 58% (Table 1, entry 8). No detectable amount of Q1 was observed in reaction devoid of catalyst, a condition that yielded the imine I1 at 30% (Table 1, entry 9). Overall, the use of solvent-free conditions and 1 mol% CX4SO<sub>3</sub>H as catalyst provided the highest Q1 yield (64%) from Povarov reaction in which 20% of the substrate aniline was converted to some byproducts (Table 1, entry 5).

By using the optimal reaction conditions, it was found that the incubation of reactions at 150, 200 or 250 °C for 5 or 10 min yielded undetectable amounts of **Q1** (Table 2, entries 1-6). Similar results was achieved when reaction were incubated at 150 °C for 15 min (Table 2, entry 7). The **Q1** yield of up to 35% was verified from incubation of reactions at 200 to 250 °C

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for 15 min (Table 2, entries 8 and 9). Longer periods of reaction incubation (20 to 25 min) at the maximum temperature of 200 °C provided the best yields for Q1 (Table 2, entries 11 and 14).

Notably, the highest yield was obtained for the model reaction carried out in an open vessel in the presence of 1 mol% CX4SO<sub>3</sub>H, under acetonitrile for 12 h and oil bath (80 °C) (Table 2, entry 16).<sup>13a</sup>

°C) (Tab	ble 2, entry 16). <sup>11</sup>	3a			
Once quinolin scope of shown acetonith Q28 for	determined the es from the Por f this reaction b in Fig. 1. Tl ile under 12 h r comparison pur	e best mild varov reacti by varying t he classica eflux <sup>13a</sup> was pose (Fig. 1	l conditions to on, we next e he aldehydes o l thermal mo adopted for ol ).	b synthesize xamined the employed as ethod using btaining Q1-	CP1
Table 2	Effect of tem	perature an	d incubation	time on the	6
producti	ion of quinoline	28 <sup>a</sup>			
	HO3S HO3S	SO3H SO3H	$\bigcirc$		
NH <sub>2</sub> Cr Br 1a 24	HO + C MW, solvent-free, time	HO H HO H, 1 mol%) me, temperature			
Entry	Temperature	Time	Yield $\mathbf{O1}(0)^{b}$	Yield	
1	150	(1111)	Q1(%)	$\frac{\Pi(\%)}{20}$	
2	200	5		20	
3	250	5		29	
4	150	10	-	33	
5	200	10	-	45	
6	250	10	-	44	
7	150	15	-	36	
8	200	15	30	30	
9	250	15	35	20	<b>Fig. 1</b> Synthesis of 2.4 disubstituted animalines apples
10	150	20	24	47	catalyst Vields in green were obtained by microware (N
11	200	20	64	20	blue were obtained by conventional beating (CH) MW
12	250	20	38	34	solvent free CH: 80 °C acetonitrile 12 h <b>O14 O28</b> sy
13	150	25	26	45	solvent-free. C.H. 80°C, accionitine, 12 n. Q14-Q28 sy $t = 1.2014^{-13a}$
14	200	25	65	16	et al, 2014.
15	250	25	42	26	The mechanism that drives Povarov reaction is st
$16^c$	80	720	65	13	in which the most widely accepted one invol

<sup>a</sup>Reagents and conditions: 4-bromoaniline/benzaldehyde/styrene (molar ratio of 1.0;1.2;1.5) in sealed tube, <sup>b</sup>Isolated yield. <sup>c</sup>Conventional heating (80 °C) in the presence of acetonitrile.

With respect to the reactions using 4-bromoaniline, similar yields were observed for obtaining Q1-Q13 using either the conventional heating or microwave approaches regardless of the aldehyde employed. However, the use of aromatic aldehydes bearing -OH or -NO2 provided the corresponding quinoline in poor yields (< 20%). Additionally, cyclohexanecarboxaldehyde furnished the corresponding quinoline (Q7) in yields equal to or lower than 35% (Fig. 1).

The use of microwave irradiation and different aniline substrates led to the formation of quinolines in better yields as attested by the results obtained for the synthesis of **O14-O28** (Fig. 1). Indeed, the microwave irradiation was determined to be in the range from 11% to 19% more efficient than conventional heating for obtaining the quinolines Q16, Q18, Q21 and Q25 while the yield of Q26 using the former approach was twice as higher as that of the latter ones (Fig. 1). Reactions under microwave irradiation furnished the desired quinolines more rapidly when compared with those under thermal heating and solvent-free conditions.



Fig. 1 Synthesis of 2,4-disubstituted quinolines employing CX4SO<sub>3</sub>H as catalyst. Yields in green were obtained by microware (MW) and yields in blue were obtained by conventional heating (CH). MW: 200 °C, 20 min, solvent-free. CH: 80 °C, acetonitrile, 12 h. Q14-Q28 synthesis by Simões et al, 2014.13

The mechanism that drives Povarov reaction is still under debate, in which the most widely accepted one involves a stepwise sequence via ionic intermediates generated by a Mannich-like reaction, followed by an intramolecular electrophilic aromatic substitution.<sup>28</sup> Based on this, a mechanism for the formation of the functionalized quinolines herein described is hypothesized in Scheme 1. The first step consists of the reaction between aniline and the activated aldehyde to provide the imine ion via Mannich reaction. The reaction with imine ion, after styrene addition, forms the corresponding carbocation. Then, an intramolecular electrophilic aromatic substitution takes place to furnish the tetrahydroquinoline, which is further oxidized to the corresponding quinoline.



Scheme 1 - Mechanistic proposal for the synthesis of quinolines from Povarov reaction.

The ion exchange capacity of the catalyst was determined by acid-base titration<sup>29</sup> using 5 mM NaOH (aq). A total of 8.6

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mmol of  $H^+$  g<sup>-1</sup> were necessary to completely titrate CX4SO<sub>3</sub>H. This result is consistent as the catalyst bears eight strong acid sites (four –SO<sub>3</sub>H and four –OH groups).

The potential of CX4SO<sub>3</sub>H to be reused in the reaction to obtain quinolines was also investigated. The CX4SO<sub>3</sub>H could be successfully used up to five successive reactions without significant loss of catalytic power (Fig. 2). The catalyst was easily recovered from the reaction by liquid-liquid extraction with water and dichloromethane followed by evaporation of aqueous phase under reduced pressure. As a result, the reaction model constituted of 4-bromoaniline (1a), benzaldehyde (2) and styrene (3) could still afford quinoline Q1 in good yield even after five cycles (Fig. 2).



**Fig. 2** Recovery and potential of CX4SO<sub>3</sub>H to be reused as catalyst in Povarov reaction for the synthesis of 2,4-disubstituted quinolines. MW, microwave heating and CH, conventional heating.

#### Antifungal activities

Because of the need to develop new structures with antifungal activity<sup>30</sup> and considering that other quinolinebearing structures have shown promising antifungal activities,<sup>31</sup> the 2,4-disubstituted quinolines synthesized were tested against some fungi of clinical interest such as *Candida albicans* and *Cryptococcus neoformans*. The *C. albicans* is the etiological agent of many opportunistic infections in immunocompromised hosts<sup>32</sup> while *C. neoformans* causes cryptococcal meningitis, one of the most important HIV-related fatal opportunistic mycosis that has killed to date over 650,000 immunocompromised patients worldwide.<sup>30c</sup> Although the incidence of HIV tends to decrease in countries with highly active anti-retroviral therapy, fungal infections is influenced by several factors in which the design of compounds with anti-cryptococcal activity is very desirable.<sup>33</sup>

To better explore the results of MIC values for inhibition of fungal growth, the quinolines synthesized were divided into three categories: i) group I, comprised of non-substituted or 2-position-substituted benzene ring quinolines; ii) II represented by **Q7** that bears a 2-cyclohexyl ring and iii) represented by **Q13** that bears a 2-furyl substituent at 2-position of the quinoline ring (Table 3). Group I was further subdivided in I.1, in which it was grouped quinolines (**Q1-Q6** and **Q8-Q12**) that possess a –Br substituent at 6-position (R<sub>2</sub>) of quinoline skeleton and a variety of substitution at 2-position (R<sub>4</sub>, R<sub>5</sub> and/or R<sub>6</sub>) of the benzene ring. The subgroup I.2, on the other hand, includes compounds (**Q14-Q28**) that present a phenyl group at 2-position of the quinoline ring with a variety of substituents at positions 5 (R<sub>1</sub>), 6 (R<sub>2</sub>)and 7 (R<sub>3</sub>) of this same core.

The MIC<sub>80</sub> and MIC<sub>50</sub> values shown in Fig. 3 were determined from experiments of fungal growth inhibition in the presence of quinolines or amphotericin B (Amph B; reference drug) in the range from 3.9 to 250  $\mu$ g mL<sup>-1</sup> (Table S1; Supplementary material). The MIC<sub>80</sub> and MIC<sub>50</sub> values correspond to the minimum concentration of a compound-test necessary to inhibit the fungus growth by 80% and 50%, respectively. Such values are well accepted in the literature as representative of the in vitro activity of target compounds.<sup>34</sup>

Quinolines **Q1-Q3**, **Q6-Q8**, **Q13-Q28** inhibited *C. albicans* or/and *C. neoformans* growth at different extents (Table 3). The *C. neoformans*, however, was shown to be more sensitive to the quinolines tested (MIC<sub>50</sub> values lower than 250  $\mu$ g mL<sup>-1</sup>) than did *C. albicans*.

**Table 3** Effect of quinolines on the growth of CandidaalbicansATCC 10231andCryptococcusneoformansATCC 32264

R1					$\square$						
R <sub>2</sub>			Br				Br				
R3	N	$\uparrow$	1 14			N	$\frown$	N N			
	(1)	$\checkmark$	Rr			(II) <sup>(</sup>	$\checkmark$		(III)		
		R <sub>6</sub>	5								
Compounds	Cotogory	D	р	D	D	р	D	C. alb	icans	C. neof	ormans
Compounds	Category	<b>K</b> <sub>1</sub>	<b>R</b> <sub>2</sub>	Кз	К4	K5	К6	MIC <sub>80</sub>	MIC <sub>50</sub>	MIC <sub>80</sub>	MIC <sub>50</sub>
Q1		Н	Br	Н	Н	Н	Н	>250.0	125.0	>250.0	31.2
Q2		Н	Br	Н	Н	Cl	Н	>250.0	250.0	>250.0	250.0
Q3		Н	Br	Н	Н	F	Н	>250.0	>250.0	>250.0	15.6
Q4		Н	Br	Н	Н	OMe	Н	>250.0	>250.0	>250.0	>250.0
Q5		Н	Br	Н	Н	Me	Н	>250.0	>250.0	>250.0	15.6
Q6	I.1	Η	Br	Н	OCH	3OCH3	OCH3	250.0	125.0	250.0	62.5
Q8		Н	Br	Н	Н	OH	Н	125.0	62.5	250.0	62.5
Q9		Н	Br	Η	Н	Н	OH	>250.0	>250.0	>250.0	>250.0
Q10		Н	Br	Н	Н	$NO_2$	Н	>250.0	>250.0	>250.0	>250.0
Q11		Н	Br	Н	Н	Н	$NO_2$	>250.0	>250.0	>250.0	>250.0
Q12		Н	Br	Н	Н	CN	Н	>250.0	>250.0	>250.0	>250.0
Q14		Η	Н	OH	Η	Н	Η	>250.0	>250.0	>250.0	15.6
Q15		Н	OH	Н	Н	Η	Η	>250.0	250.0	250.0	125.0
Q16		Н	F	Η	Н	Н	Η	>250.0	250.0	>250.0	>250.0
Q17		Н	$NO_2$	Η	Н	Н	Η	>250.0	>250.0	250.0	125.0
Q18		Н	$CF_3$	Н	Н	Н	Н	>250.0	>250.0	250.0	250.0
Q19		Н	Cl	Н	Н	Н	Н	>250.0	>250.0	>250.0	15.6
Q20		Н	$SCH_3$	Н	Н	Н	Н	>250.0	250.0	31.2	15.6
Q21	I.2	Н	CN	Н	Н	Н	Н	>250.0	250.0	250.0	125.0
Q22		H	$CO_2H$	Н	Н	Н	Н	>250.0	250.0	>250.0	250.0
Q23		H	C(CH <sub>3</sub> ) <sub>3</sub>	H	Н	H	H	>250.0	>250.0	15.6	3.9
Q24		H	H	NO <sub>2</sub>	Н	Н	H	>250.0	250.0	>250.0	125.0
Q25		H	Cl	CF <sub>3</sub>	H	H	H	>250.0	250.0	>250.0	250.0
Q26		OCH	3 UCH3	UCH:	; H	Н	H	125.0	125.0	15.6	/.8
Q2/			OCH <sub>2</sub> O	H	н	п	н	>250.0	>250.0	250.0	31.2
	п	п	UCH <sub>3</sub>	п	н	п	п	>250.0	250.0	230.0	250.0
012	11 111	-	-	-	-	-	-	>230.0	230.0	>250.0	230.0
Ammh P	- 111	-	-	-	-	-	-	125.0	02.5	230.0	02.5
Ampn B								0.12	0.00	0.25	0.12

 $\rm MIC_{80}$  and  $\rm MIC_{50}$  values correspond to the concentration of compound tested necessary to inhibit fungus growth by 80% and 50%, respectively. Values were calculated using the logistic concentration-response curve obtained by four-parameter SigmaPlot version 11 (Systat Software, Inc., San Jose, California, USA). Amph B, amphotericin B (reference drug).

To determine the influence of the substituent at 2-position of the quinoline ring, we evaluated the percentage of growth inhibition towards *C. albicans* or *C. neoformans* as a function of the concentration of **Q1**, **Q7** and **Q13**. The quinoline bearing a furan ring at 2-position (**Q13**) effectively inhibited the growth of both fungi tested acting in a concentration-dependent mode on *C. neoformans* and exhibiting maximum inhibitory effect (78%) on *C. albicans* when used at 125 µg mL<sup>-1</sup> (Fig. 3). A maximum growth inhibition of around 55% was achieved from *C. albicans* incubation with 62.5 µg mL<sup>-1</sup> **Q1** (bearing a 2-phenyl group) while a much higher concentration (4-fold) of this same quinoline was required to have similar effect on *C. neoformans*. The **Q7**, which bears a cyclohexyl at 2-position, was the less effective against *C. albicans* and *C. neoformans* (Fig. 3). Overall, the presence of a heteroaromatic ring at 2-

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position of quinolic ring boosts the antifungal activity of quinolines.



Fig. 2 Comparative antifungal activities of Q1, Q7 and Q13 against *C. albicans* ATCC 10231 (a) and *C. neoformans* ATCC 32264 (b).

When considering all the other quinolines synthesized, almost all compounds belonging to the subgroup I.1 were determined to be poor antifungal against C. albicans. On the other hand, some of them exhibited relatively low MIC<sub>50</sub> values activity against C. neoformans. Indeed, the most active compounds (MIC<sub>50</sub> values lower than 16  $\mu$ g mL<sup>-1</sup>) possess –F (Q3) or -CH<sub>3</sub> (Q5) as R<sub>5</sub> group (Table 3). Compound Q6, which presents a -p-OCH<sub>3</sub> as R<sub>5</sub> group and two other  $-OCH_3$ groups as  $R_4$  and  $R_6$ , exhibited moderate activity (MIC<sub>50</sub> lower than 65.0  $\mu$ g mL<sup>-1</sup>). Notably, the presence of -Cl, -OCH<sub>3</sub>, -NO<sub>2</sub>, and -CN as R<sub>5</sub> group and -NO<sub>2</sub> as R<sub>6</sub> group negatively affected the activity of quinolines as attested by the results of MIC values obtained for Q2, Q4, Q10, Q11 and Q12 (Table 3). On the other hand, compounds belonging to subgroup I.2 that bear  $-NO_2$  or  $-CF_3$  groups, either at 6- (R<sub>2</sub>; Q17) or 7position (R<sub>3</sub>; **Q24**) presented similar effects on *C. neoformans* (Table 3). Quinolines presenting -Cl, -SCH<sub>3</sub>, -C(CH<sub>3</sub>)<sub>3</sub>, -OCH<sub>2</sub>O or -OCH<sub>3</sub> at 6-position (**O19**, **O20**, **O23**, **O26**, **O27** and Q28) were very active against C. neoformans. Compound Q26 was also identified as the only effective against C. albicans (Table 3). The presence of -OH at 7-position (Q14) enhances 8-fold the antifungal activity against C. neoformans when compared with a non-substituted quinoline at similar position (Q15).

Overall, *C. neoformans* was more susceptible than *C. albicans* to 2,4-disubstituted quinolines and their derivatives. The **Q13** was the most active among the 2-substituted compounds (bearing –2-phenyl, 2-cyclohexyl or 2-furan) containing –Br at 6-position. Then, this opens an opportunity for the synthesis of a new series of potential antifungal quinolines bearing a furan ring. Within 6-brominated compounds (I.1 category), the most active were those solely substituted at the 2-phenyl group (**Q3** with  $R_5 = F$  and **Q5** with  $R_5 = CH_3$ ). Likewise, the 3',4',5'-OCH<sub>3</sub> derivative (**Q6**) was more active than the corresponding monosubstituted one (**Q4**).

#### Antiproliferative assays

Cell proliferation was determined using the MTT assay with absorbance measurements at 540 nm. All synthesized quinolines (Q1-Q28) were evaluated *in vitro* against the following cancer cell lines: NCI-H226 (lung), TOV-21G (ovary) and Hep-2c (Hela contaminant). Compounds were also tested against a non-cancerous human cell line (CCD 19-Lu; lung fibroblast) to determine the selective index (SI). Data (Fig. 4) were submitted to Analysis of variance (ANOVA) one factor post-*hoc* analysis using the Least Significant Difference (LSD) method, which showed statistically difference only between the control and concentrations of 1,000 and 1,500  $\mu g$  mL  $^{-1}.$ 

The concentrations of quinolines that caused cell growth inhibition by 50% (IC<sub>50</sub> values) and the selectivity indexes are summarized in Table 4. The selectivity index compares the cytotoxicity of a compound against tumor cells and non-tumor cells. The calculation provides the ratio of IC<sub>50</sub> value obtained for the non-tumor cell line to the IC<sub>50</sub> value obtained for the tumor cell line. Regarding the analysis of selectivity ratios, there is no consensus in the literature on the threshold values for tumor cell lines. However, it was found that values greater than 5 are good indicators of selectivity.<sup>35</sup>

The IC<sub>50</sub> values indicated that Tov21G cells were more resistant and that NCI-H226 cells were more susceptible to all compounds tested (Fig. 5). According to the TP53 database of the International Agency of cancer (http://p53.iarc.fr/CellLines.aspx), the NCI-H226 cell line is *TP53* mutated while the TOV21G is wild-type. This mutation could explain the differences of cell survival observed for the tumor lines studied when incubated with quinolines, as *TP53* gene encodes for a tumour suppressor protein that is responsible for regulating the expression of target genes related to DNA repair.



**Fig. 4** Tumor and non-tumor cell survival after treatment with quinolines. The y = 0 indicates the 50% survival limit, in which compounds furnishing values higher than zero show cytostatic effect while those providing values lower than zero are considered cytotoxic. Differences between groups were analyzed using a non-parametric test (Kruskal Wallis; P < 0.05).

**Table 4** Concentration of quinolines required to inhibit the proliferation of tumor cells by 50% ( $IC_{50}$ ) and respective selective index (SI)

		IC <sub>50</sub>	(µM)	SI (µM)			
0.1.11	TOV-	CCD19-	NCI-	Hep-	TOV-	NCI-	Hep-
Quinolines	21G	Lu	H220	2C	210	H226	20
Q1	530.7	668.7	43.4	128.1	1	15	5
Q2	ND	528.1	1.58	269.0	ND	356	1
Q3	570.4	ND	ND	5.0	ND	ND	ND
Q5	ND	ND	ND	175.0	ND	ND	ND
Q6	492.6	104.5	61	ND	0	1	ND
Q7	ND	489.5	91	ND	ND	5	ND
Q8	ND	104.2	8	104.8	ND	13	0
Q11	ND	ND	8.5	ND	ND	ND	ND
Q12	ND	ND	ND	98.8	ND	ND	ND
Q13	876.8	631.1	30	101.8	0	21	6
Q14	ND	ND	ND	ND	ND	ND	ND
Q24	ND	ND	558.9	ND	ND	ND	ND
Q27	ND	354.1	40	951.1	ND	8	0
0.28	110.5	924.9	57	495 5	8	16	1

Nd: not determined. The  $IC_{50}$  value was calculated using the non-linear regression obtained by OriginPro 7.0.



**Fig. 5** Concentration of quinolines required to inhibit the proliferation of some tumor (Tov21G, Hep2 and NCI-H226) and non-tumor (CCD19-Lu) cells by 50% (IC<sub>50</sub>). Asterisks indicate significant difference by ANOVA post hoc LSD test (P < 0.05).

An examination of the association between survival and IC50 values highlighted the compounds that had the most promising antitumour activities (Fig. 6). The normal cell line CCD19-Lu proved to be most susceptible to cytotoxicity with quinolines Q7, Q8, Q27 and Q28, while all were cytotoxic for NCI-H226 cells (susceptible), and none were cytotoxic for TOV21G (resistant). The NCI-H226 line was selectively susceptible for the majority of quinolines and presented the best selective indices. The NCI-H226 line is an epithelial lung cancer derived from a metastatic site. It is one of the more aggressive cancer types with a high incidence and prevalence. These compounds proved at priori to be one good source of antitumoural molecules for lung cancer. The TOV21G cells (resistant) were selectively susceptible only to the Q1, Q3 and Q28 quinolines. The Tov 21G cell line is from an epithelial ovary cancer that is classified as aggressive and very deadly among women. Thus, these compounds proved to be good agents for improving effectiveness in this cancer type. Interestingly, Q1 and Q3 were not cytotoxic against NCI-H226, suggesting that there are different biological pathways between them. The Hep2c line was susceptible to the majority of compounds as was NCI-H226, but not in a selective manner. The results for these cells were meaningless once it was shown to be a HeLa-contaminated cell line.



**Fig. 6** Association between  $IC_{50}$  values and viability of human tumor cells (Tov21G, Hep2 and NCI-H226) and non-tumor cells (CCD19-Lu). The dotted lines show the 50% survival limit, in which values are under these lines are indicative of compound cytotoxicity. Compounds whose data are under the dotted line are considered the most promising antitumor agents.

The quinoline with lower  $IC_{50}$  values was the one containing a 4-methoxyphenyl group, which indicates that methoxyl group at 4-position improved the antiproliferative activity against lung cancer. The most effective substituents on tumor cells (except for Hep2c cancer cell line) were 4-fluorophenyl, 4-nitrophenyl, 3-nitrophenyl and cyclohexane (Fig. 7). The presence of activating groups on the aromatic ring provided promising anticancer activity.



**Fig. 7** Values of  $IC_{50}$  for quinolines as a function of substituents. The asterisk shows the substituents that present lower  $IC_{50}$  values, the majority are for lung cancer. The differences among treatments were analyzed by a non- parametric test (Kruskal Wallis; *P* <0.05).

#### Scavenging of reactive nitrogen species (RNS)

The ability of the synthesized quinolines to scavenge reactive species with unpaired electrons on nitrogen atoms (RNS) was investigated using the 2,2-diphenyl-1-

picrylhydrazyl (DPPH) method.36 An initial screening was performed to select potential RNS scavengers. Compounds were tested at 160 µM against 100 µM DPPH using the plant natural product resveratrol (Resv) as a positive control. Under our experimental conditions, quinolines Q14, Q15, Q21 and Q26 were determined to be the best DPPH scavengers because they were able to scavenge 70% to 85% of the free radicals. These results are comparable to those obtained for Resv, a known free radical scavenger (Fig. 8). Quinolines Q1, Q2, Q5, Q7, Q13, Q17, Q18 and Q28 were also found to be promising DPPH-capturing compounds; they scavenged free radicals by 45% to 55% (Fig. 8). The other quinolines, except for Q11 that was almost inactive, sequestered DPPH by approximately 35-40% (Fig. 8). Overall, no significant changes were observed in the DPPH scavenging ability for compounds with structural alterations in both the quinoline core and the two phenyl rings. However, some structure-activity relationships can be addressed. For instance, the presence of a hydroxyl group at C6 (compound Q15) or C7 (compound Q14) on the quinoline moiety was determined to be essential for scavenging of DPPH. In contrast, a hydroxyl group on the phenyl ring bound to the C2 of the quinoline core is not mandatory for RNS scavenging, as Q1 (which is devoid of the corresponding –OH) is as effective as compounds Q8 and Q9. It is noteworthy that the presence of an m-NO<sub>2</sub> group on the phenyl ring bound to the C2 of the quinoline core compromises the ability of quinoline Q11 to scavenge DPP radicals. Contrary to Q11, Q10, which bears a p-NO<sub>2</sub> group instead, is able to scavenge DPPH radicals by approximately 40% (Fig. 8).



Fig. 8 Potential of quinolines as scavengers of reactive nitrogen species. The reaction medium consisted of the compound tested (160  $\mu$ M) and DPPH radicals (100  $\mu$ M). Resveratrol (Resv) was used as a positive control. Standard deviations (SD) were lower than 28.0%. Data are from three independent experiments, each performed in triplicate.

The concentration of the compound tested necessary to scavenge a free radical by 50% was determined varying the concentration of quinolines (SC<sub>50</sub>; Table 5). Compounds **Q21** and **Q26** were the most efficient DPPH scavengers, as they exhibited SC<sub>50</sub> values lower than 20  $\mu$ M. The **Q13** was also able to scavenge 50% of the DPPH present in the reaction medium when applied at 82.7  $\mu$ M (Table 5). The order of increasing potency for the other quinolines is **Q17** > **Q7** > **Q28** > **Q2** > **Q5** > **Q18** > **Q6**.

**Table 5** Concentrations of promising quinolines that are necessary to scavenge DPPH or  $O_2^-$  radicals by 50% (SC<sub>50</sub> in  $\mu$ M).

Compounda	SC <sub>50</sub> (μM)				
Compounds	DPPH	$\mathbf{O}_2$			
Q1	>250.0	>250.0			
Q2	180.3	>250.0			
Q5	197.1	104.3			
Q6	428.1	>250.0			
Q7	118.5	>250.0			
Q9	>250.0	121.4			
Q13	82.7	>250.0			
Q15	>250.0	>250.0			
Q17	104.5	>250.0			
Q18	260.6	>250.0			
Q21	19.5	220.2			
Q23	>250.0	>250.0			
Q26	18.9	>250.0			
Q28	168.5	>250.0			
Resveratrol (Resv)	8.2	255.0			

#### Scavenging of reactive oxygen species (ROS)

A screening was also performed with the synthesized quinolines to evaluate the ability of such compounds to capture the reactive oxygen species (ROS) superoxide anion  $(O_2)$ . Again, quinolines were used at 160 µM in a reaction medium in which  $O_2^-$  was artificially generated. Quinolines Q5, Q8, **09**, **015**, **017**, **020**, **021**, **023**, **026** and **027** were much more efficient in the scavenging of ROS than Resv, the positive control (Fig. 9). The efficiency of quinolines Q3, Q4, Q7, Q10, Q12 and Q22 was comparable to that of Resv (Fig. 9). In contrast to the observations with DPPH scavenging, considerable variations in the efficiency of compounds were observed toward  $O_2^-$ . The presence of a *p*-toluyl substituent at C2 boosted the activity of compound Q5 compared to the corresponding quinoline that bears a phenyl substituent at this same position (compound Q1). The positive effect observed for Q5 (Fig. 9) is likely due to the presence of benzylic hydrogens, which may favor the formation of benzyl radicals, known to be very stable. Electron-withdrawing groups (-NO<sub>2</sub> and -CN; Q17 and Q21, respectively) at C6 on the quinoline moiety, except for a 3-fluoromethyl group ( $-CF_3$ ; Q18), increased the activity of quinolines towards  $O_2^-$  (Fig. 9). In contrast, the presence of electron-withdrawing groups at C7 (-CO<sub>2</sub>H and –NO<sub>2</sub>; Q22 and Q24, respectively) and/or C6 (6-Cl and 7-CF<sub>3</sub> **Q25**) negatively affected the potential of quinolines to scavenge  $O_2$  species (Fig. 9). The presence of a hydroxyl group (-OH) at C6 (compounds Q14 and Q15) contributes to the  $O_2^-$  scavenging activity of quinolines. However, a *m*-OH group on the phenyl ring bound to the C2 of the quinoline core renders the compound twice as active as the one bearing a p-OH at the same position (Q9 and Q8, Fig. 9). The quinoline containing a thiomethyl group (-SCH<sub>3</sub>) at C7 (Q20) was 2-fold more active than the corresponding derivative bearing an - $OCH_3$  group (Q28) (Fig. 9). The presence of more than one –  $OCH_3$  group (compound Q26) or a methylenedioxy (compound Q27), however, boosted the activity of such quinolines toward  $O_2$ . Notably, quinoline Q23, which bears a tert-butyl substituent at C6, was able to scavenge 50% of the O<sub>2</sub> artificially generated in the medium. All quinolines bearing halogen atoms (Br, F and Cl; Q1, Q16 and Q19, respectively) and unsubstituted phenolic rings demonstrated a lower potential for scavenging ROS (< 10% scavenging) (Fig. 9).

The remaining quinolines were found to be poor  $O_2^-$  scavengers (Fig. 9). The most promising quinolines were used further to assess the concentration of compounds necessary to scavenge ROS by 50% (SC<sub>50</sub>). Indeed, quinolines **Q5** and **Q9** were twice as potent as Resv towards  $O_2^-$  (Table 5).



Fig. 9 Effect of quinolines on reactive oxygen species. The reaction medium included artificially-generated superoxide anion (O2<sup>-</sup>) and compounds tested at 160  $\mu$ M. Resveratrol (Resv) was used as a positive control. Standard deviations (SD) were lower than 17.0%. Data are from three independent experiments, each done in triplicate.

#### Conclusions

A new and efficient method using microwave irradiation and *p*-sulfonic acid calix[4]arene as catalyst was developed for the synthesis of 28 2,4-disubstituted quinolines. Some advantages of the approach described herein include the use of solventand metal-catalyst-free conditions, short reaction times, recycling catalyst besides the catalyst tolerance towards a wide range of functional groups. Quinolines excelled, as they have good to excellent free radical scavenging activity being notable as good antifungal and antiproliferative candidates. The 6-Br-2,4-disubstituted quinolines, in particular, are attractive for use as templates for the development of new antifungal and antitumor agents.

#### 3. Experimental Section

#### 3.1. General methods and materials

All starting materials were obtained from commercially available sources with high-grade purity and were used without further purification. The *p*-sulfonic acid calix[4]arene (CX4SO<sub>3</sub>H) was prepared according to known procedures.<sup>37</sup> Reactions did not require anhydrous conditions. The melting points (uncorrected) of synthesized quinolines were determined using a MQAPF-301 Microquímica micromelting device. Infrared spectra were obtained on a FT-IR Varian 660 Fourier Transform Infrared spectrometer. The <sup>1</sup>H and <sup>13</sup>C-NMR spectra were obtained on a Varian Mercury spectrometry at 300 MHz for <sup>1</sup>H and 75 MHz for <sup>13</sup>C, in CDCl<sub>3</sub>. Coupling constants (J) are reported in Hertz (Hz). High resolution mass spectra (HRMS) were obtained on a Shimadzu LC-IT-TOF Prominence system.

# 3.2. General procedure for the synthesis of 2,4-disubstuted quinolines

Microwave (MW) approach: A mixture of an aniline (1 mmol), aldehyde (1.2 mmol), styrene (1.5 mmol) and p-

sulfonic acid calix[4]arene (1 mol%) was stirred at 200 °C for 20 min.

Conventional heating (CH): A mixture of an aniline (1 mmol), aldehyde (1.2 mmol), styrene (1.5 mmol), p-sulfonic acid calix[4]arene and acetonitrile (1 mol%) was stirred at 80 °C for 12 h.

Treatments (MW and CH): The reaction was quenched by addition of water (10 mL) and the product was extracted with dichloromethane (3 x 10 mL). The solvent was removed under reduced pressure in a rotary evaporator. The obtained solid was obtained was purified by silica gel column chromatography (hexane/dichloromethane/acetone, 8:2.8:0.1) to afford the quinolines **Q1-Q28** in high purity (Fig. 1). All quinolines were characterized by <sup>1</sup>H and <sup>13</sup>C NMR, IR, HRMS (ESI) and melting points.

#### 3.3. Antifungal Activity

#### Microorganisms and media

For the evaluation of antifungal activity, standardized strains of *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264 from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C and then maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid). Strains were sub-cultured every 15 days to prevent pleomorphic transformations. Inocula were obtained according to reported procedures<sup>38</sup> and adjusted to 1-5 x 10<sup>3</sup> colony forming units (CFU)/mL.

#### 3.4. Fungal growth inhibition

Yeasts broth microdilutions (technique M27-A3 of CLSI) were performed in 96-well microtiter plates. For the assay, compound-test wells (CTWs) were prepared with stock solutions of each compound in DMSO (concentration  $\leq 1\%$ ) and diluted with RPMI-1640 to final concentrations in the range from 3.9 to 250.0 µg mL<sup>-1</sup>. An inoculum suspension (100  $\mu$ L) was added to each well (final volume in the well = 200 µL). A growth control well (GCW) (containing medium, inoculum and the same amount of DMSO used in a CTW) and a sterility control well (SCW) (sample, medium and sterile water) were included for each fungus tested. Microtiter trays were incubated in a moist, dark chamber at 30 °C for 48 h for both yeasts. Microtiter plates were read in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Amphotericin B was used as a positive control. Tests were performed in triplicate. Reduction of growth for each compound concentration was calculated as it follows: % inhibition = 100 - (OD 405 CTW - OD 405 SCW)/(OD 405 GCW – OD 405 SCW). The means  $\pm$  standard deviations (SD) were used for plotting dose-response curves representing % inhibition versus concentration of each compound using SigmaPlot 11.0 software.

#### MIC<sub>80</sub> and MIC<sub>50</sub> determinations

Two endpoints were defined from the experiments described above. Minimum Inhibitory Concentrations 80 ( $MIC_{80}$ ) and 50 ( $MIC_{50}$ ) were defined as the minimum concentration that inhibits 80% or 50 % of the fungal growth, respectively.

#### 3.5. Antiproliferative assay

Human tumor cell lines CCD-19Lu (ATCC<sup>®</sup> CCL-210<sup>TM</sup>), WI-26 VA4 (ATCC<sup>®</sup> CCL-95.1<sup>TM</sup>), HEp-2C (ATCC<sup>®</sup> CCL-23<sup>TM</sup>) and NCI-H226 (ATCC<sup>®</sup> CLR-5826<sup>TM</sup>) were cultured in MEN-NEA, EMEM and RPMI media, respectively (Sigma-Aldrich Co. LLC.) supplemented with 10% foetal bovine serum and 1% L-glutamine in a 37 °C humid atmosphere enriched with 5% CO<sub>2</sub>. Cells were culture on 96 well plates (1 x 10<sup>5</sup> cells/well) in the presence or absence of synthesized quinolines at concentration in the range from 1.0 to 1,000 µg mL<sup>-1</sup>. After 24 h, the samples were sensitized in a MTT assay, and their absorbances (at 540 nm) measured 3 h later using a Molecular Devices Spectramax M5E. Values, corresponding the cell viability, were used to calculate the IC<sub>50</sub> values for statistical analysis purposes.

#### 3.6. Scavenging of reactive nitrogen species

The ability of quinolines **Q1-Q28** to scavenge a 2,2diphenyl-1-picrylhydrazyl (DPPH) radical, a reactive nitrogen species (RNS), was determined according to Gülcin<sup>39</sup>, with modifications. The screening of potential candidates was performed by incubating each compound-test (200  $\mu$ g mL<sup>-1</sup>) in an ethanolic medium containing 100  $\mu$ M DPPH. The systems were maintained under stirring and absence of light for 30 min and the absorbance was measured at 517 nm. Those compounds with potential scavenging activities were then tested in the range from 0 to 160  $\mu$ g mL<sup>-1</sup> to determine the concentration necessary to scavenge DPPH radicals by 50% (IC<sub>50</sub>). The results presented are from three independent experiments, each performed in triplicate.

#### 3.7. Scavenging of reactive oxygen species

The capacity of quinolines to scavenge superoxide anions  $(O_2^-)$  was evaluated in 50 mM phosphate buffer (pH 7.8) containing 13 mM L-methionine, 75  $\mu$ M nitroblue tetrazolium, 100  $\mu$ M EDTA, 2  $\mu$ M riboflavin and compound-test at 0-200  $\mu$ g mL<sup>-1</sup>. Reaction mixtures were incubated for 10 min at 25 °C in the presence of fluorescent light to induce  $O_2^-$  formation. Controls consisted of reaction mixtures maintained at 25 °C for 10 min in the absence of light. The percentage of  $O_2^-$  scavenged by each compound-test was determined at 575 nm. The results presented are from three independent experiments, each performed in triplicate.

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