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NEW PYRIMIDINE AND FUSED PYRIMIDINE DERIVATIVES: SYNTHESIS AND ANTI HEPATITIS A VIRUS (HAV) EVALUATION

Ahmed Ali El-Sayed,[a*] Mahmoud El-Shahat,[a] Samira T. Rabie,[a] Eman M. Flefel,[a,b] Dina N. Abd-Elshafy[c]

[a] Photochemistry Department, National Research Center, Dokki, Cairo, Egypt.
[b] Chemistry Department, Faculty of Science, Taibah University, Saudi Arabia.
[c] Water pollution Department, National Research Center, Dokki, Cairo, Egypt.

***Corresponding author e-mail:** ahmedcheme4@yahoo.com

ABSTRACT

Some novel fused pyrimidine derivatives **5**–**14** were prepared starting with compound **4**. Also, Ethyl 4-(2,5-dimethoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate **2** was used for the preparation of some fused pyrimidine analogs **15**–**19**. All synthesized compounds were evaluated for their antiviral activity against HAV virus by determination of plaque infectivity count assay on HepG2 cells. Compound **7** has high antiviral activity against HAV even higher than Amentadine (positive control) and on studying its mechanism of action it showed combined action between adsorption and replication of HAV.

Keywords: Pyrimidine/ Fused Pyrimidine Derivatives/ HAV virus.

INTRODUCTION

Hepatitis A virus (HAV) is an important human pathogen causing hepatitis, Tens of millions of individuals worldwide are estimated to become infected with HAV each year with a higher incidence in developing countries. The fecal/oral route is the most important means of transmission of hepatitis A, and infection with HAV can cause sporadic and epidemic acute hepatitis in humans.[1,2] HAV infection causes fever, malaise, weakness, anorexia, nausea, vomiting, arthralgias and myalgias.[3,4]

Fulminant hepatitis is a severe complication of hepatitis A virus infection (HAV). Its mechanism is unknown but spontaneous recovery is frequent. There are no data on the level of viral replication according to the clinical form of HAV.[5] A high fatality rate among chronic hepatitis B or C patients with HAV super-infection was observed.[6]

Although there are no commercial antiviral drugs specifically licensed for treating HAV infection, ribavirin, amentadine and 2-deoxy-D-glucose are among several antiviral substances known to interfere with HAV replication.[7] Human wild-type HAV rarely grows in cell culture and requires several weeks to months in culture before it can be detected.[8] HAV has been adapted to a variety of primate[9,10] and nonprimate cell lines.[11,12] It was reported that HAV MBB strain was adapted to grow on PLC/PRF/5 cell line.[13]

HAV MBB strain was used to test antiviral activity of many compounds like some pyrrolo[2,3-*d*]pyrimidines,[14] some sugar arylglycinoyl hydrazones and their oxadiazoline derivatives,[15] synthesized triazolo[4,3-*b*]pyridazines[16] , 5-(1,2,3-triazol-1-ylmethyl)uridine derivatives[17] and from plant extracts that were examined for their anti HAV MBB activity are Dianthus caryophyllus L. and LupinustermesL. seed extracts.[18]

In the same direction and in continuation of our previous work, we test anti-HAV activity of all new synthetic pyrimidine derivatives and study the mechanism of action of active ones that might act as precursor for new anti-HAV drug.

Materials and Methods (Experimental)

Chemistry: All melting points are uncorrected and measured an electrothermal apparatus (Buchi 535, Switzerland) in an open capillary tube. IR spectra expressed in (cm^{-1}) were recorded in KBr pellets on a PA-9721 IR spectrophotometer. ^1H NMR spectrum was obtained on a Varian EM-390 (500 MHz) spectrometer, using TMS as internal reference and chemical shifts (δ) are expressed in ppm. Mass spectra were recorded on Kratos (75 eV) Ms Equipment. Elemental analyses were carried out by the microanalytical unit at National Research Centre, Giza, Egypt. All reactions were monitored by thin layer chromatography, carried out on 0.2 mm silica gel 60 F-254 (Merck) plates using UV light (245 and 365 nm) for detection.

6-(2,5-Dimethoxyphenyl)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (**1**)
A mixture of 2,5-dimethoxybenzaldehyde (1 mmol), thiourea (1 mmol) and ethyl cyanoacetate (1 mmol) in sodium ethoxide (20 ml) was stirred for 1h at room temperature. The reaction mixture was poured onto ice cold water and acidified by hydrochloric acid; the precipitated solid was filtered, dried and recrystalized from ethanol to give compound **1**.Yield 92%; m.p. 166–168°C. IR spectrum (KBr, ν, cm^{-1}): 3204, 3113 (2NH); 2256 (CN); 1720 (C=O). ^1H NMR spectrum (DMSO-d_6, δ ppm): 3.77 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 6.68–7.05 (m, 3 H, Ar-H), 10.11 (s, 1H, NH; D_2O exchangeable), 11.70 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (289, M^+, 73). Anal. Calcd. for $C_{13}H_{11}N_3O_3S$ (289.31): C, 53.97; H, 3.83; N, 14.52; S, 11.08. Found: C, 53.69; H, 3.55; N, 14.27; S, 10.81.

***General for synthesis compounds* 2 *and* 3**

A mixture of 2,5-dimethoxybenzaldehyde (1 mmol), thiourea (1 mmol) and ethyl acetoacetate or acetylacetone (1 mmol) in absolute ethanol (15 ml) containing few drops of concentrated hydrochloric acid was refluxed for 4–6 h. The formed precipitate was filtered, washed with water several time, dried and recrystallized from ethanol to give compounds **2** or **3** respectively.

Ethyl 4-(2,5-dimethoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate **(2)**
Yield 71%; m.p. 203–205°C. IR spectrum (KBr, ν, cm^{-1}): 3293, 3184 (2NH); 1705 (C=O). ^1H NMR spectrum (DMSO-d_6, δ ppm): 1.04(t, 3H, CH_3CH_2O), 2.28 (s, 3H, CH_3), 3.74 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 3.93(q, 2H, CH_3CH_2O), 5.44 (d, *J* = 3.9 Hz, 1H, Pyrimidine-H), 6.58–6.95 (m, 3 H, Ar-H), 9.18 (d, *J* = 1.5 Hz,1H, NH; D_2O exchangeable), 10.20 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (307, [M^+-C_2H_5], 56), (263, [M^+- $COOC_2H_5$], 100). Anal. Calcd. for $C_{16}H_{20}N_2O_4S$ (336.41): C, 57.13; H, 5.99; N, 8.33; S, 9.53. Found: C, 56.85; H, 5.41; N, 8.09; S, 9.25.

1-[4-(2,5-Dimethoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl]ethanone **(3)**
Yield 77%; m.p. 239–241°C. IR spectrum (KBr, ν, cm^{-1}): 3285, 3183 (2NH); 1640 (C=O). ^1H NMR spectrum (DMSO-d_6, δ ppm): 2.10 (s, 3H, CH_3), 2.27 (s, 3H, $COCH_3$), 3.66 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 5.53 (d, *J* = 3.3 Hz, 1H, Pyrimidine-H), 6.57–6.97 (m, 3H, Ar-H) ,9.28 (d, *J* = 1.6 Hz, 1H, NH; D_2O exchangeable), 10.18 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (306, M^+, 20). Anal. Calcd. for $C_{15}H_{18}N_2O_3S$ (306.38): C, 58.80; H, 5.92; N, 9.14; S; 10.47. Found: C, 58.53; H, 5.67; N, 8.81; S; 10.20.

4-(2,5-Dimethoxyphenyl)-2-hydrazinyl-6-oxo-1,6-dihydropyrimidine-5-carbonitrile **(4)**
A mixture of compound **1** (1 mmol) and hydrazine hydrate (2 mmol, 99%) was refluxed for 2 h. The resulting solid was collected after cooling by filtration and recrystallized from ethanol to give compounds **4**. Yield 67%; m.p. 231–233°C. IR spectrum (KBr, ν, cm^{-1}): 3357–3191 (NH_2 + 2NH); 2283 (CN); 1685 (C=O). ^1H NMR spectrum (DMSO-d_6, δ ppm): 3.64 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.78 (bs, 2H, NH_2, D_2O exchangeable), 6.21 (s, 1H, NH; D_2O exchangeable), 6.87–6.99 (m, 3H, Ar-H), 9.20 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (287, M^+, 65). Anal. Calcd. for $C_{13}H_{13}N_5O_3$ (287.28): C, 54.35; H, 4.56; N, 24.38. Found: C, 54.08; H, 4.29; N, 24.11.

7-(2,5-Dimethoxyphenyl)-3-mercapto-5-oxo-1,5-dihydro[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitrile **(5)**
To a warmed sodium ethoxide solution [prepared by dissolving sodium metal (1 mmol) in ethanol (50 ml)], compound **4** (1 mmol) and carbon disulphide (10 ml) were added. The mixture was heated on water bath at 80°C under reflux for 14h, and then it was poured onto water, neutralized by diluted hydrochloric acid. The formed solid was collected and recrystallized from xylene to give **5**.
Yield 32%; m.p. 218–220°C. IR spectrum (KBr, ν, cm^{-1}): 3115 (NH); 2217 (CN); 1703 (C=O). ^1H NMR spectrum (DMSO-d_6, δ ppm): 3.67 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 4.66 (s, 1H, SH; D_2O exchangeable), 6.87–6.98 (m, 3H, Ar-H), 8.94(s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (329, M^+, 53). Anal. Calcd. for $C_{14}H_{11}N_5O_3S$ (329.33): C,

51.06; H, 3.37; N, 21.27; S, 9.74. Found: C, 50.79; H, 3.09; N, 20.91; S, 9.55.

***7-(2,5-Dimethoxyphenyl)-5-oxo-1,5-dihydro[1,2,4] triazolo[4,3-a]pyrimidine-6-carbonitrile* (6)**

A mixture of compound **4** (1 mmol) and triethyl orthoformate (20 ml) was refluxed for 21 h. The reaction mixture was filtered off on hot and the separated solid was recrystallized from ethanol to give **6**. Yield 53%; m.p. 227–229°C. IR spectrum (KBr, ν, cm^{-1}): 3210 (NH); 1717 (C=O); 2211(CN). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 3.65 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 6.94–7.30 (m, 4H, 3Ar-H+Triazol-H), 8.55 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (297, M^+, 33). Anal. Calcd. for $C_{14}H_{11}N_5O_3$ (297.27): C, 56.56; H, 3.73; N, 23.56. Found: C, 56.29; H, 3.45; N, 23.31.

***7-(2,5-Dimethoxyphenyl)-3-methyl-5-oxo-1,5-dihydro[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitrile* (7)**

A mixture of compound **4** (1 mmol) and acetic anhydride (10 ml) was refluxed for **9** h. The reaction mixture was cooled and poured onto cold water. The separated solid was filtered off, dried and recrystallized from ethanol to give **7**. Yield 46%; m.p. 249–251°C. IR spectrum (KBr, ν, cm^{-1}): 3211 (NH); 1722 (C=O); 2210 (CN). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 2.22 (s, 3H, CH_3), 3.65 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 7.01–7.22 (m, 3H, Ar-H), 9.01 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (311, M^+, 45). Anal. Calcd. for $C_{15}H_{13}N_5O_3$ (311.30): C, 57.87; H, 4.21; N, 22.50; Found: C, 57.60; H, 3.95; N, 22.25.

***7-(2,5-Dimethoxyphenyl)-5-oxo-3-phenyl-1,5-dihydro[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitrile* (8)**

A mixture of compound **4** (1 mmol) and benzoyl chloride (1 mmol) in ethanolic KOH (5%, 30 ml) was refluxed for 8 h. After cooling, diluted hydrochloric acid was added , the separated solid was filtered off, washed with K_2CO_3 (1 %, 100 ml), dried and then recrystallized from methanol to give **8**. Yield 33 %; m.p. 282–284°C. IR spectrum (KBr, ν, cm^{-1}): 3195 (NH); 2217 (CN); 1705 (C=O). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 3.61 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 7.23–7.87(m, 8H, Ar-H), 9.31 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (373, M^+, 30). Analysis for $C_{20}H_{15}N_5O_3$ (373.36): C, 64.34; H, 4.05; N, 18.76. Found C, 64.08; H, 3.81; N, 18.54.

***4-(2,5-Dimethoxyphenyl)-2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile* (9)**

A mixture of compound **4** (1 mmol) and acetyl acetone (2 mmol) in ethanol (20 ml) was refluxed for 12 h. After cooling, the separated solid was filtered off, dried and then recrystallized from dioxane to give **9**. Yield 49%; m.p. 275–277°C. IR spectrum (KBr, ν, cm^{-1}): 3223 (NH); 2210 (CN); 1685 (C=O). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 2.35 (s, 3H, CH_3), 2.64 (s, 3H, CH_3), 3.65 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 5.98 (s, 1H, pyrazole–H), 7.17–7.49 (m, 3H, Ar-H), 9.65 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (351, M^+, 63). Anal. Calcd. for $C_{18}H_{17}N_5O_3$ (351.36): C, 61.53; H, 4.88; N,19.93. Found: C, 61.25; H, 4.59; N, 19.66.

***General for synthesis compounds* 10 *and* 11**

A mixture of compound **4** (1 mmol) and phenacyl bromide or chloroacetone (1 mmol) in ethanolic KOH (5%, 30 ml) was refluxed for 6–8 h. after cooling diluted hydrochloric acid was added, the separated solid was filtered off, washed with K_2CO_3 (1 %, 100 ml) then dried and recrystallized from methanol or ethanol to give **10** or **11** respectively.

***8-(2,5-Dimethoxyphenyl)-6-oxo-3-phenyl-4,6-dihydro-1H-pyrimido[2,1-c][1,2,4]triazine-7-carbonitrile* (10)**

Yield 47%; m.p. 288–291°C. IR spectrum (KBr, ν, cm^{-1}): 3262 (NH); 2226 (CN); 1692 (C=O). ^{1}H NMR spectrum ($CDCl_3$, δ ppm): 3.63 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.87 (d, *J* = 9.23Hz, 1H, CH_2), 3.91 (d, *J* = 9.22 Hz, 1H, CH_2), 7.17–7.87 (m, 8H, Ar-H), 8.75 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (387, M^+, 39). Anal. Calcd. for $C_{21}H_{17}N_5O_3$ (387.39): C, 65.11; H, 4.42; N,18.08. Found: C, 64.83; H, 4.17; N, 17.71.

***8-(2,5-Dimethoxyphenyl)-3-methyl-6-oxo-4,6-dihydro-1H-pyrimido[2,1-c][1,2,4]triazine-7-carbonitrile* (11)**

Yield 41%; m.p. 264–266°C. IR spectrum (KBr, ν, cm^{-1}): 3244 (NH); 2223 (CN); 1701(C=O). ^{1}H NMR spectrum ($CDCl_3$, δ ppm): 2.34 (s, 3H, CH_3), 3.59 (s, 3H, OCH_3), 3.68 (s, 3H, OCH_3), 3.91 (d, *J* = 9.01Hz, 1H, CH_2), 3.99 (d, *J* = 9.12 Hz, 1H, CH_2), 7.30–7.39(m, 3H, Ar-H), 8.83 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (325, M^+, 55). Anal. Calcd. for $C_{16}H_{15}N_5O_3$ (325.32): C, 59.07; H, 4.65; N, 21.53. Found: C, 58.74; H, 4.38; N, 21.27.

***General for synthesis compounds* 12–14**

To a solution of compound **4** (1 mmol) in DMF (30 ml) and bromoacetic acid or 1,2-dichloroethane and/ or chloroacetyl chloride (1 mmol) was added dropwise under stirring at room temperature. The reaction mixture was then heated for 3–6 h and after cooling, poured onto cold water with vigorous

stirring. The precipitate was collected by filtration, washed with water, dried and recrystallized from THF to give compounds **12**, **13** or **14** respectively.

***8-(2,5-Dimethoxyphenyl)-4,6-dioxo-2,3,4,6-tetra hydro-1H-pyrimido[2,1-c][1,2,4]triazine-7-carbonitrile* (12)**

Yield 36%; m.p. 234–236°C. IR spectrum (KBr, ν, cm^{-1}): 3320–3150 (2NH); 2227 (CN); 1740,1714 (2C=O). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 3.60 (s, 3H, OCH_3), 3.64 (s, 3H, OCH_3), 4.35 (d, *J* = 9.52 Hz, 1H, CH_2), 4.46 (d, *J* = 9.48 Hz, 1H, CH_2), 6.96–7.01 (m, 3H, Ar-H), 8.02 (s, 1H, NH; D_2O exchangeable), 8.63 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (327, M^+, 22). Anal. Calcd. for $C_{15}H_{13}N_5O_4$ (327.29): C, 55.05; H, 4.00; N, 21.40. Found: C, 54.81; H, 3.77; N, 21.13.

***8-(2,5-Dimethoxyphenyl)-6-oxo-2,3,4,6-tetrahydro-1H-pyrimido[2,1-c][1,2,4]triazine-7-carbonitrile* (13)**

Yield 37%; m.p. 245–247°C. IR spectrum (KBr, ν, cm^{-1}): 3320– 3170 (2NH); 2223 (CN); 1740 (C=O). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 3.60 (s, 3H, OCH_3), 3.64 (s, 3H, OCH_3), 4.01–4.38 (m, 4H, CH_2CH_2), 7.03–7.29 (m, 3H, Ar-H), 8.40 (s, 1H, NH; D_2O exchangeable), 8.66 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (313, M^+, 45). Anal. Calcd. for $C_{15}H_{15}N_5O_3$ (313.31): C, 57.50; H, 4.83; N, 22.35. Found: C, 57.22; H, 4.57; N, 22.09.

***8-(2,5-Dimethoxyphenyl)-3,6-dioxo-2,3,4,6-tetrahydro-1H-pyrimido[2,1-c][1,2,4]triazine-7-carbonitrile* (14)**

Yield 41%; m.p. 237–239°C. IR spectrum (KBr, ν, cm^{-1}): 3331– 3190 (2NH); 2229 (CN); 1738, 1688 (2C=O). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 3.63 (s, 3H, OCH_3), 3.65 (s, 3H, OCH_3), 4.64 (d, *J* = 9.36 Hz, 1H, CH_2), 4.71 (d, *J* = 9.39 Hz, 1H, CH_2), 6.98–7.11 (m, 3H, Ar-H), 7.97 (s, 1H, NH; D_2O exchangeable), 8.20 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (327, M^+, 33). Anal. Calcd. for $C_{15}H_{13}N_5O_4$ (327.29): C, 55.05; H, 4.00; N, 21.40. Found: C, 54.79; H, 3.76; N, 21.09.

***Ethyl 7-(2,5-dimethoxyphenyl)-5-methyl-3-oxo-3,7-dihydro-2H-thiazolo[3,2-a]pyrimidine-6-carboxylate* (15)**

A mixture of compound **2** (1 mmol) with bromoacetic acid (1 mmol) in acetic acid (30 ml) / acetic anhydride (15 ml) mixture in the presence of fused anhydrous sodium acetate (2g) was refluxed for 3 h. The solution was cooled, gradually poured onto cold water and the formed precipitate was washed several times with water, filtered off and recrystallized from acetic acid to give compound **15**. Yield 33%; m.p. 238–240°C. IR spectrum (KBr, ν, cm^{-1}): 1740, 1705 (2C=O). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 1.06 (t, 3H, CH_3CH_2O), 2.21 (s, 3H, CH_3), 3.67 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 4.00 (q, 2H, CH_3CH_2O), 4.19 (d, *J* = 10.36 Hz, 1H, Thiazole-H), 4.33 (d, *J* = 10.25 Hz, 1H, Thiazole-H), 5.33 (s, 1H, Pyrimidine-H), 7.05–7.32 (m, 3 H, Ar-H). Ms, *m/z* (%): (376, M^+, 12). Anal. Calcd. for $C_{18}H_{20}N_2O_5S$ (376.43): C, 57.43; H, 5.36; N, 7.44; S, 8.52. Found: C, 57.15; H, 5.09; N, 7.17; S, 8.25.

***Ethyl 7-(2,5-dimethoxyphenyl)-5-methyl-3-oxo-2-(thiophen-2-ylene)-3,7-dihydro-2H-thiazolo[3,2-a]pyrimidine-6-carboxylate* (16)**

Method A. A mixture of compounds **15** (1 mmol) and thiophene-2-carboxaldehyde (1 mmol) in acetic acid (30 ml)/acetic anhydride (15 ml) mixture was refluxed for 3 h. The solution was cooled, gradually poured onto cold water and the formed precipitate was filtered off and recrystallized from glacial acetic acid to give compound **16**.

Method B. A mixture of compound **2** (1 mmol), bromoacetic acid (1 mmol) and thiophene-2-carboxaldehyde (1 mmol) in acetic acid (30 ml)/acetic anhydride (15 ml) mixture in the presence of anhydrous sodium acetate (2g) was refluxed for 5 h. The solution was cooled, gradually poured onto cold water and the formed precipitate was washed several times with water, filtered off, and recrystallized from acetic acid to give compound **16**, the product obtained here was identical in all aspects with compound obtained from method A. Yield (39% from Method A, 30% from Method B); m.p. 277–279°C. IR spectrum (KBr, ν, cm^{-1}): 1720, 1687(2C=O). ^{1}H NMR spectrum ($CDCl_3$, δ ppm): 1.07 (t, 3H, CH_3CH_2O), 2.24 (s, 3H, CH_3), 3.69 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 4.02 (q, 2H, CH_3CH_2O), 5.37 (s, 1H, Pyrimidine-H), 6.97–7.46 (m, 6 H, Ar-H), 9.10 (s, 1H, exocyclic vinylic-H). Ms, *m/z* (%): (470, M^+, 25). Anal. Calcd. for $C_{23}H_{22}N_2O_5S_2$ (470.57): C, 58.71; H, 4.71; N, 5.95; S, 13.63. Found: C, 58.45; H, 4.44; N, 5.77; S, 13.35.

***Ethyl 2-(2,5-dimethoxyphenyl)-4-methyl-6-oxo-6,11-dihydro-2H-pyrimido[2,1-b]qunazoline-3-carboxylate* (17)**

A mixture of compound **2** (1 mmol) and anthranilic acid (1 mmol) in 2% sodium ethoxide (20 ml) was refluxed for 9 h. The reaction mixture was cooled, poured onto ice cold water and acidified by diluted hydrochloric acid. The formed precipitate was filtered, washed several times with water, dried and then recrystallized from dioxane to give compound **17**.Yield (57%); m.p. 260–262°C ; IR (KBr) (ν, cm^-

[1]): 3215 (NH); 1735, 1698 (2C=O). ^{1}H NMR (DMSO-d_6 , δ ppm): 1.11 (t, 3H, CH_3CH_2O), 2.22 (s, 3H, CH_3), 3.66 (s, 3H, OCH_3), 3.68 (s, 3H, OCH_3), 3.99 (q, 2H, CH_3CH_2O), 5.24 (s, 1H, Pyrimidine-H), 7.27–8.10 (m, 7H, Ar-H), 9.11 (s, 1H, NH; D_2O exchangeable). Ms, *m/z* (%): (421, M^+, 37). Anal. Calcd. for $C_{23}H_{23}N_3O_5$ (421.45): C, 65.55; H, 5.50; N, 9.97.Found: C, 65.29; H, 5.21; N, 9.69.

***General for synthesis compounds* 18 and 19**

A mixture compound **2** (1 mmol) and ethyl cyanoacetate or malononitrile (1 mmol) in glacial Acetic acid (25 ml) and a catalytic amount of concentrated acid (H_2SO_4) (6–8 drops) was refluxed for 3 h. The reaction mixture was then cooled diluted with H_2O (10 ml) and neutralized with ammonia solution. The obtained crude product thus obtained was collected by filtration, washed with H_2O (3x) and crystallized from ethanol to afford compounds **18** or **19** respectively.

***Diethyl 3-amino-7-(2,5-dimethoxyphenyl)-5-methyl-7H-thiazolo[3,2-a]pyrimidine-2,6-dicarboxyl-ate* (18)**

Yield 37%; m.p. 174–176°C. IR spectrum (KBr, ν, cm^{-1}): 3320–3217 (NH_2); 1735, 1720 (2C=O). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 1.09 (t, 6H, $2CH_3CH_2$ O), 2.16 (s, 3H, CH_3), 3.66 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 4.11 (q, 4H, $2CH_3CH_2O$), 4.92 (bs, 2H, NH_2; D_2O exchangeable), 5.06 (s, 1H, Pyrimidine-H), 7.14–7.40 (m, 3H, Ar-H). Ms, *m/z* (%): (447, M^+, 8). Anal. Calcd. for $C_{21}H_{25}N_3O_6S$ (447.50): C, 56.36; H, 5.63; N, 9.39; S, 7.17. Found: C, 56.07; H, 5.31; N, 9.10; S, 6.88.

***Ethyl 3-Amino-2-cyano-7-(2,5-dimethoxyphenyl)-5-methyl-7H-thiazolo[3,2-a]pyrimidine-6-carbox-ylate* (19)**

Yield 43%; m.p. 273–275°C. IR spectrum (KBr, ν, cm^{-1}): 3317– 3210 (NH_2); 2215 (CN); 1727 (C=O). ^{1}H NMR spectrum (DMSO-d_6, δ ppm): 1.06 (t, 3H, CH_3CH_2O), 2.19 (s, 3H, CH_3), 3.64 (s, 3H, OCH_3), 3.67 (s, 3H, OCH_3), 4.06 (q, 2H, CH_3CH_2O), 4.66 (bs, 2H, NH_2; D_2O exchangeable), 4.97 (s, 1H, Pyrimidine-H), 7.07–7.22 (m, 3H, Ar-H). Ms, *m/z* (%): (400, M^+, 17). Anal. Calcd. for $C_{19}H_{20}N_4O_4S$ (400.45): C, 56.99; H, 5.03; N, 13.99; S, 8.01. Found: C, 56.71; H, 4.74; N, 13.74; S, 7.78.

ANTIVIRAL BIOASSAY

Cells and Viruses: Cells: HepG2 cells were propagated in DMEM medium. They were supplemented with 10% foetal bovine serum, 1% antibiotic- antimycotic mixture.

Viruses: a cell culture adapted strain of Hepatitis A was provided from Dr. Ali Fahmy, prof of virology in VACSERA of Egypt, it is isolated from sewage sample in Egypt confirmation was made by PCR and ELISA technique. Virus was titrated to give final concentration 10^6 PFU/ml.

Synthetic compounds preparation for bioassay: 10 mg of each compound was dissolved in 10% DMSO and 90% deionized water, decontaminated with 1% antibiotic- antimycotic mixture and stored in -2^0C.

Cytotoxicity assays: The cell culture safety doses of the dissolved synthetic compounds were performed by cell morphology technique.[26] A 96 well plate was seeded with HepG2 cells and incubated overnight. Synthetic compounds were inoculated with concentration, 5, 10, 15, 20µg /100 µL and observed microscopically for any morphological changes after 24 h incubation at 37^o C in a humidified incubator with 5% CO2.

Plaque infectivity count assay: Plaque infectivity count assay is the most widely accepted method for determining the % inhibition of virus as a result of being subjected to a given material.[27] A 12 well plate was cultivated with the HepG2 cells (10^5cell/ml) and incubated for overnight at 37°C. Virus was and mixed with the safe concentrations of each compound and incubated for 1 h at 37°C.

Growth medium was removed from the multi-well plate and virus-compound mixture was inoculated in plate wells. After 1 h contact time for virus adsorption, 1 ml of 2× DMEM medium 2% agarose overlaid the cell sheet. The plates were left to solidify and incubated at 37°C until the development of the viral plaques. Formalin was added for two hours then plates were stained with crystal violet staining solution. Control virus and cells were treated identically without compounds. Viral plaques were counted and the percentage of virus reduction was calculated.

Mechanism of virus inhibition:

Possible mechanism of HAV inhibition by the compounds was studied at three different levels:

Extract affects viral particle itself (virucidal):

Virucidal Assay[28] was carried out in a 12 well plate where HepG2 cells were cultivated (10^5 cell/ml) overnight at 37^{o}C. A volume of 100µl serum frees DMEM containing 10^6 PFU HAV was added to the concentration of compound resulting in viral inhibition. After 1 hour incubation, the mixture was diluted using serum free medium 3 times, each is 10 fold, that still allows existence of viral particles to grow on HepG2 cells, but leaves nearly no extract

(100 µl of each dilution were added to the HepG2 cell monolayer). After 1 hour contact time, DMEM overlayer with 2% agarose was added to the cell monolayer. Plates were left to solidify then incubated at 37°C to allow formation of viral plaques, fixed and stained as mentioned above. Percentage reduction in plaques formation by compound was calculated in comparison to control wells, where cells were infected with virus that was not pretreated with the compound.

Extract binds to cell receptor preventing viral adsorption (early replication step):

Viral Adsorption[29] HepG2 cells were cultivated in a 12 well plate (10^5 cell/ml) and incubated overnight at 37°C. Compound was applied at different concentrations in 200 µl medium without serum and co-incubated with the cells for 2 hours at 4°C. Unabsorbed compound was removed by washing cells three successive times with serum free-medium. HAV virus (diluted to 10^4 PFU/well) was then co-incubated with the pretreated cells for 1 hour followed by adding 3 ml DMEM with 2% agarose. Plates were left to solidify then incubated at 37°C to allow formation of viral plaques, fixed and stained as above mentioned. Percentage reduction in plaques formation was calculated in comparison to control wells where untreated HepG2 cells were directly infected with HAV.

Extract affects one of the enzymes inside the cell needed by the virus to complete its replication cycle (late replication step):

Viral replication Assay[30] was carried out in a 12 well plate where HepG2 cells were cultivated (10^5 cell/ml) and incubated overnight at 37°C. Virus was diluted to give 10^4 PFU/well, applied directly to the cells and incubated for 1 hour at 37°C. Unabsorbed viral particles were removed by washing cells three successive times by serum free-medium. Compound was applied at different concentrations, after 1 hour contact time, 2 ml of DMEM medium supplemented with 2% agarose was added to the cell monolayer. Plates were left to solidify and incubated at 37°C till appearance of viral plaques and completed as previously mentioned.

RESULTS AND DISCUSSION

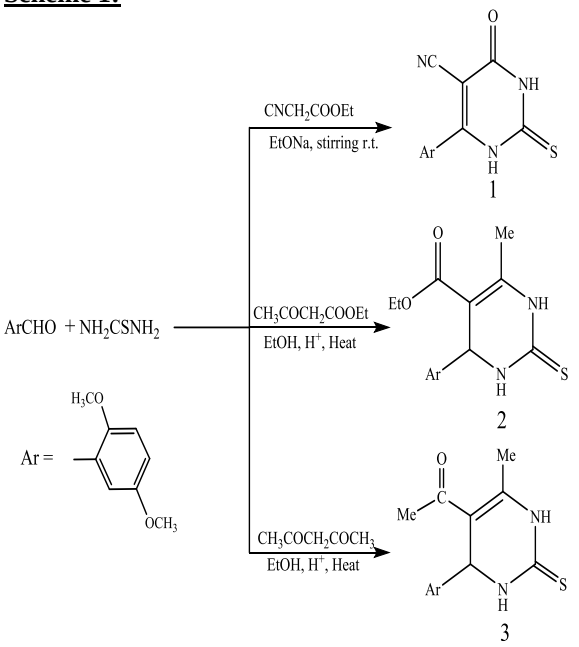
Chemistry :

Heating a mixture of 2,5-dimethoxybenzaldehyde, thiourea with different active methylene gruop (such as ethyl cyanoacetate, ethyl acetoacetate and acetylacetone) produced pyrimidine derivatives **1**–**3** (Scheme 1).[19] The spectral data of the latter compounds were in agreement with the proposed structures. (c.f. experimental).The mass spectra of compound **2** did not exhibit a molecular ion peak but it showed a peak at *m/z* 307 attributed to (M^+-C_2H_5, 56%) and base peak at *m/z* 263 (M^+-$COOC_2H_5$).

When compound **1** was treated with hydrazine hydrate, the hydrazine derivative **4** was obtained.[20] The IR and ^{1}H NMR spectra for compound **4** showed absorption bands for NH_2+ 2NH signals (c.f. experimental).

Compound **4** was utilized as a key starting material in the synthesis of many interesting pyrimidine derivatives. When compound **4** was allowed to react with carbon disulphide in sodium ethoxide solution , the product was identified as 7-(2,5-dimethoxy phenyl)-3-mercapto-5-oxo-1,5-dihydro[1,2,4]triazolo [4,3-*a*] pyrimidine-6-carbonitrile **5** (Scheme 2).[21] The absence of signal for NH_2 in both of IR and ^{1}H NMR spectra indicated compound **5** (c.f. experimental).

Scheme 1:



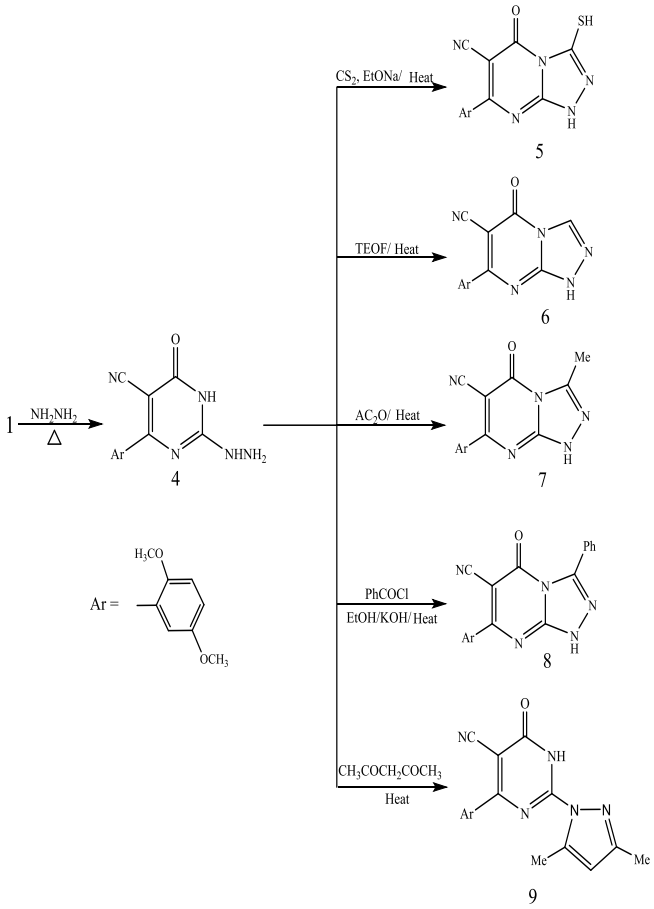
On the other hand, when compound **4** was refluxed with triethyl orthoformate or acetic anhydride and / or benzoyl chloride it afforded triazolo[4,3-*a*]pyrimidine-6-carbonitrile **6** ,**7** and **8**; respectively (scheme 2).[21] The structure of the new products was established according to their elemental and spectroscopic data. The IR spectrum of **6**,**7** and **8** revealed the absence of NH_2, NH groups and its ^{1}H NMR spectrum showed signals for triazol–CH_3 , triazol–H and /or triazol–Ph.

Condensation of compound **4** with acetyl acetone, gave 4-(2,5-dimethoxyphenyl)-2-(3,5-dimethyl-1*H*-pyrazol-1-yl)-6-oxo-1,6-dihydropyrimidine-5

carbonitrile (**9**).[21] IR spectrum of **9** revealed the absence of NH_2 group and its ^1H NMR spectrum showed signals at δ 2.35, 2.64 for two CH_3 and at δ 5.98 (s, 1H, pyrazole–H). Also, its mass spectrum showed the molecular ion peak M^+ at m/z 351.

pyrimido[2,1-*c*][1,2,4]triazine-7-carbonitrile **10** and **11** were isolated after treating compound **4** with phenacylbromide and/ or chloroacetone in ethanolic KOH (c.f., experimental). Further, reaction of compound **4** with bromoacetic acid or 1,2-dichloroethane and/or chloroacetyl chloride in DMF it afforded pyrimido[2,1-*c*] [1,2,4]triazine-7-carbonitrile **12** or **13** and / or **14** ; respectively (scheme 3) (c.f. experimental).[22]

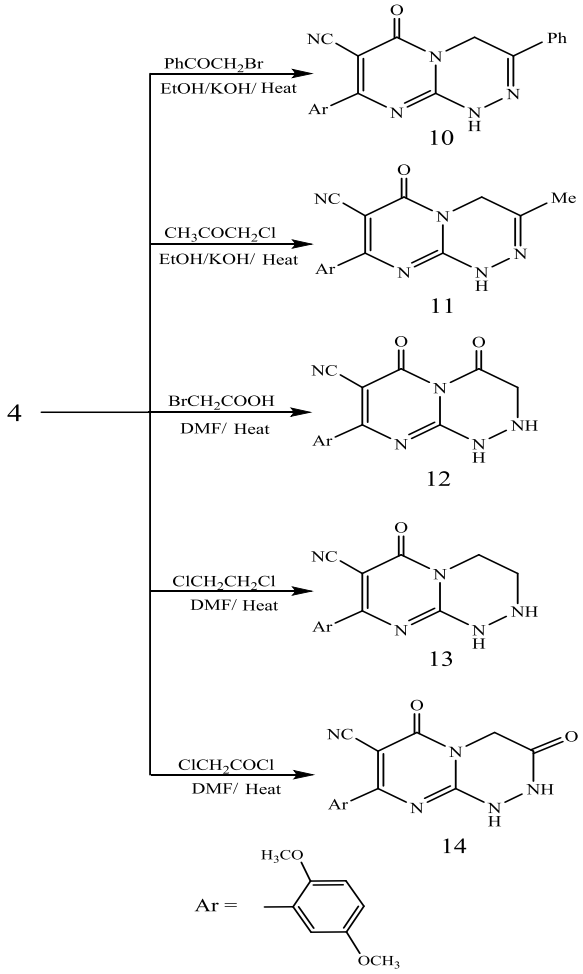
Scheme 2:



Heating of compound **2** with bromoacetic acid in a mixture of acetic acid/acetic anhydride produced thiazolo[3,2-*a*]pyrimidine-3,6-dione derivative **15**. The two protons at C–*2* appear at δ 4.19 and 4.33 ppm in ^1H-NMR spectrum.[23]

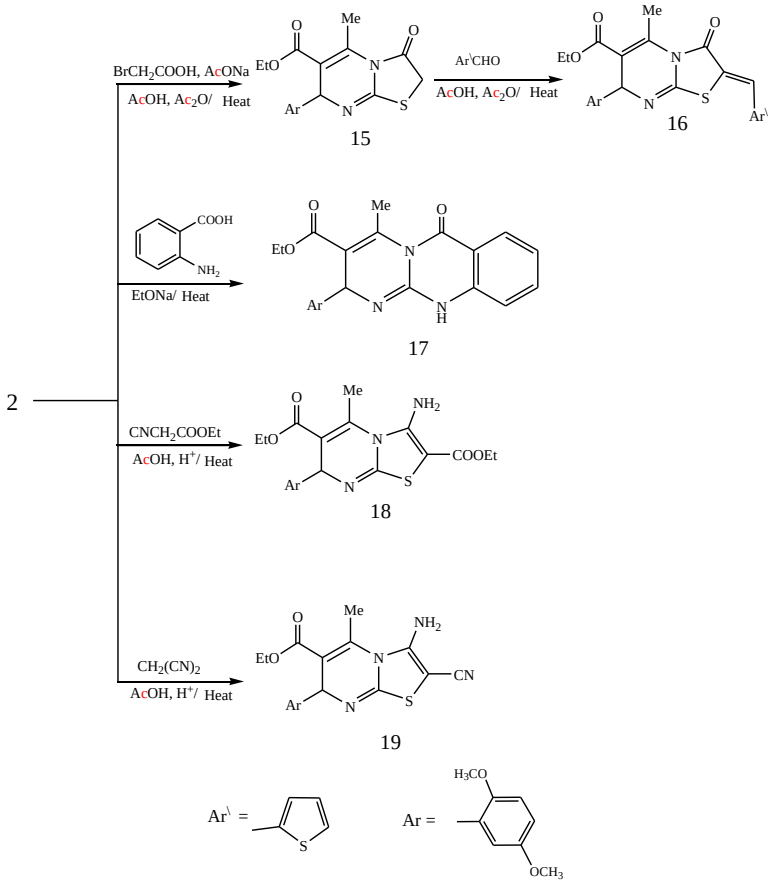
The presence of an active methylene in compound **15** could be confirmed by condensation with thiophene-2-carboxaldehyde in acetic acid/acetic anhydride mixture in the presence of anhydrous sodium acetate to produce 2-benzylidine derivative **16** (Scheme 4). The latter compound could be directly prepared from compound **2** by heating with bromoacetic acid and thiophene-2-carboxaldehyde in acetic acid/acetic anhydride mixture gave product identical in all aspects with compound **16**[23,24] (c.f. experimental).

Scheme 3:



Compound **2** was condensed with anthranilic acid in sodium ethoxide yielding pyrimido[2,1-*b*] qunazoline-3-carboxylate **17**.[19] On heating of compound **2** under reflux with cyano compounds containing active methylene group such as ethyl cyanoacetate and malononitrile using the acidified acetic acid method ($AcOH/H^+$); 3-Amino-2-substituted-7-(2,5-dimethoxyphenyl)-5-methyl-7*H*-thiazolo[3,2-*a*]pyrimidine-6-carboxylate **18** and **19** were obtained[25] (scheme 4) (c.f. experimental).

Scheme 4:



Antiviral bioassay:

In the present work we reported anti- HAV activity for **19** synthetic compounds. First of all cytotoxicity test was made to know the safe doses that can be used in antiviral assays without harming HepG2 cells **Table 1** which showed the safe doses of each compound. Safe concentrations were used in Plaque reduction assay which was made to detect any changes in viral count as a result of being treated with the compounds with respect to untreated control. Results in **Table 2** showed that compounds **3**, **4** and **15** increases viral count with respect to control, this might be as a result of a degrading effect of those compounds on the serum protein found in the growth media of the HepG2 cells subjecting more receptors for the virus to bind to and so results on an increase in viral count. Results also showed that compound **7** has high antiviral activity against HAV when compared with the effect of its different concentrations with the same concentrations of Amentadine (our positive control). Results of Figure **1** showed that compound **7** has higher inhibitory effect than Amentadine on applying the four concentrations used.

Mechanism of action of compound **7** was also studied and results in **Figure 2** showed that it has combined action as it has high effect in inhibiting HAV adsorption and also has inhibitory effect on viral replication. This can be discussed as follows that compound **7** was able to bind to HAV receptors preventing virus from entry and so prevent its adsorption causing decrease in viral count also when it enters inside the cell together with the virus it was able to affect one or more enzymes needed by the virus to complete its replicating cycle and so showed also decrease in viral count.

CONCLUSION

This work showed that 7-(2,5-Dimethoxyphenyl)-3-methyl-5-oxo-1,5-dihydro[1,2,4]triazolo[4,3-*a*] pyrimidine-6-carbonitrile has high anti HAV activity affecting both adsorption and replication of the virus, it can be used as precursor for anti HAV drug. Importance of this compound comes from the absence of antiviral drug for HAV and high fatality rate results in HAV infection of chronic HBV or HCV patients.

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Table 1. Cytotoxicity of compounds 1–19

compound	Concentrations (µg/100µl)			
	5	**10**	**15**	**20**
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	+1
7	-	-	-	+1
8	-	-	+1	+1
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	+1	+2	+3
13	-	-	-	-
14	-	-	-	-
15	-	+2	+3	+4
16	-	-	-	+1
17	-	+1	+2	+3
18	-	-	-	-
19	-	+1	+2	+3

Table 2. Results of plaque infectivity count assay showing Anti HAV activity of compounds 1–19

Compound	Concentration	% inhibition
1	15	0
	20	60
2	15	36
	20	46
3	15	0
	20	-40
4	15	-60
	20	-86
5	15	41
	20	15
6	15	0
	20	31
7	15	100
	20	100
8	15	12
	20	68
9	15	0
	20	0
10	15	0
	20	13
11	15	0

	20	0
12	5	0
	10	0
13	15	0
	20	0
14	15	34
	20	42
15	5	-40
	10	-79
16	15	9
	20	36
17	5	57
	10	41
18	15	20
	20	52
19	5	41
	10	68

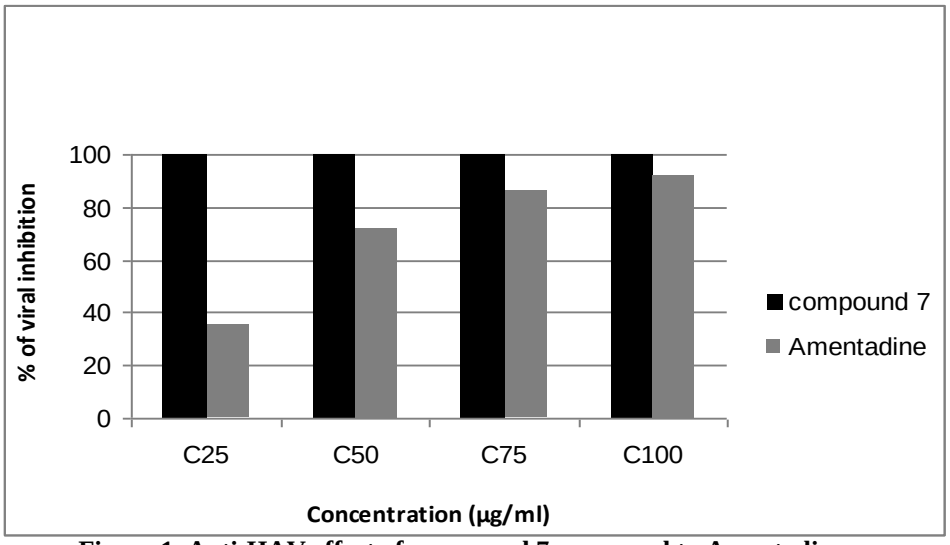

Figure 1. Anti-HAV effect of compound 7 compared to Amentadine

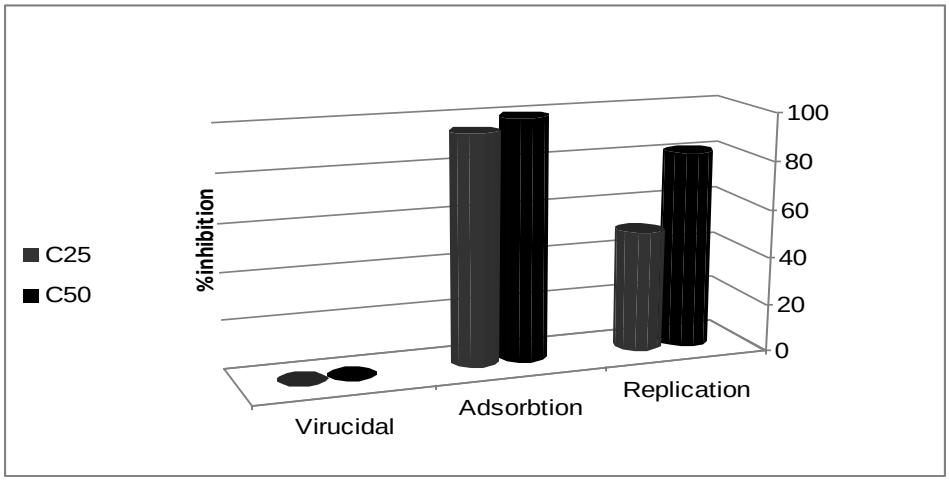

Figure 2. Mechanism of action of compound

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