Design, Synthesis, and *In Vitro* Antileishmanial and Antitumor Activities of New Tetrahydroquinolines

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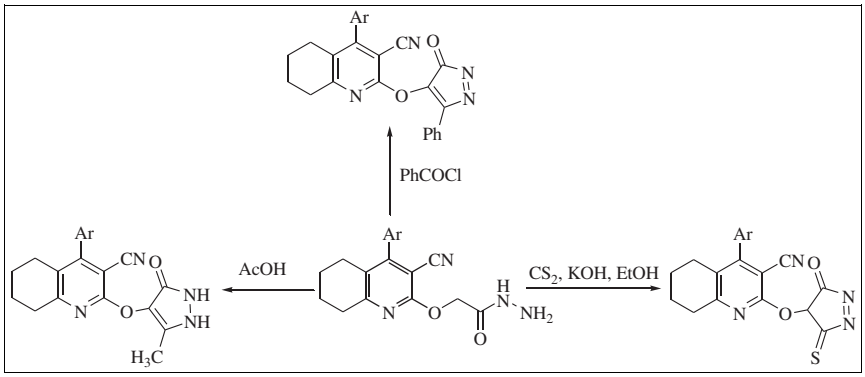


A novel series of tetrahydroquinolines containing acetohydrazide, oxopyrazole, oxothioxodihydropyrazole, and thioxotriazole have been synthesized. Antileishmanial, antitumor, and cytotoxicity activities of synthesized compounds were evaluated *in vitro*. Antileishmanial activity of the most synthesized compounds showed tremendous activity towards *Leishmania major*. Most of the test compounds exhibited significant level of tumor inhibition. The tetrahydropyrano[2,3-*b*]quinolin-2-one **6** and 4-oxo-4*H*-pyrazol-3-yloxytetrahydroquinoline-3-carbonitrile derivatives **18** showed 100% tumor inhibition comparable with standard drug vincristine (100% tumor inhibition). Tetrahydroquinolines under investigation showed cytotoxicity with LD_{50} values in the range 0.56–3.01 μg/mL compared with standard drug MS-222 with LD_{50} value of 4.30 μg/mL. The presence of a pyrazole ring markedly improved the activity profiles of tetrahydroquinoline. All newly synthesized compounds were characterized by IR, ^{1}H NMR, and MS.

INTRODUCTION

Quinoline is a heterocyclic system of remarkable importance to mankind. Several quinolones that have been isolated from natural resources or synthetically prepared are significant for the sake with respect to medicinal chemistry and biomedical use. Compounds containing quinoline moiety are most widely used as antimalarials [1–3], antibacterials [4], antifungals [5], anticancer [6], and antileismanial agents [7]. Moreover, quinoline derivatives are utilized in the design and construction of fungicides, virucides, biocides, alkaloids, rubber chemicals, and flavoring agents [5]. Because of their importance as substructures in a broad range of natural and designed products, significant efforts continue to be directed into the development of new quinoline-based structures.

Tetrahydroquinolines, as quinoline derivatives, represent an important structural subunit of natural products, and many tetrahydroquinoline derivatives show interesting biological and pharmaceutical activities [8,9], including anti-HIV [10,11], anticancer [12,13], antimalarial [14], cholesteryl ester transfer protein inhibitors [15], and antidiabetic [16]. Among various tetrahydroquinolines, biological profile of 1,2,3,4-tetrahydroquinolines and 5,6,7,8-tetrahydro-quinolines is extensively studied. Recently, the 5,6,7,8-tetrahydroquinolines have drawn considerable attention due to their interesting pharmacological applications as Rearranged during Transfection (RET) tyrosine kinase inhibitors [17], antifungal [18], anticancer [19], anti-HIV [20,21], and C5 a receptor antagonists agents [22].

Pyrazole derivatives are an important class of heterocyclic systems because of their highly appreciable biological and pharmacological activities. Several pyrazole compounds have been reported to be potential therapeutic agents for the treatment of inflammation [23,24], antitumor properties [25], and AIDS [26]. Hence, with a view of further estimate and rating the pharmacological profile of this class of heterocycles, the

authors were prompted to synthesize some new heterocycles by incorporating the tetrahydroquinolines and pyrazole moieties in a single molecular framework.

RESULTS AND DISCUSSION

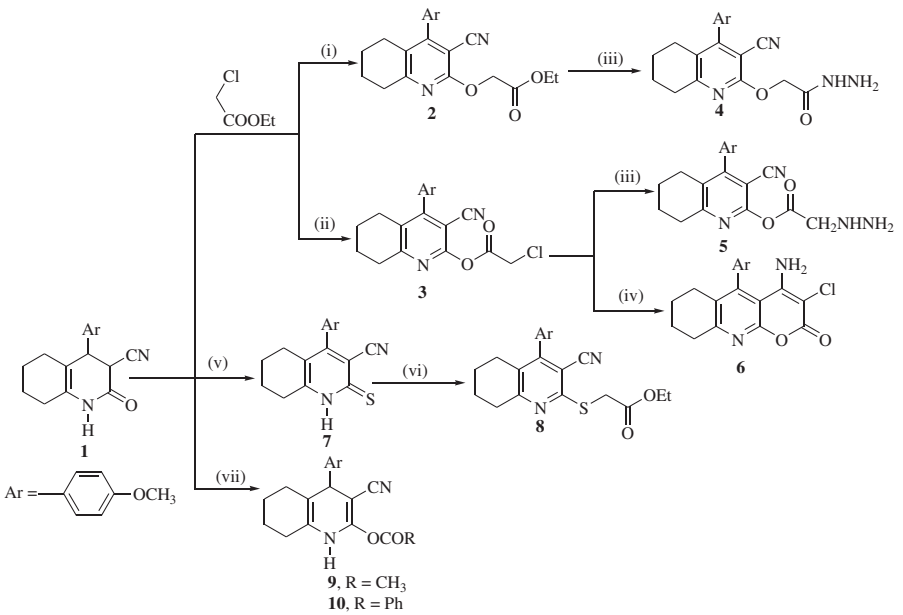
Chemistry. In continuation of our previous works [27–32], the present work aimed at utilization of the reactivity of 4-(4-methoxyphenyl)-2-oxo-1,2,3,4,5,6,7,8-octahydroquinoline-3-carbonitrile **1** towards different electrophilic and nucleophilic reagents to obtain new fused and nonfused heterocyclic systems and evaluate them for antileishmanial, antitumor, and antimicrobial activities.

The starting material 2-pyridone **1** was prepared *via* reaction of cyclohexanone, 4-methoxybenzaldehyde, ethyl cyanoacetate, and ammonium acetate in ethanol [33]. An example that illustrates the effect of changing reaction conditions on the nature of the reaction product in preparative organic chemistry was obtained when 2-pyridone **1** reacted with ethyl chloroacetate in dry acetone containing anhydrous potassium carbonate to afford *O*-alkylated product **2** (cf. Scheme 1). On the other hand, when 2-pyridone **1** was allowed to react with ethyl chloroacetate in dimethyl formamide (DMF) 3-cyano-4-(4-methoxyphenyl)-5,6,7,8-tetrahydroquinolin-2-yl 2-chloroacetate **3** was isolated. The structure of compound **3** was confirmed from spectroscopic data and qualitative and quantitative elemental analyses (Cl is present). ^{1}H NMR revealed the disappearance of NH signal and appearance of OH and CH signals of OH–C = CH–Cl at 12.28 and 3.54 ppm, respectively. The formation of products **2** and **3** could be explained *via* the polarity of the solvent used. In the presence of the less polar acetone, compound **1** reacts with the electrophilic haloester in more electrophilic center *via* SN^2 mechanism with elimination of HCl to give *O*-alkylated product **2**. In the presence of more polar DMF, 2-pyridone **1** reacts with the electrophilic haloester in less electrophilic carbonyl carbon of ester group by tetrahedral mechanism to lose off ethanol molecule to afford the 2-chloroacetate **3**.

The structure of *O*-alkylated product **2** and 2-chloroacetate **3** was chemically verified *via* hydrazinolysis with hydrazine hydrate in boiling ethanol to give the corresponding acetohydrazide derivative **4** and 2-hydrazinylacetate derivative **5**, respectively. The structural features of 2-chloroacetate **3** have been chemically proven *via* cyclization under the effect of alcoholic potassium hydroxide solution (10%) to afford tetrahydropyrano[2,3-b]quinolin-2-one **6**. The IR spectrum of **6** displayed absorption bands at 3420, 3350 cm^{-1} because of asymmetric and symmetric stretching of NH_2 group and $\nu_{C=O}$ lactone at 1738. ^{1}H NMR showed the appearance of NH_2 signal at 4.31 ppm that is exchangeable with D_2O.

Thiation of 2-pyridone **1** by phosphorous pentasulfide in dry toluene gave thione derivative **7**. The structure of thiated product **7** has been confirmed by spectral data. IR spectrum showed the disappearance of $\nu_{C=O}$ and

Scheme 1. Reactions of 2-pyridone 1 with different electrophiles.



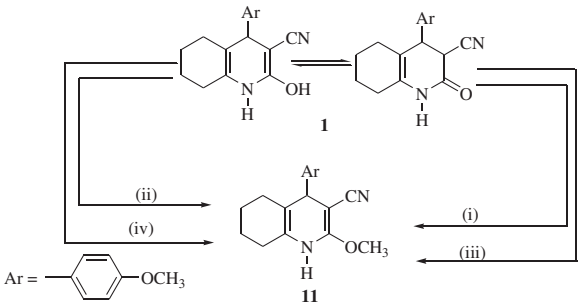
(i) K_2CO_3,acetone, reflux for 30h; (ii) K_2CO_3, DMF, reflux for 9h; (iii) $N_2H_4.H_2O$, EtOH reflux for 11h; (iv) 10% alc. KOH reflux for 6h; (v) P_2S_5 ,toluene reflux for 4h; (vi) $ClCH_2COOEt$, CH_3COONa, EtOH, reflux for 4 h; (vii) Ac_2O,and / or PhCOCl, pyridine, reflux for 5h

appearance of ν_{NH}, $\nu_{C\equiv N}$, and $\nu_{C=S}$ at 3164, 2221, and 1258 cm^{-1}, respectively. ^1H NMR showed SH signal at 5.57 ppm exchangeable with D_2O. Further, EI-fragmentation pattern showed the molecular ion peak at *m*/*z*: 296 as the base peak. Treatment of thione derivative **7** with ethyl chloroacetate in the presence of anhydrous sodium acetate furnished ethyl 2-(3-cyano-4-(4-methoxyphenyl)-3,4,5,6,7,8-hexahydroquinolin-2-ylthio) acetate **8**. Acetylation and/or benzoylation of 2-pyridone **1** with acetic anhydride and/or benzoyl chloride did not afford the expected 1-aroyl/acetyl-2-oxo-1,2-dihydropyridine derivative [34]. Surprisingly, the reaction afforded hexahydroquinoline-2-ylacetate **9** and hexahydroquinolin-2-yl benzoate **10** derivatives, respectively. The ester derivatives **9** and **10** were inferred from their IR spectra that displayed the carbonyl band expected for ester group, NH group, and disappearance of the carbonyl group of 2-pyridone. ^1H NMR showed NH and benzylic-H signals.

As a point of interest, 2**-**methoxy-4-(4-methoxyphenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carbonitrile **11** has been obtained *via* reaction of 2-pyridone **1** with three different reagents, namely, ethyl chloroacetate, chloroacetic acid, or/and chloroacetyl chloride under the same reaction conditions in absolute ethanol containing anhydrous sodium acetate. According to our speculation, *O*-methylation is preferred than *N*-methylation as *O*-methylated product is more thermodynamically stable than *N*-methylated product because of the conjugation of the formed C=C with the cyano functionality. In all cases, the reaction takes place *via* the nucleophilic attack of enolic hydroxyl group on the electronically deficient carbon of the methylene group of the reagent followed by hydrolysis to acid in case of ethyl chloroacetate and chloroacetyl chloride then decarboxylated to give *O*-methylated product **11**. The structure of **11** was chemically confirmed by comparison with an authentic sample (mp, mixed mp, and TLC) prepared from *O*-methylation of 2-pyridone **1** using methyl iodide in refluxing acetone and anhydrous K_2CO_3 (cf. Scheme 2).

Pyrazole derivatives have attracted continued interest because of their use as an important core structure in many drug substances, having a wide range of pharmacological applications [35–38]. The authors were promoted to utilize the amino functionality of acetohydrazide derivative **4** in the design and synthesis of new heterocyclic systems incorporating pyrazole nucleus. Thus, acetohydrazide derivative **4** has been allowed to react with benzoyl chloride and/or benzoic acid in the presence of phosphorous oxychloride to afford pyrazole **12** and dihydropyrazole **13** derivatives, respectively. On the other hand, acetohydrazide derivative **4** has been allowed to react with acetyl acetone, ethyl 2-cyano-3-ethoxyacrylate, and carbon disulfide, to afford pyrazole derivatives **14**–**16**, respectively.

Scheme 2. Synthesis of O-methylated product **11**.



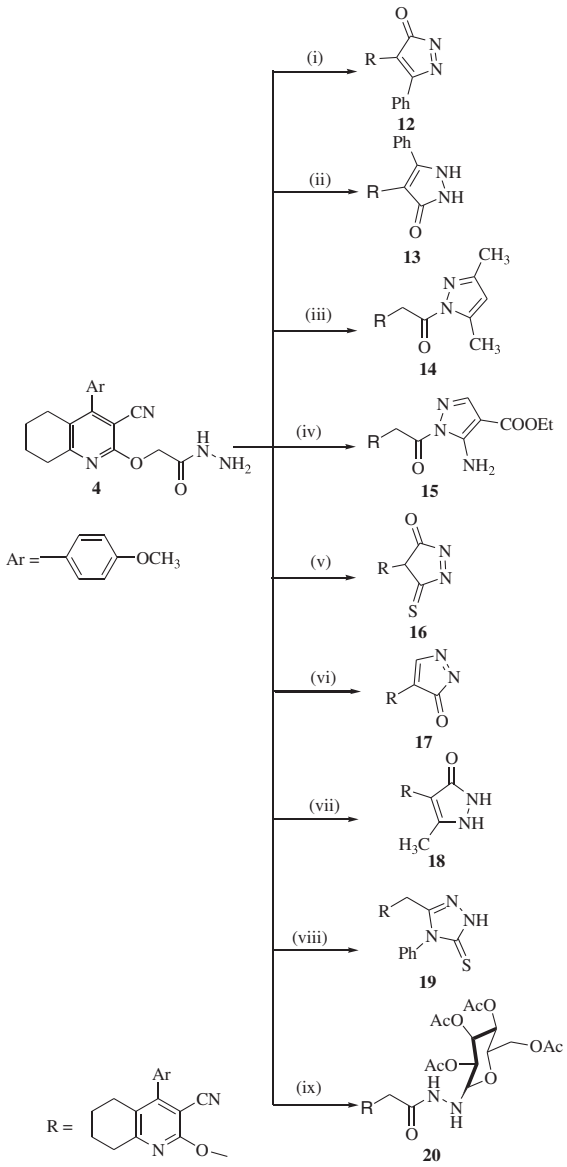
(i) $ClCH_2COOEt$, sodium acetate, EtOH, refluxfor 8h; (ii) $ClCH_2COOH$, sodium acetate, EtOH, reflux for 10h; (iii) $ClCH_2COCl$,sodium acetate, EtOH, refluxfor 13h; (iv) CH_3I, anhydrous K_2CO_3,acetone refluxfor 20h

The present work has been extended to study the effect of aliphatic acids, namely, formic and/or acetic acid on acetohydrazide derivative **4** to afford 4-oxo-4*H*-pyrazol-3-yloxytetrahydro-quinoline-3-carbonitrile derivatives **17** and **18**, respectively. When acetohydrazide derivative **4** was allowed to react with phenyl isothiocyanate in pyridine at its boiling point, and/or α-*D*-glucose pentaacetate in refluxing ethanol, 5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yltetrahydroquinoline derivative **19**, and *N*′-glucopyranosyl acetohydrazide derivative **20** were obtained, respectively (cf. Scheme 3).

Biological activity. ***Antileishmanial activity.*** In the present study on the synthesized heterocyclic compounds against *Leishmania major*, it is evidenced from Table 1 and Figure 1 that a series of heterocyclic compounds showed tremendous activity. Acetohydrazide derivative **4**, hydrazinylacetate derivative **5**, tetrahydropyrano[2,3-*b*] quinolin-2-one **6**, and oxopyrazol derivatives **12**, **14**, and **16**–**18** have shown significant activity against *L. major* with IC_{50} value of 0.59 ± 0.07 and 0.58 ± 0.08, respectively compared with amphotericin B (standard drug) IC_{50} value (0.56 ± 0.20). The presence of a pyrazole ring markedly improved the activity profiles of tetrahydroquinoline. On the other hand, further tetrahydroquinoline derivatives **2**, **3**, **11**, and hexahydroquinoline **7**–**10** showed a good activity against *L. major* with IC_{50} value of 0.60 ± 0.09 and 0.62 ± 0.09, respectively.

Antitumor activity. The crown gall is a neoplastic disease of plants that is initiated by the Gram-positive bacteria *Agrobacterium tumefaciens*. Because the mechanism of tumor induction resembles to that in animals, this test has been used previously to prescreen antitumor activity of natural and synthetic compounds. According to the data shown in Table 2 and Figure 2, most test compounds of this series showed significant level of tumor inhibition. The tetrahydropyrano[2,3-*b*]quinolin-2-

Scheme 3. Reactions of acetohydrazide derivative **4** with different electrophile reagents.



(i) Benzoyl chloride, reflux for 12h ; (ii) Benzoic acid, $POCl_3$, reflux for 10h; (iii) Acetyl acetone, EtOH, refluxfor 17h; (iv) 2-Ethoxymethylene-cyanoacetate, EtOH, reflux for 19h; (v) CS_2, KOH, EtOH, reflux for 12h; (vi) Formic acid,reflux for ; (vii) Acetic acid, reflux for 12h; (viii) Phenyl isothiocyanate, pyridine, reflux for 9h; (ix) *α–D*-Glucose pentaacetate, EtOH, reflux for 35h

one **6** and 4-oxo-4*H*-pyrazol-3-yloxytetrahydroquinoline-3-carbonitrile derivatives **18** showed 100% tumor inhibition that is comparable with standard drug vincristine (100% tumor inhibition).

Brine shrimp lethality assay. The cytotoxic activities of synthetic compounds are shown in Table 3 and Figure 3. The LD_{50} data showed that most of the compounds are cytotoxic with LD_{50} values in the range 0.56–3.01 μg/mL compared with standard drug MS-222 with LD_{50} value of 4.30 μg/mL. From Table 3, it is showed that compounds **5**, **6**, **12**, **14**, and oxopyrazol derivatives **16**–**18** are most cytotoxic to brine shrimps with LD_{50} values in the range 0.56–3.01 μg/mL comparatively with standard drug MS-222 with LD_{50} value of 4.30 μg/mL. On other hand, from Table 3, it is evidence that tetrahydroquinolines **2**–**4**, **11** and hexahydroquinolines **7**–**10** showed good LD_{50} values of 3.01–0.85 μg/mL compared with standard drug MS-222 with LD_{50} value of 4.30 μg/mL.

EXPERIMENTAL

All melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. The infrared

Table 1

Percent inhibition of synthetic compounds against *Leishmania major*

Compound no.	*L. major*
2	0.60 ± 0.09
3	0.60 ± 0.09
4	0.59 ± 0.09
5	0.59 ± 0.09
6	0.59 ± 0.09
7	0.59 ± 0.09
8	0.60 ± 0.09
9	0.62 ± 0.09
10	0.61 ± 0.09
11	0.68 ± 0.09
12	0.58 ± 0.09
14	0.58 ± 0.09
16	0.59 ± 0.09
17	0.58 ± 0.09
18	0.58 ± 0.09
Standard drug IC_{50} (μg/mL ± SD) amphotericin B	0.56 ± 0.20

Percent inhibition activity: 0.99 ± 0.00 = nonsignificant; 0.95–0.80 = low; 0.79–0.70 = moderate; 0.69–0.60 = good; <0.59–0.56 = significant.

Table 2

Antitumor activity of synthetic compounds

Compound no.	Average number of tumors[a] ± SE	Percent inhibition of tumors[b,c]
2	2.8 ± 0.89	62.66
3	1.8 ± 0.679	76.31
4	1.2 ± 0.727	85.54
5	1.0 ± 0.36	86.66
6	0.0 ± 0.0	100
7	4.8 ± 1.13	36.31
8	4.2 ± 1.04	44.59
9	3.9 ± 1.02	48.67
10	3.9 ± 1.02	48.32
11	4.1 ± 0.948	50.60
12	1.0 ± 0.36	86.66
14	0.9 ± 0.456	88.16
16	0.9 ± 0.456	88.16
17	0.8 ± 0.388	89.12
18	0.0 ± 0.0	100
Vincristine	0.0 ± 0.0	100
Vehicle control: DMSO	8.3 ± 0.931	

[a]Potato disc antitumor assay, concentration: 1000 μg/mL in DMSO.
[b]More than 20% tumor inhibition is significant.
[c]Data represent mean value of 15 replicates.

spectra were recorded using potassium bromide disks on a PyeUnicam SP-3-300 infrared spectrophotometer. ^{1}H NMR experiments were run at 300 and 400 MHz on a Varian Mercury VX-300 and a Varian Mercury VX-400 NMR spectrometer using TMS as internal standard in $CDCl_3$ or DMSO-d_6. Chemical shifts are quoted as δ. The mass spectra were recorded on Shimadzu GCMS-QP-1000EX mass spectrometers at 70 eV. All spectral measurements were carried out at Central laboratory of Ain-Shams University and Main Defense Chemical Laboratory, Egypt. All the newly synthesized compounds gave satisfactory elemental analyses. The purity of the synthesized compounds was checked by TLC.

Synthesis. ***Ethyl 2-(3-cyano-4-(4-methoxyphenyl)-5,6,7,8-tetrahydroquinolin-2-yloxy)acetate (2).*** A mixture of 2-pyridone **1** (5 mmol, 1.41 g), anhydrous K_2CO_3 (5 mmol, 0.69 g), and ethyl chloroacetate (5 mmol, 0.6 mL) in dry acetone (30 mL) was heated at reflux temperature on water bath for 30 h, left to cool, and poured into ice-cold water. The crude solid product that deposited was collected, washed with water, and recrystallized from ethanol to give tetrahydroquinolinyloxyacetate derivative **2** as yellow crystals, mp 145–147°C, yield 82%. FT-IR (KBr, cm^{-1}): 3126 ν_{CH} aromatic, 2938 ν_{CH} aliphatic, 2223 $\nu_{C\equiv N}$,1736 $\nu_{C=O}$ ester, 1631 $\nu_{C=N}$. ^{1}H NMR (400 MHz, DMSO-d_6): 7.25–6.97 (m, 4H, Ar–H), 4.88 (s, 2H, OCH_2), 4.29 (q, 2H, CH_2CH_3, J = Hz), 3.84 (s, 3H, OCH_3), 2.63–1.60 (m, 8H, cyclohexene ring protons), 1.3 (t, 3H, CH_2CH_3, J = Hz). MS m/z (%): 366 (M+, 100). *Anal.* Calcd for $C_{21}H_{22}N_2O_4$ (366.41): C, 68.84; H, 6.05; N, 7.65. Found: C, 68.67; H, 6.01; N, 7.54.

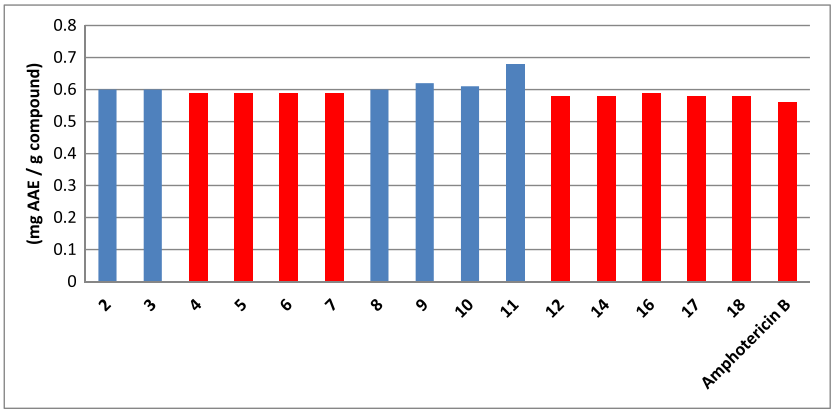


Figure 1. Percent inhibition of synthetic compounds against *Leishmania major*. [Color figure can be viewed at wileyonlinelibrary.com]

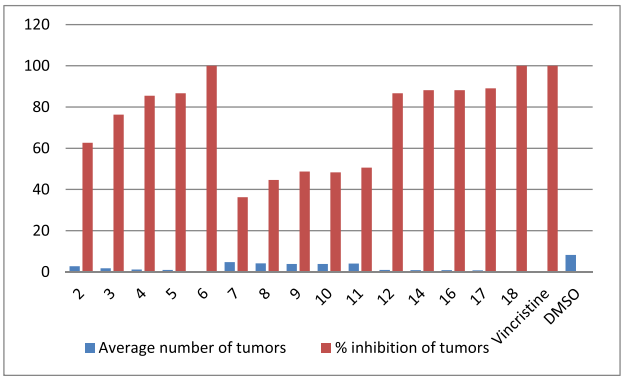


Figure 2. Antitumor activities of compounds **2–18** compared with vincristine. [Color figure can be viewed at wileyonlinelibrary.com]

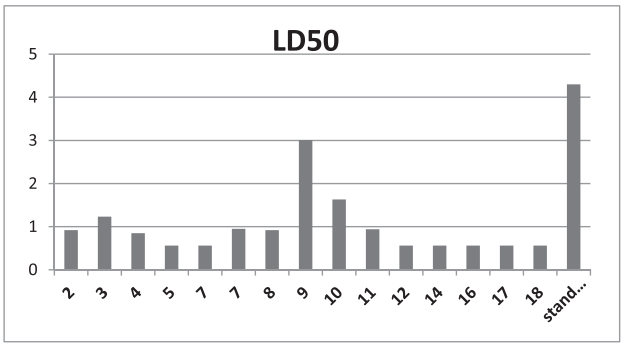


Figure 3. Brine shrimps lethality assay[1] of synthetic compounds.

Table 3

Brine shrimps lethality assay[a] of synthetic compounds

Compound no.	Numbers of shrimps killed out of 30			
Serial dilutions of compounds	1000 µg/mL	100 µg/mL	10 µg/mL	LD_{50} (µg/mL)
2	28	25	20	0.92
3	30	26	22	1.23
4	28	25	21	0.85
5	30	27	24	0.56
6	30	27	24	0.56
7	30	26	23	0.95
8	28	25	20	0.92
9	25	22	17	3.01
10	30	26	22	1.63
11	29	24	20	0.94
12	30	27	24	0.56
14	30	27	24	0.56
16	30	27	24	0.56
17	30	27	24	0.56
18	30	27	24	0.56
Control negative DMSO	00	00	00	

[a]The data are based on mean value of three replicates each of 10, 100, and 1000 µg/mL compared with the standard drug MS-222 (LD_{50} = 4.30 µg/mL).

3-Cyano-4-(4-methoxyphenyl)-5,6,7,8-tetrahydroquinolin-2-yl 2-chloroacetate (3). A mixture of 2-pyridone **1** (5 mmol, 1.41 g), anhydrous K_2CO_3(5 mmol, 0.69 g), and ethyl chloroacetate (5 mmol, 0.6 mL) in DMF (25 mL) was heated at reflux 9 h, left to cool, and poured into ice-cold water. The crude solid product was collected, washed with water, and recrystallized from petroleum ether (80–100°C) to give tetrahydroquinolin-2-yl-2-chloroacetate **3** as a pale brown crystal, mp 280–280°C, yield 62%. FT-IR (KBr, cm^{-1}): 3012 ν_{CH} aromatic, 2938 ν_{CH} aliphatic, 2222 $\nu_{C\equiv N}$,1759 $\nu_{C=O}$ ester, 1649 $\nu_{C=N}$. ^1H NMR (400 MHz, DMSO-d_6): 12.28 (s, 1H, OH, D_2O exchangeable), 7.25–7.02 (m, 4H, Ar–H), 3.79 (s, 3H, OCH_3), 3.54 (s, 1H, OH–C=CH–Cl), 2.59–1.51 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 356 (M+, 70). *Anal*. Calcd for $C_{19}H_{17}ClN_2O_3$ (356.8): C, 63.96; H, 4.80; Cl, 9.94; N, 7.85. Found: C, 63.78; H, 4.59; Cl, 9.77; N, 7.69.

Reaction of tetrahydroquinolinyloxyacetate derivative 2 and/or tetrahydroquinolin-2-yl 2-chloroacetate 3 with hydrazine hydrate. To a solution of tetrahydroquinolinyloxyacetate derivative **2** and/or tetrahydroquinolin-2-yl 2-chloroacetate **3** (5 mmol) in ethanol (30 mL), hydrazine hydrate was added (5 mmol, 0.25 mL), and the reaction mixture was heated at reflux for 11 h and left to cool, and the solid product was collected, dried, and recrystallized from dioxane to give acetohydrazide **4** and 2-hydrazinylacetate derivatives **5**, respectively.

2-(3-Cyano-4-(4-methoxyphenyl)-5,6,7,8-tetrahydroquinolin-2-yloxy)acetohydrazide (4). Yellow crystals, mp 175–177°C, yield 84%. FT-IR (KBr, cm^{-1}): 3431, 3299 ν_{NH2}, 3193 ν_{NH}, 3029 ν_{CH} aromatic, 2933 ν_{CH} aliphatic, 2219 $\nu_{C\equiv N}$, 1646 $\nu_{C=O}$, 1609 $\nu_{C=N}$. ^1H NMR (400 MHz, DMSO-d_6): 11.72 (s, 1H, NH, D_2O exchangeable), 7.25–7.06 (m, 4H, Ar–H), 4.03 (s, 2H, OCH_2CO), 3.81 (s, 3H, OCH_3), 3.36 (s, 2H, NH_2, D_2O exchangeable), 2.88–1.63 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 352 ($M^{\cdot}$+, 85). *Anal*. Calcd for $C_{19}H_{20}N_4O_3$ (352.39): C, 64.76; H, 5.72; N, 15.90. Found: C, 64.68; H, 5.81; N, 15.85.

3-Cyano-4-(4-methoxyphenyl)-5,6,7,8-tetrahydroquinolin-2-yl-2-hydrazinylacetate (5). Pale red crystals, mp over 300°C, yield 70%. FT-IR (KBr, cm^{-1}): 3431, 3299 ν_{NH2}, 3193 ν_{NH}, 3026 ν_{CH} aromatic, 2932 ν_{CH} aliphatic, 2209 $\nu_{C\equiv N}$, 1649 $\nu_{C=O}$, 1608 $\nu_{C=N}$. ^1H NMR (400 MHz, DMSO-d_6): 11.72 (s, 1H, NH, D_2O exchangeable), 7.25–7.06 (m, 4H, Ar–H), 4.03 (s, 2H, NH_2, D_2O exchangeable), 3.81 (s, 3H, OCH_3), 3.41 (s, 2H, $COCH_2NH-$), 2.88–1.64 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 352 (M+, 65). *Anal*. Calcd for $C_{19}H_{20}N_4O_3$ (352.39): C, 64.76; H, 5.72; N, 15.90. Found: C, 64.82; H, 5.65; N, 15.75.

4-Amino-3-chloro-5-(4-methoxyphenyl)-6,7,8,9-tetrahydropyrano[2,3-b]quinolin-2-one (6). A solution of tetrahydroquinolin-2-yl 2-chloroacetate **3** (2.5 mmol, 0.89 g) in alcoholic sodium hydroxide 10% (10 mL) was heated at reflux temperature for 6 h, left to cool, and neutralized by dilute hydrochloric acid. The crude solid product that deposited was collected, washed with water, dried, and recrystallized from ethanol to give tetrahydropyrano[2,3-*b*]quinolin-2-one **6** as brown crystals, mp 230–232°C, yield 62%. FT-IR (KBr, cm^{-1}): 3420, 3350 ν_{NH2}, 3071 ν_{CH} aromatic, 2935 ν_{CH} aliphatic, 1738 $\nu_{C=O}$ lactone, 1659 $\nu_{C=N}$. ^{1}H NMR (400 MHz, DMSO-d_6): 7.34–6.95 (m, 4H, Ar–H), 4.31 (s, 2H, NH_2, D_2O exchangeable), 3.81 (s, 3H, OCH_3), 2.65–1.01 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 356 (M+, 65). *Anal*. Calcd for $C_{19}H_{17}ClN_2O_3$ (356.8): C, 63.96; H, 4.80; Cl, 9.94; N, 7.85. Found: C, 63.87; H, 4.74; Cl, 9.86; N, 7.68.

4-(4-Methoxyphenyl)-2-thioxo-1,2,5,6,7,8-hexahydroquinoline-3-carbonitrile (7). A mixture of 2-pyridone **1** (5 mmol, 1.41 g) and phosphorous pentasulfide (5 mmol, 1.11 mL) in dry toluene (25 mL) was refluxed for 4 h, then collect the solid on hot, dried, and recrystallized from ethanol to give hexahydroquinoline **7** as pale orange crystals, mp 182–183°C, yield 43%. FT-IR (KBr, cm^{-1}): 3164 ν_{NH}, 3068 ν_{CH} aromatic, 2938 ν_{CH} aliphatic, 2221 $\nu_{C°N}$, disappearance of $\nu_{C=O}$, 1636 $\nu_{C=N}$, 1258 $\nu_{C=S}$. ^{1}H NMR (300 MHz, $CDCl_3$): 7.36–7.01 (m, 4H, Ar–H), 5.57 (s, 1H, SH, D_2O exchangeable),3.87 (s, 3H, OCH_3), 3.04–1.73 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 296 (M+, 100). *Anal*. Calcd for $C_{17}H_{16}N_2OS$ (296.39): C, 68.89; H, 5.44; N, 9.45; S, 10.82. Found: C, 68.74; H, 5.32; N, 9.39; S, 10.75.

Ethyl 2-(3-cyano-4-(4-methoxyphenyl)-3,4,5,6,7,8-hexahydroquinolin-2-ylthio)acetate (8). To a mixture of hexahydroquinoline **7** (5 mmol, 1.48 g) and ethyl chloroacetate (5 mmol, 0.6 mL) in absolute ethanol (20 mL) fused sodium acetate (10 mmol, 0.82 g) was added, heated at reflux temperature for 4 h, and left to cool; the solid product was collected by filtration, dried, and recrystallized from petroleum ether 80–100°C to give acetate derivative **8** as yellow crystals, mp 150–152°C, yield 58%. FT-IR (KBr, cm^{-1}): 3070 ν_{CH} aromatic, 2973 ν_{CH} aliphatic, 2218 $\nu_{C°N}$, 1738 $\nu_{C=O}$ ester,1651 $\nu_{C=N}$. ^{1}H NMR (300 MHz, $CDCl_3$): 7.27–6.99 (m, 4H, Ar–H), 4.23 (q, 2H, CH_2CH_3, J = 6.9 Hz), 4.01 (s, 2H, −S–CH_2), 3.86 (s, 3H, OCH_3), 2.45–1.34 (m, 8H, cyclohexene ring protons), 1.32 (t, 3H, CH_2CH_3, J = 6.9 Hz). MS *m*/*z* (%): 384 (M + 2)+, 1). *Anal*. Calcd for $C_{21}H_{22}N_2O_3S$ (382.48): C, 65.95; H, 5.80; N, 7.32; S, 8.38. Found: C, 65.78; H, 5.68; N, 7.41; S, 8.22.

3-Cyano-4-(methoxyphenyl)1,4,5,6,7,8-hexahydroquinoline-2-ylacetate (9). A solution of 2-pyridone **1** (5 mmol, 1.41 g) in acetic anhydride (10 mL) was heated at reflux for 5 h, then left to cool and poured into ice-cold water with stirring. The crude solid product that deposited was collected by filtration, washed with water, dried, and recrystallized from benzene to givehexahydroquinoline-2-ylacetate **9** as pale brown crystals, mp 125–127°C, yield 75%. FT-IR (KBr, cm^{-1}): 3298 ν_{NH}, 3015 ν_{CH} aromatic, 2936 ν_{CH} aliphatic, 2224 $\nu_{C°N}$,1783 $\nu_{C=O}$ ester, 1648 $\nu_{C=N}$. ^{1}H NMR (300 MHz, DMSO-d_6): 12.30 (s, 1H, NH, D_2O exchangeable), 7.27–6.92 (m, 4H, Ar–H), 3.82 (s, 3H, OCH_3, benzylic-H), 3.75 (s, 3H, $OCOCH_3$), 2.62–1.55 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 322 (M-2)+, 5). *Anal*. Calcd for $C_{19}H_{20}N_2O_3$ (324.37): C, 70.35; H, 6.21; N, 8.64. Found: C, 70.21; H, 6.32; N, 8.57.

3-Cyano-4-(4-methoxyphenyl)-1,4,5,6,7,8-hexahydroquinolin-2-yl benzoate (10). A mixture of 2-pyridone **1** (5 mmol, 1.41 g) and benzoyl chloride (5 mmol, 0.6 mL) in dry pyridine (20 mL) was heated on water bath 5 h, then left to cool and neutralized by diluted hydrochloric acid for complete precipitation. The separated material was collected by filtration, washed with water, dried, and recrystallized from ethanol to afford hexahydroquinolin-2-yl benzoate **10** as pale yellow crystals, mp 159–161°C, yield 63%. FT-IR (KBr, cm^{-1}): 3340 ν_{NH}, 3011 ν_{CH} aromatic, 2938 ν_{CH} aliphatic, 2227 $\nu_{C°N}$, 1738 $\nu_{C=O}$ ester, 1647 $\nu_{C=N}$. ^{1}H NMR (300 MHz, DMSO-d_6): 12.31 (s, 1H, NH, D_2O exchangeable), 7.27–7.04 (m, 9H, Ar–H), 3.81 (s, 3H, OCH_3, benzylic-H), 2.62–1.55 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 384 (M-2)+, 4). *Anal*. Calcd for $C_{24}H_{22}N_2O_3$ (386.44): C, 74.59; H, 5.74; N, 7.25. Found: C, 74.42; H, 5.66; N, 7.34.

2-Methoxy-4-(4-methoxyphenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carbonitrile (11). **Method A.** To a solution of 2-pyridone **1** (5 mmol, 1.41 g), ethyl chloroacetate, 2-chloroacetyl chloride, or/and chloroacetic acid (5 mmol), in absolute ethanol (25 mL), anhydrous sodium acetate (5 mmol, 0.82 g) was added, then the reaction mixture was refluxed for 8–13 h. The reaction mixture was left to cool and neutralized by diluted hydrochloric acid for complete precipitation. The separated product was collected by filtration, washed with water, dried, and recrystallized from ethanol to give hexahydroquinoline **11** as yellow crystals, mp 295–296°C, yield 52%, 61%, and 49%, respectively. FT-IR (KBr, cm^{-1}): 3290 ν_{NH}, 3125 ν_{CH} aromatic, 2933 ν_{CH} aliphatic, 2218 $\nu_{C°N}$,1646 $\nu_{C=N}$. ^{1}H NMR (300 MHz, DMSO-d_6): 12.27 (s, 1H, NH, D_2O exchangeable), 7.27–7.04 (m, 4H, Ar–H), 3.82 (s, 6H, $2OCH_3$), 3.57 (s, 1H, benzylic-H), 2.62–1.55 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 294 (M-2)+, 1). *Anal*. Calcd for $C_{18}H_{20}N_2O_2$ (296.36): C, 72.95; H, 6.80; N, 9.45. Found: C, 72.81; H, 6.69; N, 9.38.

Method B. To a solution of 2-pyridone **1** (5 mmol, 1.41 g), methyl iodide (5 mmol, 0.71 g), and anhydrous K_2CO_3 (5 mmol, 0.69 g), in dry acetone (30 mL) was heated at reflux temperature on water bath for 20 h. The reaction mixture was left to cool and poured into the water. The separated product was collected by filtration, washed with water, dried well, and recrystallized from ethanol to givehexahydroquinoline**11** as yellow crystals, mp 295–296°C, yield 66%.

4-(4-Methoxyphenyl)-2-(3-oxo-5-phenyl-3H-pyrazol-4-yloxy)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (12). A mixture of acetohydrazide derivative **4** (1.3 mmol, 0.46 g) and benzoyl chloride (7.2 mmol, 10 mL) in pyridine (5 mL) was heated on water bath for 12 h, left to cool, and neutralized by cooled dilute hydrochloric acid. The solid product that formed was collected by filtration, washed with water, dried, and recrystallized from methanol to give pyrazoltetrahydroquinoline **12** as yellow crystals, mp 95–97°C, yield 77%. FT-IR (KBr, cm^{-1}): 3070 ν_{CH} aromatic, 2885 ν_{CH} aliphatic, 2213 $\nu_{C\equiv N}$, 1686 $\nu_{C=O}$. ^1H NMR (400 MHz, $CDCl_3$): 8.13–7.46 (m, 9H, Ar–H), 3.77 (s, 3H, OCH_3), 2.55–1.63 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 436 (M+, 4). *Anal*. Calcd for $C_{26}H_{20}N_4O_3$ (436.46): C, 71.55; H, 4.62; N, 12.84. Found: C, 71.41; H, 4.45; N, 12.78.

4-(4-Methoxyphenyl)-2-(3-oxo-5-phenyl-2,3-dihydro-1H-pyrazol-4-yloxy)-5,6,7,8-tetrahydro-quinoline-3-carbonitrile (13). A mixture of acetohydrazide derivative **4** (1.3 mmol, 0.46 g) and benzoic acid (1.3 mmol, 0.18 g) in phosphorous oxychloride (15 mL) was heated on water bath for 10 h, then left to cool, and the solid product was collected, dried, and recrystallized from ethanol to give dihydropyrazole-tetrahydroquinoline **13** as pale brown crystals, mp 222–224°C, yield 69%. FT-IR (KBr, cm^{-1}): 3410 ν_{NH}, 3064 ν_{CH} aromatic, 2941 ν_{CH} aliphatic, 2241 $\nu_{C\equiv N}$, 1643 $\nu_{C=O}$. ^1H NMR (400 MHz, DMSO-d_6): 9.98 and 9.76 (2 s, 2H, 2NH, D_2O exchangeable), 8.13–7.11 (m, 9H, Ar–H), 3.77 (s, 3H, OCH_3), 2.55–1.63 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 438 (M+, 76). *Anal*. Calcd for $C_{26}H_{22}N_4O_3$ (438.48): C, 71.22; H, 5.06; N, 12.78. Found: C, 71.34; H, 5.00; N, 12.67.

2-(2-(3,5-dimethyl-1H-pyrazol-1-yl)-2-oxoethoxy)-4-(4-methoxyphenyl)-5,6,7,8-tetrahydro-quinoline-3-carbonitrile (14). To a solution of acetohydrazide derivative **4** (2.5 mmol, 0.88 g) in absolute ethanol (20 mL), acetylacetone (2.5 mmol, 0.25 mL) was added, and the reaction mixture was heated at reflux for 17 h, then left to cool at room temperature. The crude solid product that obtained was collected, dried, and recrystallized from ethanol to give pyrazoltetrahydroquinoline **14** as pale brown crystals, mp 187–189°C, yield 63%. FT-IR (KBr, cm^{-1}): 3145 ν_{CH} aromatic, 2934 ν_{CH} aliphatic, 2206 $\nu_{C\equiv N}$, 1648 $\nu_{C=O}$, 1619 $\nu_{C=N}$. ^1H NMR (400 MHz, $CDCl_3$): 7.42–6.99 (m, 5H, Ar–H), 4.77 (s, 2H, OCH_2CO), 3.87 (s, 3H, OCH_3), 2.77–1.67 (m, 8H, cyclohexene ring protons), 1.25 (s, 6H, $2CH_3$). MS *m*/*z* (%): 416 ($M^{\cdot}$+, 81). *Anal*. Calcd for $C_{24}H_{24}N_4O_3$ (416.47): C, 69.21; H, 5.81; N, 13.45. Found: C, 69.12; H, 5.74; N, 13.38.

Ethyl 5-amino-1-(2-(3-cyano-4-(4-methoxyphenyl)-5,6,7,8-tetrahydroquinolin-2-yloxy)acetyl)-1H–pyrazole-4-carboxylate (15). A mixture of acetohydrazide derivative **4** (5 mmol, 1.76 g) and ethyl 2-cyano-3-ethoxyacrylate (5 mmol, 0.62 g) in ethanol (15 mL) was heated at reflux for 19 h and left to cool, and the solid product was collected by filtration, dried, and recrystallized from methanol to give enaminoester **15** as yellow crystals, mp 114–116°C, yield 58%. FT-IR (KBr, cm^{-1}): 3375, 3291 ν_{NH2}, 3136 ν_{CH} aromatic, 2979 ν_{CH} aliphatic, 2212 $\nu_{C\equiv N}$, 1688 $\nu_{C=O}$ ester, 1617 $\nu_{C=N}$. ^1H NMR (400 MHz, DMSO-d_6): 13.22 (s, 2H, NH_2, D_2O exchangeable), 8.78–7.05 (m, 5H, Ar–H), 4.34 (s, 2H, OCH_2CO), 4.06 (q, 2H, $\underline{CH_2}CH_3$), 3.82 (s, 3H, OCH_3), 2.97–1.67 (m, 8H, cyclohexene ring protons), 1.20 (t, 3H, $CH_2\underline{CH_3}$). MS *m*/*z* (%): 475 (M+, 68). *Anal*. Calcd for $C_{25}H_{25}N_5O_5$ (475.5): C, 63.15; H, 5.30; N, 14.73. Found: C, 63.04; H, 5.15; N, 14.67.

4-(4-Methoxyphenyl)-2-(3-oxo-5-thioxo-4,5-dihydro-3H-pyrazol-4-yloxy)-5,6,7,8-tetrahydro-quinoline-3-carbonitrile (16). To a solution of acetohydrazide derivative **4** (1.3 mmol, 0.46 g) and carbon disulfide (6.6 mmol, 5 mL) in ethanol (20 mL), alcoholic potassium hydroxide 10% was added. The reaction mixture was heated at reflux on water bath for 10 h, left to cool, and neutralized by dilute hydrochloric acid. The solid product that formed was collected, washed with water, dried, and recrystallized from ethanol to give pyrazol derivative **16** as pale red crystals, mp 205–207°C, yield 70%. FT-IR (KBr, cm^{-1}): 3067 ν_{CH} aromatic, 2933 ν_{CH} aliphatic, 2222 $\nu_{C\equiv N}$, 1642 $\nu_{C=O}$, 1607 $\nu_{C=N}$, 1249 $\nu_{C=S}$. ^1H NMR (400 MHz, DMSO-d_6): 7.37–7.09 (m, 4H, Ar–H), 3.83 (s, 3H, OCH_3), 3.00 (s, 1H, COCHCS), 1.85–1.04 (m, 8H, cyclohexene ring protons). MS *m*/*z* (%): 392 (M+, 49). *Anal*. Calcd for $C_{20}H_{16}N_4O_3S$ (392.43): C, 61.21; H, 4.11; N, 14.28; S, 8.17. Found: C, 61.34; H, 4.25; N, 14.12; S, 8.28.

Reaction of 2-(3-cyano-4-(4-methoxyphenyl)-5,6,7,8-tetrahydroquinolin-2-yloxy)acetohydrazide with formic acid and/or glacial acetic acid. A mixture of acetohydrazide derivative **4** (5 mmol, 1.76 g) in formic acid and/or glacial acetic acid (20 mL) was heated at reflux for 12 h, then the mixture was left to cool at room temperature and poured into ice-cold water, and the separated material was collected by filtration, washed with water, dried well, and recrystallized to afford pyrazolyloxytetrahydroquinoline derivatives **17** and **18**, respectively.

4-(4-Methoxyphenyl)-2-(3-oxo-3H-pyrazol-4-yloxy)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (17). Pyrazolyloxytetrahydroquinoline derivative **17** recrystallized from methanol to afford pale brown crystals, mp 130–132°C, yield 58%. FT-IR (KBr, cm^{-1}): 3070 ν_{CH} aromatic, 2935 ν_{CH} aliphatic, 2219 $\nu_{C\equiv N}$, 1685 $\nu_{C=O}$, 1648 $\nu_{C=N}$. ^{1}H NMR (400 MHz, $CDCl_3$): 7.83–6.88 (m, 5H, Ar–H), 3.74 (s, 3H, OCH_3), 2.61–1.76 (m, 8H, cyclohexene ring protons). MS *m/z* (%): 360 (M+, 50). *Anal*. Calcd for $C_{20}H_{16}N_4O_3$ (360.37): C, 66.66; H, 4.48; N, 15.55. Found: C, 66.45; H, 4.34; N, 15.62.

4-(4-Methoxyphenyl)-2-(3-methyl-5-oxo-2,5-dihydro-1H-pyrazol-4-yloxy)-5,6,7,8-tetrahydro-quinoline-3-carbonitrile (18). Pyrazolyloxytetrahydroquinoline derivative **18** recrystallized from ethanol to brown crystals, mp 199–201°C, yield 62%. FT-IR (KBr, cm^{-1}): 3202 ν_{NH}, 3067 ν_{CH} aromatic, 2932 ν_{CH} aliphatic, 2221 $\nu_{C\equiv N}$, 1665 $\nu_{C=O}$, 1607 $\nu_{C=N}$. ^{1}H NMR (400 MHz, DMSO-d_6): 13.04, 9.22 (2 s, 2H, 2NH, D_2O exchangeable), 7.34–6.95 (m, 4H, Ar–H), 3.79 (s, 3H, OCH_3), 2.97–1.66 (m, 8H, cyclohexene ring protons), 1.43 (s, 3H, CH_3). MS *m/z* (%): 376 (M+, 3). *Anal*. Calcd for $C_{20}H_{18}N_4O_3$ (362.38): C, 66.29; H, 5.01; N, 15.46. Found: C, 66.14; H, 5.24; N, 15.56.

4-(4-Methoxyphenyl)-2-((4-phenyl-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)methoxy)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (19). A mixture of acetohydrazide derivative **4** (2.5 mmol, 1.76 g) and phenyl isothiocyanate (2.5 mmol, 0.34 mL) in pyridine (20 mL) was refluxed for 9 h, left to cool, and neutralized by cooled dilute hydrochloric acid. The solid product that formed was collected, washed with water, dried, and recrystallized from dioxane to give triazoltetrahydroquinoline **19** as brown crystals, mp 241–243°C, yield 74%. FT-IR (KBr, cm^{-1}): 3375 ν_{NH}, 3057 ν_{CH} aromatic, 2932 ν_{CH} aliphatic, 2222 $\nu_{C\equiv N}$, 1650 $\nu_{C=N}$, 1249 $\nu_{C=S}$. ^{1}H NMR (400 MHz, $CDCl_3$): 7.89 (s, 1H, NH, D_2O exchangeable), 7.53–6.99 (m, 9H, Ar–H), 3.90 (s, 2H, OCH_2), 3.74 (s, 3H, OCH_3), 2.69–1.63 (m, 8H, cyclohexene ring protons). MS *m/z* (%): 469 (M+, 36). *Anal*. Calcd for $C_{26}H_{23}N_5O_2S$ (469.56): C, 66.50; H, 4.94; N, 14.91; S, 6.83. Found: C, 66.41; H, 4.84; N, 14.82; S, 6.75.

(2R,3R,4R,5R,6S)-2-(Acetoxymethyl)-6-(2-(2-((3-cyano-4-(4-methoxyphenyl)-5,6,7,8-tetrahydro-quinolin-2-yl)oxy)acetyl) hydrazinyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (20). A mixture of acetohydrazide derivative **4** (2.5 mmol, 1.76 g) and α-D-glucose pentaacetate (2.5 mmol, 0.98 g) in absolute ethanol (20 mL) was heated at reflux for 35 h, then the mixture was left to cool at room temperature and the solid product that formed was collected, washed with water, dried, and recrystallized from ethanol to give **20** as dark brown crystals, mp over 300°C, yield 55%.FT-IR (KBr, cm^{-1}): 3306 ν_{NH}, 3072 ν_{CH} aromatic, 2939 ν_{CH} aliphatic, 2221 $\nu_{C\equiv N}$, 1753 $\nu_{C=O}$, 1656 $\nu_{C=O}$. The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum exhibited the following signals(δ/ppm): 8.35 (s, 1H, CONH, D_2O exchangeable), 7.22–6.98 (m, 4H, Ar–H), 6.33 (d, 1H, −NH–CH–O−), 5.46–5.07 (m, 6H, 3CHOCO, O–CH–CH_2–$OCOCH_3$), 4.27 (s, 2H, $-OCH_2CO-$), 3.89 (s, 3H, OCH_3), 2.17–1.69 (m, 8H, cyclohexene ring protons), 1.85 (s, 1H, NHCH, D_2O exchangeable), 1.29 (s, 12H, $4COCH_3$). MS *m/z* (%): 682 (M+, 15). *Anal*. Calcd for $C_{33}H_{38}N_4O_{12}$ (682.67): C, 58.06; H, 5.61; N, 8.21. Found: C, 58.14; H, 5.50; N, 8.32.

Biological activity. ***Antileishmanial activity.*** All the synthesized heterocyclic compounds were initially screened for their antileishmanial activity in contrast to culture of *L. major* [31,39].

One milligram of every compound was dissolved using solvent DMSO (1 mL), and 1 mg of amphotericin B was also solubilized in 1 mL of DMSO; 180 µL of medium was poured in various wells of 96-well plates; and 20 µL of the tetrahydroquinoline derivatives solution was then added in medium and diluted serially. Parasites at log phase for 3 min were centrifuged at 3000 rpm. Fresh culture medium was used for diluting the parasites to a final density of 2×10^6 cells/mL. In all wells, 100 µL of parasite culture was added. Negative and standard drug were maintained having DMSO and amphotericin B. For positive and negative controls, three rows were left empty. DMSO was diluted serially in medium in case of negative controls, while in positive control, there were different concentrations of standard antileishmanial compound, for example, amphotericin B. Micro titer plates were incubated for 72 h at 24°C. Every assay was in duplicate; 15 µL of test culture were then transferred after 72 h, to enhanced Neubauer counting chamber, and live promastigotes were counted using light microscope.

Crown gall tumor inhibition (potato disc) assay.

Preparation of agar plates and treatment. These potato discs were then transferred to petri plates each containing 25 mL of 1.5% agar (1.5 g agar/100 mL distilled water). Five potato discs were placed on each plate, and three plates were used for each test sample along with same number of plates for vehicle control (DMSO) and reference drug (vincristine). As a stock solution, 10 mg of each compound was dissolved in 1 mL of DMSO in separate test tubes. Then 0.5 mL of stock (10 mg/mL) of the test sample was added to 2 mL of a broth culture of *A. tumefaciens* (At-10, a 48-h culture containing 5 × 109 cells/mL) and 2.5 mL of autoclaved distilled water to make 1000 µg/mL final concentration. One drop (10 µL) was drawn from these test tubes using a sterile pipette, and it was used to inoculate each potato disc, spreading it over the disc surface. The process starting from the cutting of the potatoes to the inoculation was completed in 30 min in order to avoid contamination. The lids of the petri plates were taped down with parafilm to minimize moisture loss.

Incubation and analysis. The petri plates were incubated at 28°C for 21 days, and the number of tumors was counted with the aid of dissecting microscope after staining with Lugol's solution (5% I_2, 10% KI in distilled water). The numbers of tumors in vehicle control (DMSO) were used as a reference for activity. The results were derived from the number of tumors on test discs versus those on the vehicle control disc. Twenty percent or more inhibition was considered as significant activity.

Cytotoxicity activity by brine shrimp lethality assay. The procedure described by Singh et al. was used to perform the brine shrimp cytotoxicity assay [40]. Artificial sea water was prepared by dissolving 34 g of sea salt in 1000 mL of distilled water with regular stirring. The solution was aerified for about 2 h in an open beaker with vigorous shaking on magnetic stirrer. Shallow rectangular dish (22 × 32 cm) previously filled with prepared seawater was used for hatching of *Artemiasalina* (brine shrimp eggs) that is composed of two compartments: One compartment was large, and the other compartment was small. Both compartments were separated by a wall consisted of several holes of 2 mm in diameter. The eggs were spread on the surface of artificial sea water in the large compartment. The large compartment was covered with aluminum foil so that there was darkness in that compartment. The smaller compartment was lighted up with a lamp. After 24–26 h, the hatching process started and the newly hatched nauplii (brine shrimp larvae) traveled towards the smaller compartment because of presence of light. The nauplii were collected in a beaker with the help of Pasteur pipette. Three concentrations of 10, 100, and 1000 µg/mL were used against brine shrimp larvae and then the rate of death at all concentrations was observed. To check the number of deaths, each sample quantity taken was 0.5 mL in 20 mL vial. Furthermore, additional 2 mL artificial sea water was added to evaporate the solvent. After this, 30 shrimps were transferred to each vial with final volume adjusted to 5 mL. They were kept under florescence light at a temperature of 25°C for a time period of 24 h. Finney computer program was used to evaluate ED_{50} after the percentage survival was calculated.

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