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Original article

Synthesis of new acridines and hydrazones derived from cyclic β-diketone for cytotoxic and antiviral evaluation

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ABSTRACT

Cyclic β-diketone namely, dimedone was utilized to prepare different chemical entities whether cyclic such as acridines, thiadiazole and triazole or acyclic systems as hydrazide, hydrazones, thiosemicarbazide and semicarbazide. The structures of the novel compounds were determined using elemental analyses and various spectroscopic methods. Most acyclic derivatives especially semicarbazide **19**, hydrazide **9** and thiosemicarbazide **16** showed a higher *in vitro* cytotoxic activity against hepatoma cell line (HepG2) than the cyclized acridine derivatives. The antiviral activity of the new compounds against *Hepatitis A Virus* (HAV) using the plague infectivity reduction assay revealed that the acridine **4** and the hydrazone **12** were more active than the reference drug amantadine.

1. Introduction

Malignant tumor and viral infections are the most serious threats against human health in the world. *Hepatitis A Virus* (HAV) is a picornavirus, a common causative agent of acute self-limited hepatitis that sometimes leads to fulminant hepatic failure [1,2].

Therefore, treatment of severe infections [1] is still an issue of major concern especially the clinically used drugs remains relatively poor.

Furthermore, there exist a number of acridine (amsacrine) [3–7], thiadiazole [8,9] and triazole (ribavirin) [10–12] derivatives having anticancer and antiviral activities. Moreover, some hydrazones [13,14] and thiosemicarbazone [15] derivatives were reported to exhibit anticancer and antiviral properties, respectively.

Based on the above findings and coupled with our interest of the chemistry of cyclic β-diketone especially dimedone gave us the opportunity for preparation of diverse heterocyclic ring systems such as acridine, thiadiazole and triazole derivatives in addition to acyclic systems e.g. hydrazone, thiosemicarbazide as well as semicarbazide derivatives with the aim to evaluate their cytotoxic and antiviral activities.

2. Results and discussions

2.1. Chemistry

In this work, the novel derivatives **4**–**19** were designed and prepared as illustrated in Schemes 1 and 2.

The key intermediate ethyl 2-(5,5-dimethyl-3-oxocyclohex-1-enyl amino) acetate (**3**) was prepared by condensation of equimolar amounts of 5,5-dimethyl-1,3-cyclohexandione (**1**) with ethyl glycinate HCl (**2**) in the presence of anhydrous sodium acetate adopting two procedures either via heating the reactants under reflux in toluene for 15 h to produce 65% yield [16–19] or in ethanol for 2.5 h giving 85% yield. The latter method was preferred than the former one due to it afforded pure product with higher yield in shorter time.

The novel nonclassical acridine derivatives (**4**–**8**) were obtained in one-pot synthesis reaction via cyclocondensation of enaminone **3** with half equivalent of aromatic aldehyde through heating the reactants at reflux for 4 h in glacial acetic acid (Scheme 1). This method gave the acridines in one step without passing through the formation of bis intermediates. Moreover, acetic acid was found to be the solvent of choice for conducting the cyclocondensation whereas it provided the reaction with a higher temperature necessary for its completion and also maintained the expelled amine residue, thus it favors the formation of acridines.

The structures of the novel acridines were confirmed using IR spectra which showed the disappearance of NH stretching band at

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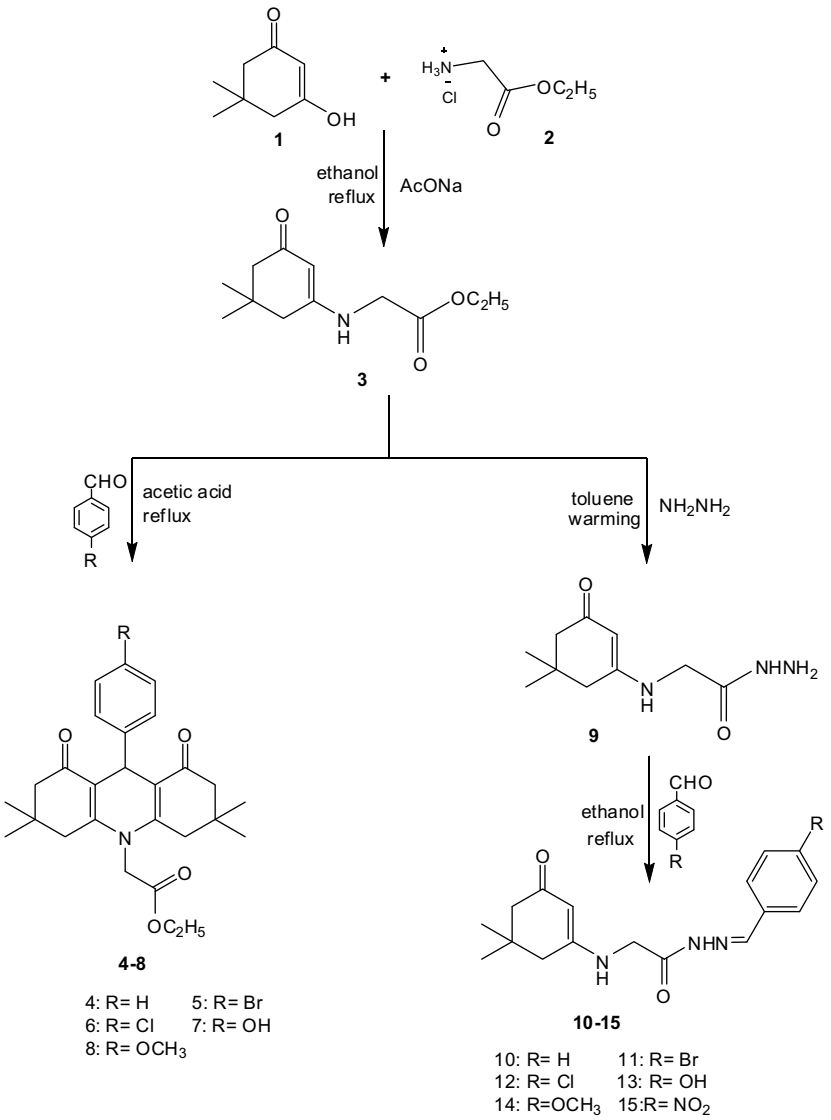


Scheme 1. Synthetic pathway for compounds **3**–**15**.

$\upsilon = 3260\ cm^{-1}$ for the starting enaminone. ^1H NMR proved the absence of vinylic H singlet peak around $\delta = 4.992$ ppm as well as NH singlet peak around $\delta = 7.25$ ppm for the starting enaminone. In addition, the appearance of singlet signal at $\delta = 5.168$ ppm assignable to 9-H of acridine nucleus.

An interesting observation is the appearance of geminal methyl groups at different chemical shifts (0.954 and 1.065). This observation of the different values for geminal methyl groups may be due to either the nonbonding interaction between the aromatic residue at position 9 and the 1,8-dioxo functions.

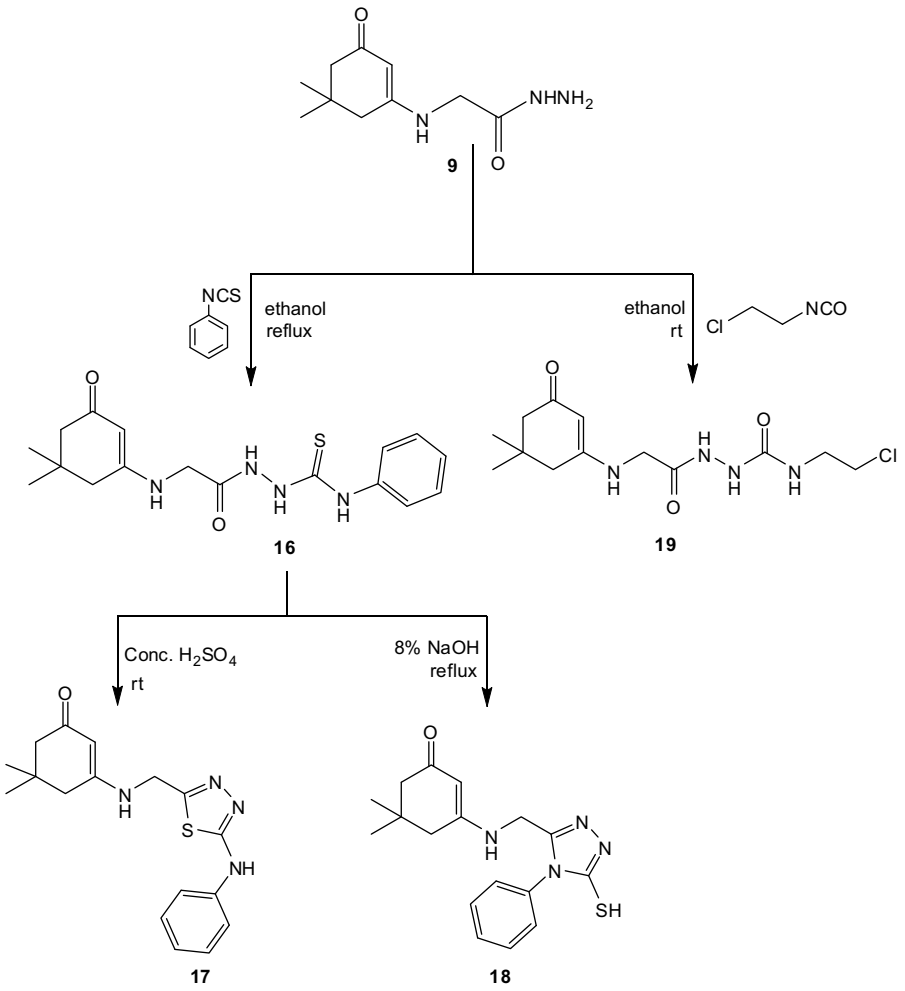
This may allow the former to occupy angular position to the tricyclic plane of the acridine skeleton. The other possibility for the distinguished chemical shifts of the methyls may be due to the limited free rotation of the phenyl group at position 9. One of the geminal methyl may also be affected by the anisotropy of the ring current. Thus, it appears at higher chemical shift value which was supported by constructing models for these compounds.

Moreover, the acid hydrazide **9** was synthesized through the reaction of enaminone ester **3** with hydrazine hydrate either in ethanol by heating the reactants at reflux for 3 h (50% yield) or by just warming them in toluene with continuous stirring for 10 min only (83% yield). The latter method was characterized by affording pure product in an excellent yield.

Condensation of the hydrazide **9** with different aromatic aldehydes was conducted by heating the reactants in ethanol containing catalytic amount of glacial acetic acid to afford the novel hydrazone derivatives **10**–**15**.

The structures of these hydrazones were confirmed using ^1H NMR spectra which showed the disappearance of NH_2 singlet broad peak around $\delta = 4.322$ ppm for the starting hydrazide. In addition, the appearance of singlet signal at $\delta = 7.939$–8.031 ppm assignable to azomethine proton as well as multiplet peaks around $\delta = 6.827$–7.794 ppm due to aromatic residue. Furthermore, the presence of vinylic H singlet around $\delta = 4.7$ ppm as well as NH broad singlet around $\delta = 7.0$ ppm of enaminone system exclude the formation of acridine nucleus under the reaction condition.

The formation of the novel thiosemicarbazide derivative **16** was accomplished through the reaction of equimolar amounts of hydrazide **9** and phenyl isothiocyanate in refluxing ethanol. In addition, the thiosemicarbazide **16** was cyclized either under acidic



Scheme 2. Synthetic pathway for compounds **16**–**19**.

condition (conc. H_2SO_4) to afford the novel thiadiazole derivative **17** or in basic media (8% NaOH) to give the triazole derivative **18** (Scheme 2).

Furthermore, the acid hydrazide **9** was allowed to react with 2-chloroethylisocyanate by stirring the reactants together in ethanol at room temperature for 12 h [20] in order to produce the novel 1-(2-(5,5-dimethyl-1-oxo-2-cyclohexen-3-ylamino)acetyl)-4-(2-chloroethyl)semicarbazide (**19**).

The new compounds were characterized using melting points and thin layer chromatography techniques in different solvent systems. Moreover, the structures of the novel compounds were determined using elemental analyses and various spectroscopic methods.

2.2. Biological study

Compounds **3**–**19** were evaluated *in vitro* to explore their cytotoxic effect using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay and also their antiviral activities were studied adopting plaque reduction assay.

2.2.1. Cytotoxicity test

Cytotoxicity of the new compounds was tested against hepatoma cell line (HepG2) at 10 μmol/ml using MTT assay [21,22]. The results were calculated and expressed as percentage reduction in cell proliferation (Table 1).

All tested compounds showed marked and significant cytotoxic effect when the treated cells incubated for 24 h except compound **4**. Unfortunately, compounds **10**, **12**, **15** and **17** cannot be determined due to solubility problems.

First of all, through analysis of the data presented in Table 1 and illustrated in Fig. 1, it was observed that the ester **3** showed 50% reduction in the cell growth. It can be modified to acyclic derivatives or cyclized to nonclassical acridines.

Table 1
Effect of test compounds on cell growth of hepatoma cell line (HepG2) incubated for 24 h *in vitro*.

Test compounds	Absorbance	Cell growth	%Age reduction
Control	0.724	100	0.00
T 3	0.360	50	50
T 4	0.700	96.68	3.32
T 5	0.368	50.83	49.17
T 6	0.332	45.86	54.14
T 7	0.328	45.3	54.7
T 8	0.449	62.01	37.99
T 9	0.307	42.6	57.4
T 11	0.434	59.94	40.06
T 13	0.432	59.68	40.32
T 14	0.601	83.01	16.99
T 16	0.327	45.16	54.84
T 18	0.466	64.36	35.64
T 19	0.302	41.71	58.29

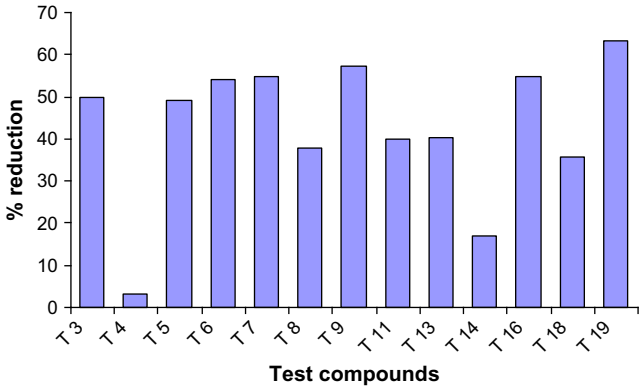


Fig. 1. Effect of new compounds on cell growth of hepatoma cell line (HepG2) incubated for 24 h *in vitro* expressed as %age reduction.

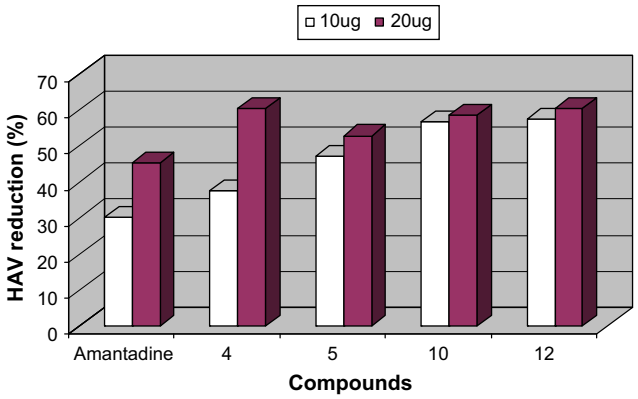


Fig. 2. Effect of some compounds (**4**, **5**, **10**, **12**) on *Hepatitis A Virus* in comparison with the reference drug amantadine at concentrations 10 and 20 μg/ml.

- Regarding the acyclic derivatives (**9**, **11**, **13**, **14**, **16** and **19**), it was observed that semicarbazide (**19**), hydrazide (**9**) and thiosemicarbazide (**16**) showed higher cytotoxic activity (58.29, 57.4 and 54.84%, respectively) than the hydrazone derivatives (**11**, **13**, **14**). The higher activity of the semicarbazide (**19**) may be attributed to the presence of chloroethylurea moiety in the semicarbazide which can form aziridinium cation alkylating DNA inhibiting cell growth [20].

Table 2
Antiviral activity for amantadine and compounds **3**–**19** against *Hepatitis A Virus* at concentrations 10 and 20 μg/ml.

Compound No	Concentration (μg/ml)	Initial virus count (PFU/ml)[a]	Virus count (PFU/ml)	%Age reduction
Amantadine[b]	10 μg	1.06×10^7	0.74×10^7	30.1
	20 μg		0.58×10^7	45.2
3	10 μg		1.00×10^7	5.6
	20 μg		0.9×10^7	15
4	10 μg		0.66×10^7	37.7
	20 μg		0.42×10^7	60.3
5	10 μg		0.56×10^7	47.1
	20 μg		0.5×10^7	52.8
6	10 μg		0.74×10^7	30.1
	20 μg		0.56×10^7	47.1
7	10 μg		0.74×10^7	30.1
	20 μg		0.55×10^7	48.1
8	10 μg		0.8×10^7	24.5
	20 μg		0.58×10^7	45.2
9	10 μg		0.7×10^7	33.9
	20 μg		0.69×10^7	34.9
10	10 μg		0.46×10^7	56.6
	20 μg		0.44×10^7	58.4
11	10 μg		0.6×10^7	43.3
	20 μg		0.77×10^7	27.3
12	10 μg		0.45×10^7	57.5
	20 μg		0.42×10^7	60.3
13	10 μg		0.96×10^7	9.4
	20 μg		0.9×10^7	15
14	10 μg		0.8×10^7	24.5
	20 μg		0.65×10^7	36.2
15	10 μg		0.64×10^7	39.6
	20 μg		0.6×10^7	43.3
16	10 μg		0.86×10^7	18.8
	20 μg		0.64×10^7	39.6
17	10 μg		0.58×10^7	45.2
	20 μg		0.54×10^7	49
18	10 μg		0.76×10^7	28.3
	20 μg		0.74×10^7	30.1
19	10 μg		0.61×10^7	42.4
	20 μg		0.63×10^7	40.5

[a] PFU: Plaque forming unit.
[b] Amantadine: positive control.

- It was also noted that cyclization of the thiosemicarbazide **16** to the triazole **18** led to decrease in the cytotoxic activity from 54.84 to 35.64% (Table 1 and Fig. 1).
- It was found also that the acyclic semicarbazide **19** was the most cytotoxic derivative (58.29%) while the cyclized parent acridine **4** bearing 9-unsubstitutedphenyl group (3.32%) was the least active one. Replacement of the phenyl group of acridine **4** by *p*-substituted phenyl led to the enhancement of the cytotoxic activity in general to reach 54.7% for compound **7** (Table 1). These acridines may act either through binding tightly but reversibly to DNA by intercalation between the base pairs of the double helix or by blocking topoisomerase binding to DNA. The latter mechanism of action may be of great interest in the development of anticancer or antiviral agents [4–6].

Thus, it was noted that the acyclic derivatives (**9**, **16**, **19**) were relatively more active than the cyclized acridine derivatives (**4**–**8**).

2.2.2. Evaluation of the antiviral activity

All the synthesized compounds **3**–**19** were examined *in vitro* against *Hepatitis A Virus* (HAV) to evaluate their antiviral activity using plague infectivity reduction assay [23]. The known drug, amantadine, commonly utilized for therapeutic treatment of HAV was used as a reference drug. The results of the antiviral evaluation recorded in Table 2 and illustrated in Fig. 2 revealed the following:

The ester **3** showed very weak antiviral activity which was improved through its cyclization to nonclassical acridines or its conversion to acyclic derivatives.

1. For the acridine derivatives **4**–**8**, the most active one at a dose of 20 μg was the parent compound **4** bearing unsubstitutedphenyl group whereas it caused 60.3% reduction in the virus titer (Fig. 2) while compound **8** bearing a *p*-methoxyphenyl group showed the least antiviral activity at both conc. 10 μg and 20 μg. The order of antiviral activity for acridines at a dose of 20 μg/ml is **4** > **5** > **6** > **7** > **8**.
2. Regarding the acyclic derivatives, the hydrazone bearing the *p*-chlorophenyl moiety (**12**) was the most active one whereas it caused 60.3% reduction in the virus titer at 20 μg/ml (Fig. 2) while most derivatives showed moderate antiviral activity.

The rank order of antiviral activity is **12** > **4** > **10** > **5**. The parent acridine **4** bearing 9-unsubstitutedphenyl group showed very weak *in vitro* cytotoxic activity (3.32%) against hepatoma cell line (HepG2) and in the same time it exhibited the highest antiviral activity (60.3%) at concentration 20 μg/ml against *Hepatitis A Virus*, so, it can be subjected to future optimization to be used as a safe antiviral agent.

3. Conclusion

From cyclic β-diketone, heterocyclic derivatives such as acridines and thiadiazole or acyclic systems as hydrazones and semicarbazide were synthesized. The acyclic semicarbazide **19** showed a higher *in vitro* cytotoxic activity against hepatoma cell line (HepG2) than the cyclized acridine derivatives. The antiviral study revealed that the acridine **4** and the hydrazone **12** were more active against *Hepatitis A Virus* than the reference drug amantadine upon using the plague infectivity reduction assay.

4. Experimental protocols

4.1. Chemistry

Melting points were determined with a *Gallenkamp* digital melting point apparatus in open capillaries and are uncorrected. IR spectra (KBr) were recorded using *Bruker* spectrophotometer. ^{1}H NMR spectra were determined on *Varian Gemini* 200 MHz using *TMS* as an internal standard (chemical shifts in δ, ppm). Mass spectra were measured on a GCMS-QP1000EX-*SHIMADZU* with ionization energy 70 eV. Elemental analyses were determined using Heraeus (Elemntar), CHNS analyzer (Germany) at Microanalytical Center, Faculty of Science, University of Cairo, Giza, Egypt. Their results corresponded to the calculated values within experimental error. TLC was performed on silica gel G (*Fluka*) and spots were visualized by iodine vapors or irradiation with UV light (254 nm). The starting materials were purchased from *Sigma–Aldrich*.

4.1.1. Ethyl 2-(5,5-dimethyl-3-oxocyclohex-1-enylamino)acetate (**3**)

A mixture of equimolar amounts (21.42 mmol) of 5,5-dimethyl-1,3-cyclohexanedione (dimedone, **1**), ethyl glycinate HCl (**2**) and anhydrous sodium acetate was heated under reflux either in toluene (50 ml) for 15 h (method 1) or in ethanol (50 ml) for 2.5 h (method 2). The reaction mixture was filtered, concentrated and then allowed to cool to room temperature. The reaction mixture was mixed with petroleum ether (60–80) with continuous stirring. The obtained crystalline product was filtered, dried and crystallized from petroleum ether (60–80).

Yield: 65% (method 1); 85% (method 2); m.p.: 101–103 °C; IR: $\upsilon = 3282, 3260$ (NH), 3093 (CH, aromatic), 2953 (CH, aliphatic), 1743 (C═O, ester), 1602 (C═O, cyclic) cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta = 1.067$ (s, 6H, $2CH_3$), 1.272 (t, 3H, $CH_2\underline{CH_3}$), 2.182 (s, 2H, CH_2), 2.238 (s, 2H, CH_2), 3.801 (s, 1H, CH_2CO), 3.824 (s, 1H, CH_2CO), 4.243–4.279 (q, 2H, $\underline{CH_2}CH_3$), 4.992 (s, 1H, vinylic H), 7.25 (s, 1H, NH, exch.) ppm. Anal. calcd for $C_{12}H_{19}NO_3$:C, 63.98; H, 8.50; N, 6.22. Found: C, 63.93; H, 8.45; N, 6.42%.

4.1.2. General procedure for preparation of compounds **4–8**

A solution of enaminone **3** (10 mmol) and half equivalent of aromatic aldehyde (5 mmol) in glacial acetic acid (15 ml) were heated at reflux for 4 h. The reaction mixture was allowed to cool to room temperature and then diluted with water. The separated product was filtered, washed with water, dried and crystallized with ethanol/H_2O (5:2).

4.1.2.1. Ethyl 2-[9-(phenyl)-1,2,3,4,5,6,7,8-octahydro-3,3,6,6-tetramethyl-1,8-dioxoacridin-10(9H)-yl]acetate (**4**).

Yield: 56%; m.p.: 202–204 °C; IR: $\upsilon = 3063$ (CH, aromatic), 2960 (CH, aliphatic), 1750 (CO, ester), 1632 (CO, cyclic) cm^{-1}; MS: *m*/*z* (rel. int.) = 435 (M^+, 19.8), 358 (100.0), 330 (23.0), 274 (4.7), 218 (2.0), 165 (2.0), 115 (1.9), 77 (9.8). Anal. calcd for $C_{27}H_{33}NO_4$: C, 74.45; H, 7.64; N, 3.22. Found: C, 74.55; H, 7.40; N, 3.25%.

4.1.2.2. Ethyl 2-[9-(4-bromophenyl)-1,2,3,4,5,6,7,8-octahydro-3,3,6,6-tetramethyl-1,8-dioxoacridin-10(9H)-yl]acetate (**5**).

Yield: 53%; m.p.: 208–210 °C; MS: *m*/*z* (rel. int.) = 516 ($M^+ + 1$, 4.7), 515 (M^+, 5.6), 458 (3.9), 426 (9.9), 358 (64.4), 270 (16.3), 201 (14.6), 154 (69.5), 90 (25.3), 55 (100.0). Anal. calcd for $C_{27}H_{32}BrNO_4$: C, 63.04; H, 6.27; N, 2.72. Found: C, 63.07; H, 6.33; N, 2.74%.

4.1.2.3. Ethyl 2-[9-(4-chlorophenyl)-1,2,3,4,5,6,7,8-octahydro-3,3,6,6-tetramethyl-1,8-dioxoacridin-10(9H)-yl]acetate (**6**).

Yield: 50%; m.p.: 195–197 °C; ^{1}H NMR ($CDCl_3$): $\delta = 0.954$ (s, 6H, $2CH_3$), 1.065 (s, 6H, $2CH_3$), 1.319 (t, 3H, $CH_2\underline{CH_3}$), 2.187 (s, 4H, $2CH_2$), 2.309 (d, 4H, $2CH_2$), 4.275–4.385 (m, 4H, $\underline{CH_2}CH_3 + CH_2CO$), 5.168 (s, 1H, C_9-acridine H), 7.115 (d, 2H, $J = 8.6$ Hz, ArH), 7.291 (d, 2H, $J = 8.4$ Hz, ArH) ppm. Anal. calcd for $C_{27}H_{32}ClNO_4$: C, 69.00; H, 6.86; N, 2.98. Found: C, 69.13; H, 6.75; N, 3.11%.

4.1.2.4. Ethyl 2-[9-(4-hydroxyphenyl)-1,2,3,4,5,6,7,8-octahydro-3,3,6,6-tetra methyl-1,8-dioxoacridin-10(9H)-yl]acetate (**7**).

Yield: 52%; m.p.: 252–254 °C; IR: $\upsilon = 3335$ (OH), 3022 (CH, aromatic), 2960 (CH, aliphatic), 1738 (CO, ester), 1628 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta = 0.966$ (s, 6H, $2CH_3$), 1.055 (s, 6H, $2CH_3$), 1.316 (t, 3H, $CH_2\underline{CH_3}$), 1.714 (br s,1H, OH, exch.), 2.198 (s, 4H, $2CH_2$), 2.295–2.367 (m, 4H, $2CH_2$), 4.257–4.386 (m, 4H, $\underline{CH_2}CH_3 + CH_2CO$), 5.125 (s, 1H, C_9-acridine H), 6.514 (d, 2H, $J = 8.4$ Hz ArH), 7.095 (d, 2H, $J = 8.6$ Hz ArH) ppm. Anal. calcd for $C_{27}H_{33}NO_5$: C, 71.82; H, 7.37; N, 3.10. Found: C, 71.66; H, 7.16; N, 3.13%.

4.1.2.5. Ethyl 2-[9-(4-methoxyphenyl)-1,2,3,4,5,6,7,8-octahydro-3,3,6,6-tetramethyl-1,8-dioxoacridin-10(9H)-yl]acetate (**8**).

Yield: 58%; m.p.: 239–241 °C; IR: $\upsilon = 3066$ (CH, aromatic), 2959 (CH, aliphatic), 1744 (CO, ester), 1629 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$): $\delta = 0.969$ (s, 6H, $2CH_3$), 1.067 (s, 6H, $2CH_3$), 1.319 (t, 3H, $CH_2\underline{CH_3}$), 2.195 (s, 4H, $2CH_2$), 2.305 (d, 4H, $2CH_2$), 3.716 (s, 3H, OCH_3), 4.278–4.372 (m, 4H, $\underline{CH_2}CH_3 + CH_2CO$), 5.163 (s, 1H, C_9-acridine H), 6.695 (d, 2H, $J = 8.6$ Hz, ArH), 7.242 (d, 2H, $J = 8.4$ Hz, ArH) ppm. Anal. calcd for $C_{28}H_{35}NO_5$: C, 72.23; H, 7.58; N,3.01. Found: C, 72.22; H, 7.36; N, 3.08%.

4.1.3. 2-(5,5-Dimethyl-3-oxocyclohex-1-enylamino)acetohydrazide (**9**)

4.1.3.1. Method 1. A mixture of ester **3** (10 mmol) and hydrazine hydrate (15 mmol) in ethanol (50 ml) was refluxed for 3 h. The solvent was removed by distillation under reduced pressure and the separated product was filtered, and then crystallized from ethanol.

4.1.3.2. Method 2. A mixture of ester **3** (10 mmol) and hydrazine hydrate (15 mmol) in toluene (50 ml) was just warmed with continuous stirring for 10 min only. The reaction mixture was allowed to cool to room temperature and the separated product was filtered, washed with toluene and then crystallized.

Yield: 50% (method 1); 83% (method 2); m.p.: 214–216 °C; IR: $\upsilon = 3304$, 3249 (NH_2, 2NH), 3086 (CH, aromatic), 2954 (CH, aliphatic), 1658 (CONH), 1609 (C═O, cyclic) cm^{-1};^{1}H NMR (DMSO-d_6): $\delta = 0.980$ (s, 6H, $2CH_3$), 1.969 (s, 2H, CH_2), 2.237 (s, 2H, CH_2), 3.599 (s, 1H, CH_2CO), 3.628 (s, 1H, CH_2CO), 4.322 (br s, 2H, NH_2, exch.), 4.750 (s, 1H, vinylic H), 7.115 (br s, 1H, NH, exch.), 9.188 (br s, 1H, NH, exch.) ppm. Anal. calcd for $C_{10}H_{17}N_3O_2$: C, 56.85; H, 8.11; N, 19.89. Found: C, 57.00; H, 8.10; N, 19.98%.

4.1.4. General procedure for preparation of compounds **10–15**

A mixture of equimolar amounts (5 mmol) of hydrazide **9** and the appropriate aldehyde was refluxed in ethanol (30 ml) containing acetic acid (1 ml) for 2 h. After cooling, the reaction mixture was treated with least amount of cold H_2O and the separated product was filtered and then crystallized from the appropriate solvent.

*4.1.4.1. 2-(5,5-Dimethyl-3-oxocyclohex-1-enylamino)-N′-benzylideneacetohydrazide (**10**).* Yield: 66%; m.p.: 211–213 °C; crystallized from ethanol/H_2O; MS: *m*/*z* (rel. int.) = 299 (M^+, 39.1), 284 (53.0), 269 (35.7), 207 (20.0), 180 (20.9), 152 (46.1), 138 (20.0), 124 (27.8), 106 (53.0), 95 (32.2), 83 (59.1), 67 (100.0).Anal. calcd for $C_{17}H_{21}N_3O_2$:C, 68.20; H, 7.07; N, 14.04. Found: C, 68.40; H, 6.92; N, 14.24%.

*4.1.4.2. 2-(5,5-Dimethyl-3-oxocyclohex-1-enylamino)-N′-(4-bromobenzylidene)acetohydrazide (**11**).* Yield: 85%; m.p.: 245–247 °C; crystallized from dioxane/H_2O; IR: $\upsilon = 3264$ (NH), 3088 (CH, aromatic), 2952 (CH, aliphatic),1693 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 0.974$ (s, 6H, $2CH_3$), 1.967 (s, 2H, CH_2), 2.279 (s, 2H, CH_2), 3.808 (s, 1H, CH_2CO), 4.216 (s, 1H, CH_2CO), 4.743 (s, 1H, vinylic H), 7.082 (br s, 1H, $\underline{NH}CH_2$, exch.), 7.648–7.655 (m, H, ArH), 7.980 (s, 1H, CH═N), 11.666 (s,1H, NHCO exch.) ppm. Anal. calcd for $C_{17}H_{20}BrN_3O_2$: C, 53.98; H, 5.33; N, 11.11. Found: C, 53.97; H, 5.22; N, 11.42%.

*4.1.4.3. 2-(5,5-Dimethyl-3-oxocyclohex-1-enylamino)-N′-(4-chlorobenzylidene)acetohydrazide (**12**).* Yield: 90%; m.p.: 236–237 °C; crystallized from ethanol/H_2O; IR: $\upsilon = 3259$ (NH), 3087 (CH, aromatic), 2953 (CH, aliphatic), 1692 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 1.007$ (s, 6H, $2CH_3$), 1.999 (s, 2H, CH_2), 2.309 (s, 2H, CH_2), 3.809 (d, 1H, $J = 5.6$ Hz, CH_2CO), 4.231 (d, 1H, $J = 5.4$ Hz, CH_2 CO), 4.782 (s, 1H, vinylic H), 7.066 (s, 1H, $\underline{NH}CH_2$, exch.), 7.506 (d, 2H, $J = 8.6$ Hz, ArH), 7.752 (d, 2H, $J = 8.4$ Hz, ArH), 8.031 (s, 1H, CH═N), 11.652 (s, 1H, NHCO exch.) ppm. Anal. calcd for $C_{17}H_{20}ClN_3O_2$: C, 61.17; H, 6.04; N, 12.59. Found: C, 61.18; H, 6.11; N, 12.65%.

*4.1.4.4. 2-(5,5-Dimethyl-3-oxocyclohex-1-enylamino)-N′-(4-hydroxybenzylidene)acetohydrazide (**13**).* Yield: 80%; m.p.: 247–249 °C; crystallized from ethanol; IR: $\upsilon = 3397$ (OH), 3300, 3175 (NH), 3078 (CH, aromatic), 2960 (CH, aliphatic), 1686 (CONH), 1609 (C═O, cyclic) cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 1.007$ (s, 6H, $2CH_3$), 2.002 (s, 2H, CH_2), 2.286 (d, 2H, CH_2), 3.773 (d, 1H, $J = 5.4$ Hz, CH_2CO), 4.189 (d, 1H, $J = 5.2$ Hz, CH_2CO), 4.789 (s, 1H, vinylic H), 6.827 (d, 2H, $J = 7.8$ Hz, ArH), 7.037 (br s, 1H, NH exch.), 7.535 (d, 2H, $J = 8.6$ Hz, ArH), 7.939 (s, 1H, N═CH), 9.919 (s, 1H, OH, exch.), 11.393 (s, 1H, NH, exch.) ppm. Anal. calcd for $C_{17}H_{21}N_3O_3$: C, 64.74; H, 6.71; N, 13.32. Found: C, 64.88; H, 6.90; N, 13.42%.

*4.1.4.5. 2-(5,5-Dimethyl-3-oxocyclohex-1-enylamino)-N′-(4-methoxybenzylidene) acetohydrazide (**14**).* Yield: 66%; m.p.: 235–236 °C; crystallized from ethanol; IR: $\upsilon = 3371$, 3206 (NH), 3068 (CH, aromatic), 2957 (CH, aliphatic), 1688, 1607 (CO) cm^{-1}; MS: *m*/*z* (rel. int.) = 330 ($M^+ + 1$, 2.2), 329 (M^+, 2.6), 134 (38.0), 67 (100.0). Anal. calcd for $C_{18}H_{23}N_3O_3$: C, 65.63; H, 7.04; N, 12.76. Found: C, 65.78; H, 7.02; N, 12.66%.

*4.1.4.6. 2-(5,5-Dimethyl-3-oxocyclohex-1-enylamino)-N′-(4-nitrobenzylidene) acetohydrazide (**15**).* Yield: 85%; m.p.: 260–261 °C; crystallized from ethanol; IR: $\upsilon = 3366$, 3256 (NH), 3082 (CH, aromatic), 2957 (CH, aliphatic), 1695 (CO), 1520, 1343 (NO_2) cm^{-1}. MS: *m*/*z* (rel. int.) = 345 (M^+, 7.2), 344 (26), 343 (9.9), 330 (5.3), 330 (5.3), 329 (26.3), 67 (100.0). Anal. calcd for $C_{17}H_{20}N_4O_4$: C, 59.29; H, 5.58; N, 16.27. Found: C, 59.42; H, 5.70; N, 16.37%.

*4.1.5. N^1-[2-(5,5-Dimethyl-3-oxocyclohex-1-enylamino)acetyl]-N^4-phenyl thiosemicarbazide (**16**)*

A mixture of the hydrazide **9** (5 mmol) and phenyl isothiocyanate (5 mmol) in 40 ml ethanol was heated at reflux for 1.5 h. After cooling, the separated product was filtered and crystallized from dioxane/H_2O.

Yield: 90%; m.p.: 192–193 °C; IR: $\upsilon = 3286$, 3255, 3228 (NH), 3019 (CH, aromatic), 2956 (CH, aliphatic), 1701, 1680 (CO) cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 1.001$ (s, 6H, $2CH_3$), 1.996 (s, 2H, CH_2), 2.289 (s, 2H, CH_2), 3.826 (d, 2H, CH_2CO), 4.841 (s, 1H, vinylic H), 7.100 (br s, 1H, $\underline{NH}CH_2$ exch.), 7.169–7.471 (m, 5H, ArH), 9.613 (br s, 1H, NHCS, exch.), 9.723 (s, 1H, NHPh, exch.), 10.170 (s,1H, NHCO, exch.) ppm. Anal. calcd for $C_{17}H_{22}N_4O_2S$: C, 58.94; H, 6.40; N, 16.17. Found: C, 58.89; H, 6.55; N, 16.41%.

*4.1.6. 5,5-Dimethyl-3-[(5-mercapto-4-phenyl-4H-1,2,4-triazol-3-yl)methylamino] cyclohex-2-enone (**17**)*

Thiosemicarbazide **16** (5 mmol) was heated at reflux in NaOH (8%, 20 ml) for 2 h. The solution was cooled, filtered and acidified with 2 N HCl. The separated solid was filtered, dried and crystallized from ethanol/H_2O.

Yield: 60%; m.p.: 296–297 °C; IR: $\upsilon = 3269$ (NH), 3064 (CH, aromatic), 2949 (CH, aliphatic), 1600 (CO, cyclic) cm^{-1}. ^1H NMR (DMSO-d_6): $\delta = 0.887$ (s, 6H, $2CH_3$), 1.908 (s, 2H, CH_2), 1.980 (s, 2H, CH_2), 4.106 (d, 2H, N–CH_2), 4.713 (s, 1H, vinylic H), 7.057–7.533 (m, 7H, ArH + 2NH) ppm. Anal. calcd for $C_{17}H_{20}N_4OS$: C, 62.17; H, 6.14; N, 17.06. Found: C, 61.85; H, 5.77; N, 17.52%.

*4.1.7. 5,5-Dimethyl-3-[(5-(phenylamino)-1,3,4-thiadiazol-2-yl)methylamino] cyclohex-2-enone (**18**)*

Thiosemicarbazide 16 (3 mmol) was dissolved in 10 ml concentrated H_2SO_4 while cooling and then allowed to stand for 1 h. The reaction mixture was poured on crushed ice with continuous stirring and then neutralized with NH_4OH. The separated product was filtered, washed with water and crystallized from dioxane/H_2O.

Yield: 50%; m.p.: 252–254 °C; MS: *m*/*z* (rel. int.) = 330 ($M^+ + 2$, 5.5), 329 ($M^+ + 1$, 23.3), 328 (M^+, 64.1), 210 (11.2), 190 (56.5), 136 (41.1), 118 (28.7), 77 (100.0). Anal. calcd for $C_{17}H_{20}N_4OS$: C, 62.17; H, 6.14; N, 17.06. Found: C, 62.17; H, 6.21; N, 17.40%.

*4.1.8. N^1-[2-(5,5-Dimethyl-3-oxocyclohex-1-enylamino)acetyl]-N^4-(2-chlor oethyl)semicarbazide (**19**)*

A mixture of the hydrazide **9** (5 mmol) and 2-chloroethylisocyanate (5 mmol) in ethanol (25 ml) was stirred at room temperature for 12 h. The separated product was filtered and crystallized from ethanol.

Yield: 75%; m.p.: 168–169 °C; ^1H NMR (DMSO-d_6): $\delta = 0.983$ (s, 6H, $2CH_3$), 1.979 (s, 2H, cyclic 4-CH_2), 2.256 (s, 2H, cyclic 6-CH_2), 3.320–3.350 (m, 2H, CH_2NH), 3.549–3.611 (m, 2H, CH_2CO), 3.707–3.737 (m, 2H, CH_2Cl), 4.765 (s, 1H, vinylic H), 6.617 (t, 1H, $NHCH_2$, exch.), 7.124 (br s, 1H, NH, exch.), 8.07 (s, 1H, NH, exch.) 9.756 (s, 1H, NH, exch.) ppm. Anal. calcd for $C_{13}H_{21}ClN_4O_3$: C, 49.29; H, 6.68; N, 17.69. Found: C, 49.40; H, 6.69; N, 17.80%.

4.2. Biological study

4.2.1. Cytotoxicity test

4.2.1.1. Reagents. MTT solution (0.5 mg/ml) in complete medium RPMI-1640; Phosphate buffered saline (PBS, 0.15 mM pH 7.4); Dimethyl sulphoxide (DMSO).

4.2.1.2. Cell culture. Tumor Liver cell line (HepG2), was supplied by Naval American Research Unit – Egypt (NAmRU). Cells were propagated and maintained in RPMI-1640 medium with L-glutamine (Sigma) and supplemented with 10% fetal calf serum (Sigma) for growth and 2% for maintenance medium; 1% of 4% sodium bicarbonate (Merck) and 1% antibiotic mixture (1,000,000 units of penicillin G sodium and 1,000,000 μg streptomycin sulfate); in 100 ml deionized water.

4.2.1.3. MTT test. The cytotoxic effect of compounds **3–19** was done according to Mosmann [21]. The cells at approximately 80% confluence (i.e., logarithmically growing cells) were selected for trypsinization. The cell suspension was prepared and cells were seeded in 96-well microplates (3×10^3 cells/well) in 100 μl RPMI-1640 culture medium and incubated at 37 °C and 5% CO_2 overnight. After overnight incubation, the cells were treated with the corresponding newly synthesized compounds and then incubated for further 24 h. The medium was discarded and the cells were washed with sterile PBS and then 100 μl of the MTT (0.5 mg/ml) solution was added to each well. Cells were incubated for 4 h and then the blue crystals were dissolved in 100 μl DMSO and the optical density (OD) was measured at 570 nm (reference filter 690 nm) using an automatic microplate reader. The results were recorded and expressed as %age reduction in cell growth of hepatoma cell line (HepG2) compared with the control (Table 1).

4.2.1.4. Statistics. Statistical evaluation of the results was done using MSTAT-C.

4.2.2. Evaluation of the antiviral activity

4.2.2.1. Cells. Hepatoma cell line (HepG2): The cells were propagated in Dulbecco's minimum essential medium (DMEM) supplemented with 10% foetal bovine serum and 1% antibiotic–antimycotic mixture (10,000 units penicillin G sodium, 10.000 mg streptomycin sulfate and 250 mg amphotericin B). The pH was adjusted at 7.2–7.4 by 7.5% sodium bicarbonate solution.

4.2.2.2. Virus. Hepatitis A Virus cell culture adapted strain (HAV-MBB) kindly provided by Prof. Dr. Verena Gauss-Muller, Luebeck University of Medicine, Institute of Molecular Virology, Germany.

4.2.2.3. Preparation of compounds for assay. The tested compounds were dissolved as 100 mg each in 1 ml of 10% DMSO in water. The final concentration was 100 μg/μl (Stock solution). The dissolved stock solutions were sterilized by addition of 50 μg/ml antibiotic–antimycotic mixture.

4.2.2.4. Plaque reduction assay. A 96-well plate was cultivated with hepatoma (HepG2) cell culture (10^5 cells/ml) and incubated for 2 days at 37 °C. HAV was diluted to give 10^4 PFU/ml (Plaque forming unit/ml) final concentrations and then mixed with the tested compounds at 10 and 20 μg/10^5 cells concentration and incubated overnight at 4 °C. Growth medium was removed from the multi-well plate and then virus–compound mixture was inoculated (100 μl/well). After 1 h contact time, the inoculum was aspirated and 3 ml of MEM with 1% agarose was overlaid the cell sheets. The plates were left to solidify and incubated at 37 °C until the development of virus plaques. Cell sheets were fixed in 10% formalin solution for 2 h, and stained with crystal violet. Control virus and cells were treated identically without the test compound. Virus plaques were counted and the % age reduction was calculated.

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