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# Phytochemical Investigation and Differential Effects of *Cestrum elegans* Isolated Compounds as Antimicrobial and Virucidal against Hepatitis A Virus

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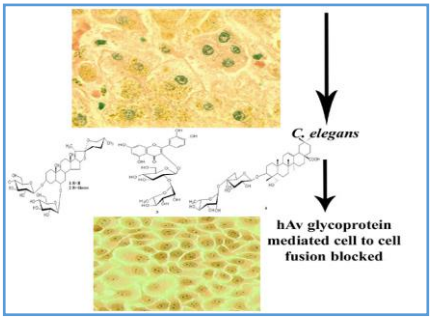
## ABSTRACT

A new steroidal saponin, named as (25R)-6α-[(β-D-glucopyranosyl) oxy]-5α-spirostan-3β-yl β-D-glucopyranosyl (1"→3')-*O*-β-D-glucopyranosid (2) was isolated from the flowers of *Cestrum elegans*. In addition, a known steroidal saponin (1), a flavonoid compound (3) along with a triterpene saponin (4) were isolated for the first time in genus *Cestrum* and identified based on their chromatographic properties, chemical and spectral data (HR-ESI-MS, $^{1}$H, $^{13}$C NMR, $^{1}$H-$^{1}$H COSY, HSQC, and HMBC). The four compounds were evaluated for their antibacterial, antifungal and antiviral activities. The maximum non-toxic concentration (MNTC) on vero cell line was 1.56 µg/mL for all compounds. While, compound 1 and 3 showed antiviral activity of 34.3% and 25%, respectively.




**Key words:** *Cestrum elegans*, Antiviral, Antimicrobial, Cytotoxicity, Steroidal saponin

## INTRODUCTION

Genus *Cestrum* (Family Solanaceae) comprises more than 300 species and is widely distributed in subtropical and tropical areas around the world like, India, Bangladesh, United States, Australia, Southern China and South America [1]. In folk medicine, *Cestrum* species have a long history for the treatment of several diseases and health disorders [2]. Different species belonging to genus *Cestrum* were previously investigated for their chemical constituents, e.g., parquine, carboxyparquine and steroid. Glycosides were also identified in *C. diurnum* [3], whereas saponins were identified in *C. parqui* [4, 5]. In Chinese folk medicine, *C. nocturnum* used for the treatment of some health problems [6]. The plant showed noticeable biological activities including; pesticidal [7], insecticidal [8], antimicrobial [9], cytotoxic [10], hepatoprotective [11], anticonvulsant [12], antidiabetic [13], larvicidal [14], anti-inflammatory, analgesic [15], antitumour [16], and wound healing [17]. Microbial and viral infections are still major risks to human health since there is a dramatic growth in microbial resistance to existence antibiotics. This situation encourages scientists to discover new naturally occurring antimicrobial agents to treat the infectious disease [18, 19]. The antimicrobial power of natural compounds is due to the ability of these compounds to interfere with the metabolism, receptor attach mechanism, reproduction and attenuation of microorganisms. In continuous to our work on *C. elegans* [3] therefore, the present study was designed to evaluate the cytotoxic, antiviral, antimicrobial activities of compounds isolated from *Cestrum elegans* (Family: Solanaceae), as well as isolation and identification of certain compounds from this plant using different chromatographic techniques and spectroscopic analyses.

### Material and Methods

### I- General

NMR spectra were recorded at 300 & 500 ($^{1}$H), and 75 & 125 ($^{13}$C) MHz, on Varian Mercury 300 and JEOL spectrometers. The δ-values were reported as ppm relative to TMS. HRESI-MS analyses were run on an LTQ-FT-MS (Thermo Electron, Germany).

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Optical rotation values were measured by use of an ATAGO POLAX-D, No. 936216, AEAGO Co., LTD. (Japan) polarimeter with a 1dm cell (ATAGO 901048). Column chromatography was performed using Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and silica gel G 60. Thin layer chromatography (TLC) was performed using silica gel plates F254 plates for TLC (Merck, Germany).

**II- Plant material**

Flowers of *Cestrum elegans* (C.E) were collected from Paraghel, Giza, Egypt in February, 2014. The plant was kindly identified by Dr. Threase Labib, Consultant in Orman Botanical Garden and National Gene Bank. A voucher specimen (No.C14/3/21) was kept at the herbarium of the garden.

**III- Extraction and isolation**

Dried flowers of *C. elegans* (190 g) were extracted with 85% MeOH (3 X 3L) at room temperature (each time for 7 days), and then concentrated under reduced pressure. The resulting residue (52 g) was dissolved in $H_2O$ (250 mL), extracted with $CHCl_3$ (250 mL X 3), EtOAc (250 mL X 3), followed by *n*-BuOH (150 mL X 3). The *n*-BuOH extract was washed with 100 mL distilled water for three times, and then concentrated to dryness by removing the solvent in a rotary evaporator. The *n*-BuOH extract (25 g) was subjected to CC. (5 X 100 cm) separation over silica gel (250 g) using a gradient of light petroleum ether (60-80ºC): $CHCl_3$ (1:0, 10:1, 5:1, 1:1 and 0:1, each 250 mL), and then with $CHCl_3$: MeOH (9:1, 8:2, 7:3, 1:1, 3:7 and 0:1, each 250 mL). Four fractions (Fr1-Fr4) were obtained according to the differences in composition indicated by TLC analysis. Fr2 ($CHCl_3$: MeOH, 8:2, 3 g) was purified by CC. over silica gel, eluting with MeOH: EtOAc (2:8), to give two main subfractions. The first subfraction was then separately chromatographed on Sephadex LH-20 with MeOH as eluent, whereby a pure sample of compound 1 (39 mg) was isolated, and has represented the major component of this subfraction. Crude 2 was crystallized from Fr3 ($CHCl_3$: MeOH; 7:3, 5 g) and purified by repeated crystallization from MeOH to yield a pure sample of compound 2 (55 mg). Repeated preparative TLC of the mother liquor using $CHCl_3$: MeOH: $H_2O$ (65:35:2; v/v/v) as the development eluent yielded a pure sample of compound 3 (49 mg). Fr4 ($CHCl_3$: MeOH, 1:1, 3 g) was rechromatographed on Sephadex LH-20 with MeOH to afford a pure sample of compound 4 (45 mg). All separation processes were followed up by Co-TLC using solvent systems: MeOH: $CHCl_3$ (3:7), MeOH: EtOAc: $CHCl_3$: $H_2O$ (35:32:28:2), $CHCl_3$: MeOH: $H_2O$ (65:35:3) and *n*-BuOH: EtOAc: $H_2O$ (4:1:1; v/v/v).

**IV- Acid hydrolysis**

Ten mg of compounds 1 and 2 were separately hydrolyzed with 2N HCl in EtOH under reflux at 90 ºC for 3 h. The solvent was then evaporated until most of EtOH eliminated, the residue diluted with $H_2O$ (15 mL) and neutralized with $NaHCO_3$, followed by extraction with $CHCl_3$ (100 mL). The sapogenin was identified in $CHCl_3$ layer by Comp-TLC (MeOH: $CHCl_3$; 1.5: 9.5). The $H_2O$-layer was then concentrated and subjected to Comparative-PC (EtOAc: $C_5H_5N$ : $H_2O$; 12:5:4) against authentic sugar samples.

**V- Antimicrobial activity evaluation**

The tested compounds were prepared by dissolving 2mg in 2ml of DMSO and then 100µl (containing 100µg) were used in this test. The antimicrobial activity of different samples was investigated by the agar cup plate method. Four different test microbes namely: *Staphylococcus aureus* (G+ve), *Pseudomonas aeruginosa* (G-ve), *Candida albicans* (yeast) and *Aspergillus niger* (fungus) were used. Nutrient agar plates were heavily seeded uniformly with 0.1ml of $10^5$-$10^6$ cells/ml in case of bacteria and yeast. A Czapek-Dox agar plate seeded by 0.1 ml the fungal inoculum was used to evaluate the antifungal activities. Then a hole (1cm diameter) was made in media by gel cutter (Cork borer) in sterile condition. Then one drop of melted agar was poured into hole and allowed to solidify to make a base layer. After that specific amount of the tested sample (0.1 ml) was poured into the hole. Then plates were kept at low temperature (4°C) for 2-4 hours to allow maximum diffusion. The plates were then incubated at 37°C for 24 hours for bacteria and at 30ºC for 48 hours in upright position to allow maximum growth of the organisms. The antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter (mm). The experiment was carried out more than once and mean of reading was recorded, while the positive control were Penicillin, Streptomycin, Neomycin, and Cyclohexamide respectively for the tested microbes by concentration 100 µg per cup [20].

**VI- Determination of the maximum non-toxic concentration [MNTC] of compounds on Vero cell**

Different concentrations from tested samples were prepared and growth medium was decanted from 96 well micro titer plates after confluent sheet of vero cell was formed. The cell monolayer was washed twice with wash media and double-fold dilutions of tested sample were made in DMEM. 0.1 mL of each dilution was tested in different wells leaving 3 wells as control, receiving only maintenance medium. Plate

was incubated at 37°C and examined frequently for up to 2 days. Cells were checked for any physical signs of toxicity, e.g. partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was prepared as (5mg/ml in PBS) (Bio Basic Canada Inc). 20µl MTT solution was added to each well and then placed on a shaking table, 150rpm for 5 minutes to thoroughly mix the MTT into the media. Mixture was incubated at (37°C, 5% $CO_2$) for 4 hours to allow the MTT to be metabolized. MTT metabolic product was resuspended in 200 µl DMSO, and rested on a shaking table, 150rpm for 5 minutes to thoroughly mix the formazan into the solvent. Optical density was readed at 560nm and subtracts background at 620nm. The maximum non-toxic concentration [MNTC] of each fraction was determined and used for further biological studies [3]

**VII- In vitro antiviral assay on hepatitis A Virus by MTT Assay**

Ten thousand cells were plated in 200 µl media per well in a 96 well plate. Leave 3 wells empty for blank controls and then incubated at (37°C, 5% $CO_2$) overnight to allow the cells to attach to the wells equal volume (1:1 v/v) of non-lethal dilution of tested sample and the virus suspension for one hour. 100µl were added from viral/ sample suspension and placed on a shaking table at150rpm for 5 minutes, and then at (37°C, 5% $CO_2$) for 1- days to allow the virus to take effect. 2ml or more of MTT solution per 96 well plate at 5mg/ml in PBS and 20ul MTT solution to each well. Then reshaked on table at 150rpm for 5 minutes, to thoroughly mix the MTT into the media. Finally, incubated at (37°C, 5% $CO_2$) for 1-5 hours to allow the MTT to be metabolized. The media was dumped and formazan (MTT metabolic product) resuspend in 200ul DMSO and shaked at 150rpm for 5 minutes to thoroughly mix the formazan into the solvent. The optical density evaluated at 560nm and subtracts background at 620nm. Optical density should be directly correlated with cell quantity [21]. In each paragraph and clicking the required style on the drop-down menus.

## Results and Discussion

### A.I- Chromatographic Isolation of Pure Compounds

The *n*-butanol soluble phase of *C. elegans* methanolic extract gave two steroidal glycosides (1, 2) after a series of chromatographic separations. Based on chemical and physicochemical analyses, compounds 1, 3, 4 were identified as three known metabolites (25*R*)-6α-[(β-D-glucopyranosyl) oxy]-5α-spirostane-3β-yl β-D-glucoopyranoside (1) [22]. Quercetin 3-*O*-neohesperidoside (3) [23], and 3, 23-di-hydroxy Olean 12-en 28-oic acid 3-*O*-α-$^1C_4$ rhamnopyranosyl (1"→6')-*O*-β-$^4C_1$ glucopyranoside (4) [24, 25] (Figure 1).

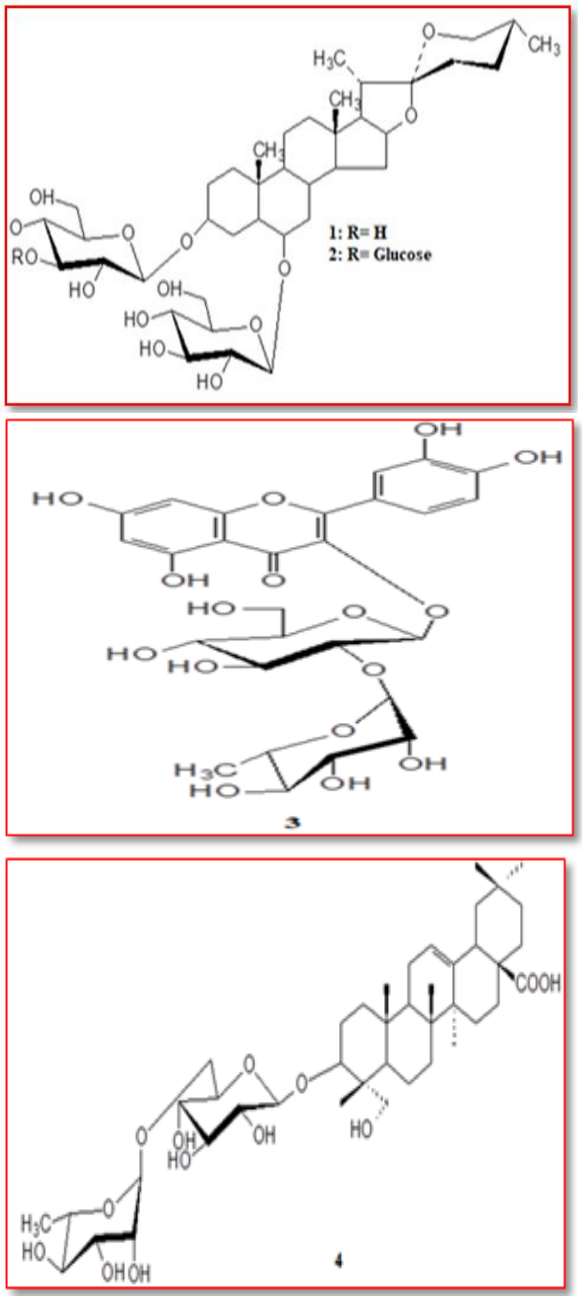



**Figure 1:** Chemical structures of the isolated compounds.

### A.II- Structural Elucidation of New Compound (2)

Compound 2 exhibited a chromatographic behavior indicative of a steroidal saponin structure (brown color with sulfuric acid spray reagent, followed by heating at 120ºC) and gave a positive Liebermann-Burchard test. The negative HRESI-MS showed a molecular ion peak at *m/z* 917 $[M-H]^-$ corresponding to $C_{45}H_{74}O_{19}$. The MS -spectrum gave a negative fragment at *m/z* 755 $[M-H-162]^-$ (loss of hexose). The

MS fragmentation led to an ion at *m/z* 593 [M-H-2x162]$^-$ (loss of 2 hexose). The fragment ion peak at *m/z* 431 [aglycone-H]$^-$, attributed to the loss of an extra 162 due to a third hexose. These data, together with acid hydrolysis that afforded glucose in the aqueous phase (Co-PC) were compatible with the structure of three *O*-sugar units (Co- TLC and PC with authentic samples) with a saturated dihydroxy spirostane. The spirostane structure of 2 was supported by the $^1$H NMR spectrum that exhibited signals at $\delta_H$ 0.71 and 0.77 ppm assigned to $CH_3$-18 and 19 and two secondary methyl signals for $CH_3$-27 and 21 as two doublets at $\delta_H$ 0.73 ($J$ = 6.5 0Hz) and .0.89 ($J$ = 6.5 Hz), respectively. These correlated with $^{13}$C resonances at $\delta_C$ 12.1, 14.8, 16.2 and 17.0 signals, 21 and 27 ppm, respectively in the HSQC spectrum. Additional evidence for the spirostane skeleton was obtained from the $^{13}$C NMR spectrum, which exhibited 45 carbon moieties 27 of which were for the aglycone moiety and 18 for three sugar unit and an acetylic quaternary carbon at $\delta_C$ 108.8 ppm (Table 1). The equatorial orientation of the C-27 methyl was verified by the axial-axial coupling of H-26*ax* at $\delta_H$ 3.32 and H-25*ax*, (*J*26*ax*, 25ax = 10.5 Hz) in the $^1$HNMR spectrum. This structural feature was further supported by the $^{13}$C shift at $\delta_C$ 17.0 ppm assignable to C-27, which is characteristic of a (25*R*) spirostane [26]. 1D and 2D NMR spectroscopic experiments, including the proton multiplicities, confirmed that 2 had the usual 5α ring junctions of B/C *trans*, C/D *trans*, D/E *cis*, C-22α, C-3ß, C-6α, and a C-25*R* configuration. The resonances of C-3 at $\delta_C$ 77.0 and C-6 at $\delta_C$ 79.4 ppm were characteristic of a bisdesmosidic aglycone with 3ß and 6α-hydroxy substituents. This evidence was further confirmed by the long-range three bond HMBC correlations exhibited between H-1' ($\delta_H$ 4.20) and C-3 (δC 77.0) of the aglycone. Similarly, there were correlations between H-1''' ($\delta_H$ 4.71) and C-6 (δC 79.4) of the aglycone Thus, the structure of the aglycone was assigned as (25*R*)-3β, 6α-dihydroxy-5α-spirostane) [27]. Three anomeric proton signals were assigned at δH 4.20 (d, $J$ = 7.5Hz, β-glucosyl) 4.41, (d, $J$ = 8 Hz, β-glucosyl) and 4.71 (d, $J$ =7 Hz, β-glucosyl), in the $^1$HNMR spectrum through their direct one bond coupling in the HSQC spectrum with anomeric carbon signals at δC 100.8, 102.4 and 103.3 ppm, respectively, The glucose moieties were deduced to adopt β-$^4C_1$-pyranose stereo structures based on δ-values of their $^1$H and $^{13}$C-resonances and *J*-values of the anomeric protons (Table 1). The deshielded location of C-3' of glucose at $\delta_C$ 85.0 suggesting the (1''→3') interglycosidic link. This evidence was further conformed by observation of the three bond correlation peak between the H-1'' proton signal at $\delta_H$ 4.41 ppm and the C-3 signal at $\delta_H$ 85.0 ppm in the HMBC spectrum. All $^1$H and $^{13}$C-resonances of the sugar moieties were assigned with the aid of $^1$H-$^1$H-COSY and HSQC correlation peaks and by comparison with corresponding data of structurally related compounds [28]. This compound isolated for the first time in nature and identified as (25R)-6α-[(β-D-glucopyranosyl) oxy]-5α-spirostan-3β-yl β-D-glucopyranosyl (1''→3')-*O*-β-D-glucopyranosid.

**B. Biological Activities Assessment**

**B.I- Antimicrobial deferential effects:**

The antimicrobial activities for the four compounds evaluated by cup technique and clear zone in compare to the referenced specific antibiotics [29]. The results revealed that compound 1 has a vital activity against Gram-positive *S. aureus*, Gram-negative *P. aeruginosa* and pathogenic dimorphic yeast *C. albicans*, while compound 2 shows varied antimicrobial effects and mostly against mycotoxins fungus *A. niger*. Compound 3 shows a very high antibacterial effect against *S. aureus* and antifungal for *A. niger*. Compound 4 shows a very high anti-yeast effect against *C. albicans* and antibacterial for *S. aureus* as stated in table 2 and figure 2.

**B.II- Significantly blocks hAv entry into Vero cells:**

Viral infections play a crucial role in human diseases, and up to date outbreaks despite the progress made in immunization and drug development. Thus, identifying novel antiviral drugs is of critical importance and natural products are a superb source for such discoveries. Hepatitis A virus is usually spread by eating food or drinking water contaminated with infected feces [30]. This study identifies a potent anti-hAv activity extracts from dried flowers of *Cestrum elegans* plant. Significantly inhibited hAv entry and viral glycoprotein mediated cell-cell fusion in a cell culture model. Most of the studies on antiviral activities use MTT as say to check the cytotoxic concentration of any new molecule synthetic or natural [31].
This assay gives an idea about the maximum nontoxi c dose of the compounds. The *in vitro* cytotoxicity analysis was carried out by the extracted compounds to determine the maximum non-toxic dose (MNTD) to vero cell line. In this study we found that, (MNTC) statistically achieved after triplet optical density reads was 1.56 ug/ml for all compounds as shown in figure (3), while extract concentrations up to 1000 ug/ml didn't impair at all the cell viability. Compounds 1 and 3 possess the highest antiviral activities against hepatitis A virus grown on vero cell line were the activities 34.4 and 25 respectively (Table 4, Figure 4 & 5).

**Table 1:** $^{13}C$ &$^{1}HNMR$ spectrum of compound 1(500⁄ 125MHz, DMSO-$d_6$.) δ in ppm and *J* values (Hz), are given in parentheses. All carbon and proton resonances were assigned on the basis of 2D ($^{1}H$-$^{1}H$ COSY, HSQC and HMBC)

| No. | $\delta_C$ | $\delta_H$ | No. No. | $\delta_C$ | $\delta_H$ |
|---|---|---|---|---|---|
| 1 | 38.1 | | 24 | 28.2 | |
| 2 | 32.0 | | 25 | 28.5 | |
| 3 | 77.0 | | 26 | 66.6 | |
| 4 | 31.4 | | 27 | 17.0 | 0.73 |
| 5 | 53.6 | | 1' | 100.8 | 4.20, d, *J* = (7.5 Hz) |
| 6 | 79.4 | | 2' | 73.8 | |
| 7 | 40.0 | | 3' | 85.0 | |
| 8 | 34.6 | | 4' | 69.7 | |
| 9 | 53.6 | | 5' | 76.4 | |
| 10 | 36.4 | | 6' | 60.7 | |
| 11 | 22.4 | | 1'' | 102.4 | 4.41, d, (*J* = 7 Hz) |
| 12 | 39.2 | | 2'' | 73.8 | |
| 13 | 39.0 | | 3'' | 76.5 | |
| 14 | 55.3 | | 4'' | 69.7 | |
| 15 | 30.7 | | 5'' | 76.5 | |
| 16 | 80.2 | | 6'' | 61.3 | |
| 17 | 60.7 | | 1''' | 103.3 | 4.71, d,( *J* = 8 Hz) |
| 18 | 12 .1 | 0.71 | 2''' | 73.8 | |
| 19 | 14.8 | 0.77 | 3''' | 76.4 | |
| 20 | 41.2 | | 4''' | 69.4 | |
| 21 | 16.2 | 0.89 | 5''' | 76.5 | |
| 22 | 108.1 | | 6''' | 61.3 | |
| 23 | 29.5 | | | | |

Interestingly, the blocking activity of *C. elegans* was only seen when hepatitis A virions were pre-incubated with the extract as compared to pre-incubation with target cells. This result suggested two important points: (1) the tested concentrations of *C. elegans* weren't cytotoxic to the cells because the viral entry was quantified when compounds pre-incubated with the target cells; and (2) the inhibitory effect of *C. elegans* targets the virions as virucidal effect was seen when the extract was incubated with the virus (Figure 4). Future studies are going to be needed to isolate, detect and characterize the active molecule present in *C. elegans* which is liable for anti-hepatitis A activity. In addition, activity could also be evaluated against individual hAv glycoprotein. Further characterization of the molecule and its toxicity will aid within the development of the therapeutic agents against hepatitis A infection.

**B.III- Vero treatment inhibits hAv glycoprotein mediated cell to cell fusion and receptor attach configuration**

Finally, we tested the effect of *C. elegans* at hAv glycoprotein mediated cell–cell fusion during virus-cell interactions as means of viral spread. We sought to work out whether interaction between compounds extracted and hAv envelope glycoproteins that are essential for viral entry affects cell–cell fusion. In parallel, the control untreated effector cells co-cultured with target cells had high fusion (Figure. 4). Fusion of the membrane between virion envelop and cell wall of the target cell requires glycoproteins (gB, gD, gH-gL) or together of all of them [32]. Our results indicate that the presence of *C. elegans* significantly reduced the viral attachment and penetration. We therefore propose that *C. elegans* extracts possibly interferes with hAv envelope glycoproteins, thereby preventing the binding and fusion process.

**B.IV- Data analysis**

The data represent mean of 3 replicates ± variance (SD) and results were edited to further analysis of variance, and the mean comparisons were performed by using SPSS version 20.0 (Statistical Package for the Social Sciences). Variations between means were considered significant at p-value < 0.05.

**Table 2:** Antimicrobial evaluation by cup technique and clear zone

| Compound/ Antibiotic | Clear zone (ϕmm) | | | |
|---|---|---|---|---|
| | ***S. aureus*** | ***P. aeruginosa*** | ***C. albicans*** | ***A. niger*** |
| 1 | 23 | 19 | 19 | 15 |
| 2 | 22 | 17 | 18 | 16 |
| 3 | 28 | 16 | 18 | 17 |
| 4 | 25 | 17 | 34 | 14 |
| Penicillin | 29 | 21 | 27 | - |
| Streptomycin | 18 | 23 | - | - |
| Neomycin | 19 | 21 | 22 | - |
| Cyclohexamide | - | - | - | 26 |

**Table 3:** Determination of samples cytotoxicity and maximum non-toxic concentration (MNTC) on vero cell line

| ID | ug/ml | O.D | | | Mean O.D | Viability % | Toxicity % | SEM ± | $IC_{50}$ | MNTC |
|---|---|---|---|---|---|---|---|---|---|---|
| Vero | --- | 0.325 | 0.339 | 0.335 | 0.333 | 100 | 0 | 0.004163 | µg | µg/ml |
| 1 | 50 | 0.017 | 0.02 | 0.019 | 0.018667 | 5.605606 | 94.39439 | 0.000882 | 3.48 | 1.56 |
| | 25 | 0.019 | 0.03 | 0.027 | 0.025333 | 7.607608 | 92.39239 | 0.003283 | | |
| | 12.5 | 0.024 | 0.019 | 0.033 | 0.025333 | 7.607608 | 92.39239 | 0.004096 | | |
| | 6.25 | 0.034 | 0.04 | 0.036 | 0.036667 | 11.01101 | 88.98899 | 0.001764 | | |
| | 3.12 | 0.124 | 0.136 | 0.129 | 0.129667 | 38.93894 | 61.06106 | 0.00348 | | |
| | 1.56 | 0.304 | 0.311 | 0.309 | 0.308 | 92.49249 | 7.507508 | 0.002082 | | |
| 2 | 50 | 0.026 | 0.022 | 0.024 | 0.024 | 7.207207 | 92.79279 | 0.001155 | 4.71 | 1.56 |
| | 25 | 0.019 | 0.017 | 0.018 | 0.018 | 5.405405 | 94.59459 | 0.000577 | | |
| | 12.5 | 0.034 | 0.039 | 0.033 | 0.035333 | 10.61061 | 89.38939 | 0.001856 | | |
| | 6.25 | 0.102 | 0.099 | 0.115 | 0.105333 | 31.63163 | 68.36837 | 0.00491 | | |
| | 3.12 | 0.215 | 0.216 | 0.209 | 0.213333 | 64.06406 | 35.93594 | 0.002186 | | |
| | 1.56 | 0.326 | 0.331 | 0.325 | 0.327333 | 98.2983 | 1.701702 | 0.001856 | | |
| 3 | 50 | 0.018 | 0.019 | 0.019 | 0.018667 | 5.605606 | 94.39439 | 0.000333 | 3.67 | 1.56 |
| | 25 | 0.018 | 0.018 | 0.026 | 0.020667 | 6.206206 | 93.79379 | 0.002667 | | |
| | 12.5 | 0.017 | 0.025 | 0.023 | 0.021667 | 6.506507 | 93.49349 | 0.002404 | | |
| | 6.25 | 0.035 | 0.041 | 0.047 | 0.041 | 12.31231 | 87.68769 | 0.003464 | | |
| | 3.12 | 0.162 | 0.154 | 0.159 | 0.158333 | 47.54755 | 52.45245 | 0.002333 | | |
| | 1.56 | 0.31 | 0.299 | 0.307 | 0.305333 | 91.69169 | 8.308308 | 0.003283 | | |
| 4 | 50 | 0.019 | 0.02 | 0.019 | 0.019333 | 5.805806 | 94.19419 | 0.000333 | 5.57 | 1.56 |
| | 25 | 0.019 | 0.017 | 0.018 | 0.018 | 5.405405 | 94.59459 | 0.000577 | | |
| | 12.5 | 0.045 | 0.039 | 0.057 | 0.047 | 14.11411 | 85.88589 | 0.005292 | | |
| | 6.25 | 0.142 | 0.124 | 0.133 | 0.133 | 39.93994 | 60.06006 | 0.005196 | | |
| | 3.12 | 0.295 | 0.274 | 0.289 | 0.286 | 85.88589 | 14.11411 | 0.006245 | | |
| | 1.56 | 0.334 | 0.326 | 0.333 | 0.331 | 99.3994 | 0.600601 | 0.002517 | | |

**Table 4:** Hepatitis A Viral activities on vero cell line and antiviral activities by the isolated compounds

| | ug/ml | O.D | | | Mean | Viability | Toxicity | Viral | Anti-viral |
|---|---|---|---|---|---|---|---|---|---|
| **Control vero** | | | | | **O.D** | | | **Activity %** | **Effect %** |
| | | 0.322 | 0.341 | 0.327 | 0.33 | 100 | 0 | | |
| HAV | | 0.142 | 0.136 | 0.136 | 0.138 | 41.81818 | 58.18182 | 100 | 0 |
| Compound 1 | 1.56 | 0.274 | 0.295 | 0.268 | 0.279 | 84.54545 | 15.45455 | 26.56251 | 73.4378917 |
| | 0.78 | 0.198 | 0.182 | 0.179 | 0.186333 | 56.46465 | 43.53535 | 74.82641 | 25.17398773 |
| | 0.39 | 0.153 | 0.147 | 0.14 | 0.146667 | 44.44444 | 55.55556 | 95.48614 | 4.514259049 |
| Compound 2 | 1.56 | 0.186 | 0.167 | 0.177 | 0.176667 | 53.53535 | 46.46465 | 79.86114 | 20.13926393 |
| | 0.78 | 0.132 | 0.142 | 0.144 | 0.139333 | 42.22222 | 57.77778 | 99.30559 | 0.694813411 |
| | 0.39 | 0.141 | 0.138 | 0.139 | 0.139333 | 42.22222 | 57.77778 | 99.30559 | 0.694813411 |
| Compound 3 | 1.56 | 0.241 | 0.275 | 0.269 | 0.261667 | 79.29293 | 20.70707 | 35.59029 | 64.4101111 |
| | 0.78 | 0.162 | 0.157 | 0.148 | 0.155667 | 47.17172 | 52.82828 | 90.79864 | 9.201760514 |
| | 0.39 | 0.133 | 0.152 | 0.139 | 0.141333 | 42.82828 | 57.17172 | 98.26392 | 1.736480404 |
| Compound 4 | 1.56 | 0.157 | 0.146 | 0.14 | 0.147667 | 44.74747 | 55.25253 | 94.96531 | 5.035092546 |
| | 0.78 | 0.14 | 0.138 | 0.137 | 0.138333 | 41.91919 | 58.08081 | 99.82642 | 0.173979915 |
| | 0.39 | 0.136 | 0.139 | 0.139 | 0.138 | 41.81818 | 58.18182 | 100 | 0 |

**Figure 2:** Antimicrobial inhibition zones of the isolated compounds (1-4).

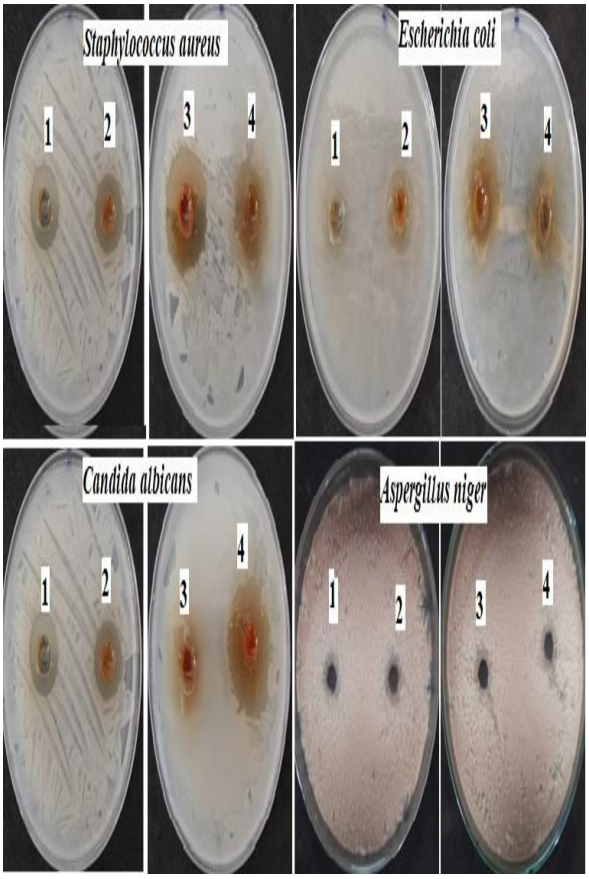



**Figure 3:** The maximum non-toxic concentration (MNTC) reads 1.56 ug/ml for the isolated compounds on vero cell line.

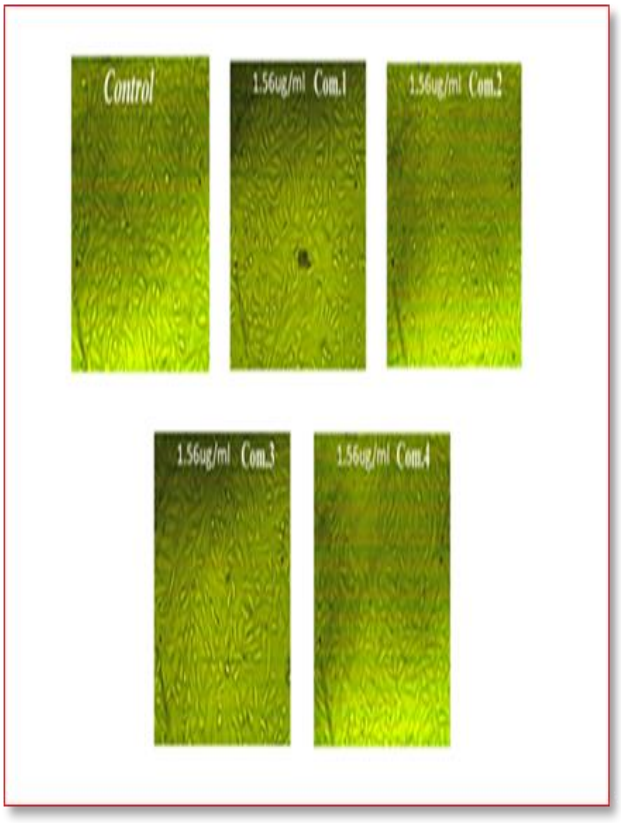


**Figure 4:** Effect of HAV on vero cells, **A**: Control viral activity irregular outline and show cytoplasmic projections and intense cytoplasmic vacuolization, Nuclear membrane beings to disintegrate. **B**: the antiviral activity of compound 1 against HAV show high effect, **C**: the antiviral activity of compound 2 against HAV, **D**: the antiviral activity of compound 3 against HAV show high effect, **E**: the antiviral activity of compound 4 against hAv.

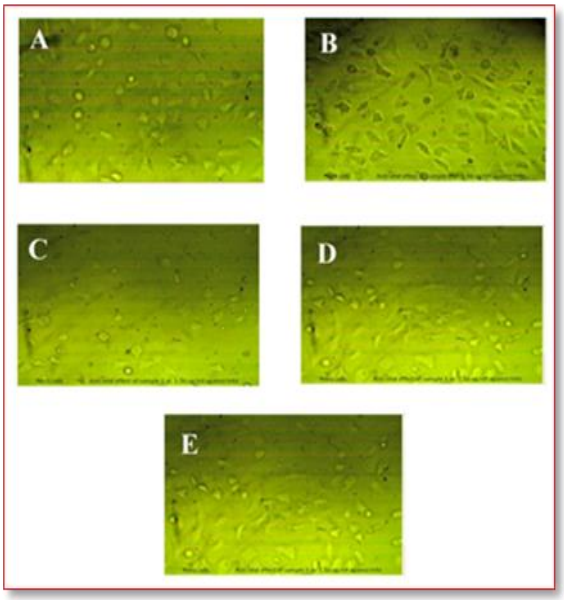
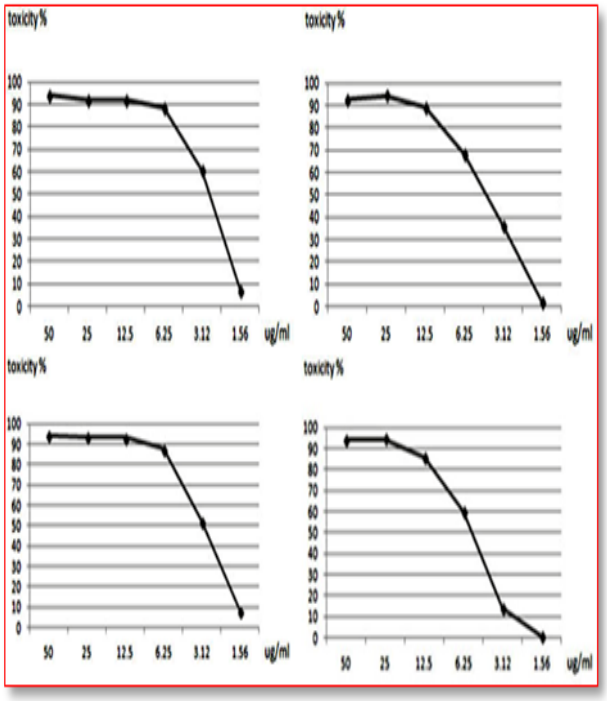



**Figure 5**: Cytotoxicity of *C. elegans* on Vero cells monolayers. Data are expressed as percent cytotoxicity ($OD_{560}$test/control) for four extracted compounds respectively. The MNTD concentration at or below 1.56 µg/ml) was not significantly toxic to cells.




## C- Conclusion

Bio guided isolation of *C. elegans* flowers extract led to identification of four compounds were categorized as saponins and flavonoids. The isolated compounds showed appreciable *in vitro* antimicrobial activity against four pathogenic microbial strains in comparison with standard antibiotics alongside antiviral activity against hepatitis A virus. Compounds 1 and 3 exhibited substantial antiviral activity of 34.3% and 25%, respectively. In conclusion, *C. elegans* extract could be considered as a potential candidate, for further evaluation as a remedy for fighting infectious diseases and its associated signs.

## D- Acknowledgment

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## E- Declaration of Competing Interest

The authors declare no conflict of interest.

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