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Isolation of some chemical constituents from licorice and their evaluation as anticancer and antiviral agents

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Abstract

Ten compounds were isolated from the methanol extract of *Glycyrrhiza glabra* L., they were identified by spectroscopic analysis as 2-(3',5'-dihydroxy phenyl- 4'-methoxy)-7-*O*-*β*-D-xylopyranosyl-6-methoxybenzofuran [**1**], erosta-7,22-dien-3β,5α,6β,9α-tetraol [**2**], 5,2',4'-trihydroxy-8,3'-dimethoxy-2''-hydroxyisopropyl-dihydrofurano [4,5:5',6']-flavanol [**3**], isolicoflavanol [**4**], 7-*O*-*β*-D-xylopyranosyl-(1—>4)-*β*-D-glucopyranosyl-6-O-isobutyl isoflavone [**5**], licorice saponin L3 [**6**], lupinifolin [**7**], licochalcone B [**8**], isoliquirtin [**9**] and isoliquiritigenin [**10**]. Compounds **1**, **3** and **5** are new, while compounds **2** and **7** were isolated for the first time from licorice. Eight compounds were evaluated for anticancer and antiviral activities; they were tested on three cytokines of the human ascites fluid, TNF-alpha, Interferons-gamma and NO. All tested compounds showed significant curative effect compared to control. Compound **8** showed significant decrease compared with control ($p<0.01$) in both HCV and malignant HCV cases in the three cytokines of the human ascites fluid after 24 and 48 hours followed by compound **3**.

KEYWORDS: GLYCYRRHIZA GLABRA L., ANTIVIRAL, ANTICANCER, CYTOKINES

Introduction

Licorice is derived from the roots and rhizomes of *Glycyrrhiza glabra* L. plant (family Fabaceae), it grows in Europe, Middle East and Asia, and it has been used by human being for at least 4000 years in China, Egypt, India, Grease and Rome (1). The genus name Glycyrrhiza is derived from the ancient Greek word for sweet root (Gr. Glykos (Sweet) + rhiza (root), which was later Latinized to liqurite and eventually to licorice.

In traditional medicine it used as antiulcer (2), antitumor (3), antibacterial (4) and antimicrobial agent (5), it also used as flavoring sweetening agent, demulcent, protective action for hepatotoxicity and expectorant (6). Administration of licorice prevented liver cancer and helped in treating stomach cancer (7). More than sixty phenolic compounds were isolated from Glycyrrhiza species (8).

Several oleanane type triterpenes were isolated from the plant species (9). The chemical constituents of licorice include glycyrrhizin which is the most active ingredient in licorice, it constitutes up to 14% of the total soluble solids content and responsible for the characteristic sweet taste of the licorice root, it is about 50-170 times sweeter than sucrose but toxic at high dose, its aglycones, glycyrrhetinic acid which isolated from the aqueous extract is used in treatment of hyperlipemia, viral diseases, inflammation and hepatotoxicity (10,11).

The ethanol extract of Chinese licorice root, *Glycyrrhiza uralensis* showed estrogenic effect and can inhibit cell proliferation in the MCF-7 human breast cancer cell line (12). Flavonoides isolated from the plant exhibit strong antioxidant and anticarcinogenic activities (13).

In this study, ten compounds were isolated from methanol extract of licorice and identified by using chemical and spectroscopic analysis; eight isolated compounds showed anticancer and antiviral activities tested on three cytokines of the human ascites fluid, TNF-alpha, Interferons-gamma and NO.

Methods

Experimental

Equipment: ^{1}H-NMR (δ [ppm], J [Hz]) and ^{13}C-NMR spectra were recorded in CD3OD, operating at 500 MHz for proton and 125 MHz for carbon 13 spectrometer on Varian Mercury 500. Chemical shifts (δ) are reported in parts per million, using TMS as internal standard. Mass spectra were recorded on a Finnigan TSQ 700 GC/MS equipped with a Finnigan electrospray source (ESI-MS). Paper chromatography sheet Whattman 1 (Maidstone, England), using 15 % Acetic acid as solvent system, the chromatograms were visualized under UV light Vilber Lourmat (VL -6LC France) at 254 and 365 nm. Column chromatography was performed using a glass column 120 x 7 cm and using polyamide 6S (Riedel de Darmstadt, Germany) as a stationary phase.

Plant material and Extraction

The dry powder of licorice plant (3 Kg) were purchased from the local market and extracted by 70 % methanol for four weeks (9 L X 5), evaporated under reduced pressure to give 90 gm of crude methanol extract. 60 gm of the crude extract was subjected to polyamide column chromatography eluted with water, water: Methanol with gradient ratios, then pure methanol. Fractions (250 ml each) were collected and the similar fractions grouped together according to paper chromatogram (PC). Five groups [A-E] were collected. The first group [A] contains sugar and traces of undetected compounds. Compounds 1-3 were isolated from group B (7.5 gm) with elution system 30 % Methanol, group B subjected to Sephadex LH-20 sub-column eluted with 30-60 % methanol to give compounds 1 (14 mg), 2 (20 mg) and 3 (33 mg). The group C (1.5 gm), (50% methanol) gives compound 4 and purified with crystallization with organic solvents [EtOAc and Acetone] to give pure compound (50 mg). Compound 5 was isolated from group D (2.3 gm) with elution system 80 % methanol and purified on sephadex LH-20 column chromatography eluted with 60-80 % methanol to give (43 mg). Compound 6 (66 mg), was isolated from group E (1.9 gm) with

pure methanol.

The separation processes were carried out by 2D-PC and Co-C by using whatman No. 1 paper with 15% aqueous AcOH (S1) and BIW (n-BuOH/2-propanol/H2O, 4:1:5, organic layer) (S2) as solvent systems for the pure compounds 1-6. 30 gm of the crude methanol extract was subjected to silica gel column chromatography eluted with chloroform, chloroform: methanol with different ratios then finally with methanol to give four fraction groups [I-IV]. Compound 7 (35 mg) was isolated from fraction II (1.3 gm) with elution system [CHCl3: MeOH, 50:50 v/v]. It was purified with organic solvent EtoAc. Compound 8 (10 mg) obtained from fraction III (0.9 gm) with elution system [CHCl3: MeOH, 20:80 v/v]. Compound 9 (24 mg) &10 (43 mg) was isolated from fraction IV (2.6 gm) with pure methanol and subjected to silica gel [70-230 mesh, Merk] sub-column using solvent system [CHCl3: MeOH, 20:80 v/v] to pure methanol. For compounds 7-10, all the separation processes were followed up by Co-TLC with solvent systems: [CHCl3: MeOH, 6: 4] (S3), [MeOH: EtOAc: CHCl3: H2O, 35:32:28:7] (S4) and [n-BuOH: MeOH: H2O, 4:1:1] (S5).

Acid hydrolysis

Glycoside compounds (2 mg) each was refluxed in 10 ml of 2 N HCl for 4 hrs. The aglycone was extracted with diethyl ether, and then evaporated to dryness. The sugars in the aqueous layer were identified by co-paper chromatography (PC) with authentic samples using solvent system [n-BuOH–AcOH–H2O, 4:1:5, upper layer] by using aniline phthalate spray as detection reagent.

Chemicals

Human IFN-gamma ELISA Kit and Human TNF-alpha ELISA Kit were purchased from Koma Biotech Inc. (Korea). Nitrate reductase (from Aspergillus species), Silica gel 60 GF254 (Merck) for TLC, Silica gel (70-230 mesh) (Merck) for column chromatography, Sephadex LH-20 (Sigma), Whatmann filter paper No.1 and 3 mm for paper chromatography (Maidstone, England). Sulphuric acid (40%) Aniline phthalate reagent, Phosphate Buffered saline (Sigma), NADPH (Nicotinamide Adenine Dinucleotide Phosphate) (Sigma), FAD (Flavin Adenine Dinucleotide) (sigma), sulphanilamide and N-(1-naphthyl) ethylendiamine in 5% H3PO4.

Patients

Two patients were included in this study. They referred to the inpatient tropical medicine department of Theodor Bilharz Research Institute [TBRI]. Their ages were 52 and 63 years old. The first patient was diagnosed with hepatitis C (HCV), liver cirrhosis and the second patient was diagnosed with hepatocellular carcinoma (HCC), liver cirrhosis and hepatitis C (HCV). The patients were subjected to detailed history taking, clinical examination, biochemical investigations, cystoscopy and biopsy.

Application of plant extracts to ascetic fluid culture

-Culture of ascetic cells according to Kohler and Milstein (1975) (14):

The ascetic fluids were obtained from TBRI inpatient unit for patients suffered from HCV and HCV with cancer. The obtained ascetic fluids were centrifuged, supernatants were decanted, then pellets were taken and resuspended in 1 ml complete culture growth medium (SCM).

-**Preparation of ascetic fluids complete medium**

Reagents

RPMI, (Rose well Park Memorial Institute), 1640 medium (Sigma, Chem. Co, St Louis, MO, USA). HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Seromed, Biochrome KG, Berlin, Germany). Sodium bicarbonate, Penicillin- Streptomycin (P/S), 10000 unites (Sigma). L-glutamine (Gibco Lab, Grand Island, NY, USA). Fetal calf serum (FCS) (Hyclone, Logan, Utah, USA).

Method

Serum free medium prepared from RPMI 1640 medium PH 7.2-7.4 was supplemented with 20 ml/l HEPES, 3 ml/l sod. bicarb. 7.5%, 10 ml/l P/S (10000

units) and 10 ml/l L-glutamine. Fetal calf serum (FCS) was heated inactivated at 56°C for 30 min and 20% FCS were added to SFM.

The ascetic cells were cultured in growth medium (consisting of FCS and SFM at about 1:10 ratio) with starting cultures of $1X10^6$ cells/ml in 24 wells culture plates. 50 µg of each pure compound were dissolved in serum free medium. The different compounds were applied to the cultured ascetic cells and dropped as 500 ul in each well and tissue culture plates were incubated for 24 hours at 37°C in humidified atmosphere with 5% CO2.

After this primary incubation period, 0.5 ml aliquot from each well was removed by aspiration and kept in vials at – 2°C then after another 24 hours incubation at 37°C humidified atmosphere with 5% CO2, and aliquot of 0.5 ml was aspirated in vials and freezed at -20°C. Immunological parameters were applied for NO, IFN-γ, TNF-α concentration in ascetic fluids before and after 24 and 48 hrs incubation with pure compounds different dilutions, also without plant application as control samples.

Nitrite assay

Ascites fluid sample were taken, collected in sterile 50 ml tubes. Nitric oxide was assayed in patient's ascites according to Tracey *et al.*, (1995) (15) NO concentrations were determined using the Griess reaction with the following modification. Six µl of ascites fluid were mixed with 44 µl pure water, 20 µl 0.31 M PBS (pH 7.5), 10 µl 0.86 mM NADPH, 10 µl 0.11 mM FAD, 10 µl Nitrate reductase (1 µl) in individual wells for 1hour at room temperature in dark. 200 µl Griess reagent and 20 µl Nitrate reductase from Aspergillus species was added to determine the concentration of both nitrites and nitrates in ascites (µM/ml), since the enzyme reduces nitrates to nitrites.

Absorbance was measured at 450 nm using a Microplate reader and converted to NO concentrations by using the following equation:

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Concentration(µl)=Optical Density × 200(constant)
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Gamma interferon assay

Interferon gamma was assayed in patient ascites according to De maeyer *et al.*, (1992) (16), using IFN-gamma enzyme immunoassay kit (Koma Biotech Inc. Korea). The plate washed three times using 300 µl of washing solution per well, the plate inverted to remove residual solution. 100 µl of sample added to well, covered and incubated at room temperature for 2 hours. 100 µl of the diluted detection antibody (0.25 µg/ml) added per well. 100 µl of the diluted Color Development Enzyme (1:20) added per well, incubated for 30 min at room temperature. 100 µl of Color Development solution added to each well, incubated at room temperature for a proper color development (5-15 min). To stop the color reaction; 100 µl of the stop solution added to each well, using a microtiter plate reader, the absorbance was read at 450 nm wavelength.

Tumor necrosis factor alpha assay

Tumor necrosis factor-alpha was assayed in patient ascites according to Damas et al., (1989) (17) using TNF-alpha enzyme immunoassay kit (Koma Biotech Inc. Korea). The plate washed using 300 µl of washing solution per well, then 100 µl of sample added to well, covered with sealer and incubated at room temperature for 2 hours. 100 µl of the diluted detection antibody (0.25µg/ml) added per well, covered and incubated at room temperature for 2 hours. 100 µl of the diluted Color Development Enzyme (1:20) was added per well. 100 µl of Color Development solution added to each well, incubated at room temperature for a proper color development (5-15 min). To stop the color reaction; 100 µl of the stop solution added to each well. The absorbance was read at 450 nm wavelength.

Statistical analysis

Data were analyzed using the computer system (STATA). One way analysis of variance (ANOVA) was used to test the significance of difference between multiple means for each cytokine. Scheffe's test was done to determine which group was responsible for the significant difference in the multiple analyses.

Results

2-(3',5'-dihydroxyphenyl-4'-methoxy)-7-O-β-D-xylopyranosyl-6-methoxybenzofuran [1]: Amorphous white powder, Rf=0.74 (S1), 0.70 (S2). Positive ESI-MS : m/z 434.07 $[M]^+$ and m/z 301.53 [M– xylose], ^{1}H NMR (500 MHz, DMSO-d6): δ 3.57 & 3.33 (6H, s, 2 OMe), 6.95 (1H, s, H-3), 7.06 (2H, br s, H-2',6'), 7.16 (2H, br s, H-5,8) and 4.09 (1H, d, J=7.0 Hz, H-1 xyl). ^{13}C NMR spectrum show carbon-13 signals (table 1).

Ergosta-7,22-dien-3β,5α,6β,9α-tetraol [2]: White powder, Rf=0.56 (S1), 0.51 (S2). Positive ESI-MS: 434.6 $[M]^+$, 416.8, 327.8, 255.2, 192.4, 125.8. ^{1}H NMR (500 MHz, DMSO-d6): δ 0.67 (3H, s, H-18), 0.82 (3H, d, J=6.8 Hz, H-26), 0.85 (3H, d, J=6.4 Hz, H-27), 0.93 (3H, d, J=6.8 Hz, H-28), 1.10 (3H, d, J=6.8 Hz, H-21), 1.4 (3H, s, H-19), 2.47 (1H, dd, 13.6, 5.6 Hz, H-4eq), 3.52 (1H, dd, J=12.8, 13.6 Hz, H-4ax), 4.2 (1H, d, J=5.0 Hz, H-6), 4.4 (1H, br dd, 5.6, 12.8 Hz, H-3), 5.31 (1H, dd, J=16.4, 8.0 Hz, H-22) and 5.04 (1H, dd, J=16.4, 7.6 Hz, H-23). ^{13}C NMR spectrum (table 2).

5,2',4'-trihydroxy-8,3'-dimethoxy-2''-hydroxyisopropyl-dihydrofurano[4,5:5',6']-flavanol [3]: White needle crystals, Rf=0.65 (S1), 0.59 (S2). Positive ESI-MS: m/z 448 $[M+2H]^+$, ^{1}H NMR (500 MHz, DMSO-d6): δ 6.91 (1H, d, J=8.4 Hz, H-6), 6.58 (1H, d, J=8.4 Hz, H-7), 8.4 (4'-OH), 3.23, 3.33 (6 H, s, 2 OMe), 3.1 (t, H-2"), 4.3 (1H,d, J=7.5 Hz, H-3") and 1.19, 1.6 (6 H, s, 4", 5" Me). ^{13}C NMR spectrum (table 1).

Isolicoflavanol [4]: Amorphous yellow powder, Rf=0.54 (S1), 0.49 (S2). Positive ESI-MS: m/z 355 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d6): δ 1.45, 1.6 (6 H, s, 2 Me), 6.1 (1H,t, J=7 Hz, H-2"), 6.22 (1H, d, J=2 Hz, H-6), 6.55(1H, d, J=2 Hz, H-8), 7.01(1H, d, J=8 Hz, H-5'), 7.91 (1H, d, J=2, 8 Hz, H-6') and 7.99 (1H, d, J=2 Hz, H-2').

7-O-β-D-xylopyranosyl-(1—>4)-β-D-glucopyranosyl 6-O-isobutyl isoflavone [5]: Crystalline powder, Rf=0.72 (S1), 0.67 (S2). Positive ESI-MS: m/z 617.87 $[M+H]^+$, 486.40 [M +H –xylose]$^+$, m/z 324.33 [M+H –(xylose + glucose)]$^+$. ^{1}H NMR (500 MHz, DMSO-d6): δ 8.25 (1H, s, H-2), 7.86 (1H, s, H-8), 7.94 (1H,s, H-5), 6.9-7.3 (5H, m, H-2',6'), 5.3 (1H, d, J=7.6 Hz, H"-glu), 3.78 (2H, dd, J=14.5, 2 Hz, 2H-6" glu), 4.02 (1H, d, J=4.6 Hz, H-1"' xyl), 3.73, 3.75 (2H, dd, J=12.2, 4 Hz, 2H-5"' xyl), 3.47 (m, H-4"'), 3.31 (m, H-3"'), 3.24 (m, H-2"'), 4.48 (2H, d, J=7.5 Hz, 2H-1""), 5.58 (1H, t, J=7.2 Hz, H-2""), 1.19 (s, 3H-4"") and 1.23 (s, 3H-5""). ^{13}C NMR spectrum (table 1).

Licorice saponin L3 [6]: Amorphous colourless powder, Rf=0.77 (S1), 0.72 (S2). Positive ESI-MS: m/z 950.80 $[M]^+$, 706.87, 530.0, 528.4. ^{1}H NMR (500 MHz, DMSO-d6): δ 0.89, 0.96, 1.02, 1.16, 1.33, 1.43 (3H, S), 1.46 (3H, d, Rh), 2.01 (3H, s, acetoxyl CH3), 4.95 (1H, d, J=7 Hz, H-1'), 5.4 (1H, br s, H-1'') and 5.5 (1H, d, J=7.0 Hz, H-1"'). ^{13}C NMR spectrum (table 2).

Lupinifolin [7]: Colourless crystals, Rf=0.57 (S3). Positive ESI-MS: m/z 406 $[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d6): δ prenyl gp. 1.65, 1.66 (2 x 3H, s Me), 3.39 (2H, d, H-1'") and 5.5 (1H, t, H-2"'), 6.3, 5.7 (each 1H, d, H-3'', H-4") and 12.2 (1H, s, 5-OH). ^{13}C NMR spectrum (table 1).

licochalcone B [8]: Colourless needles, Rf=0.60 (S4). Positive ESI-MS: 287 $[M+H]^+$, 255. ^{1}H NMR (500 MHz, DMSO-d6): δ 3.5 (3H, s, OMe), 6.7 (1H, d, J=8 Hz, H-2', 6'), 6.8 (2H, d, J=8 Hz, H-5 & H-6), 7.22 (1H, d, 8 Hz, H-5'), 7.56 (1H, d, J=15 Hz, H-α), 7.9 (1H, d, J=15 Hz, H-β) and 8.1 (1H, d, J=8 Hz, H-3', 5').

Isoliquirtin [9]: Off-white amorphous powder, Rf=0.71 (S5). Positive ESI-MS: m/z 418$[M]^+$. ^{1}H NMR (500 MHz, DMSO-d6): δ 6.3 (1H, d, J=2 Hz, H-3'), 6.5 (1H, dd, J= 8, 2 Hz, H-5'), 6.8 (2H, d, J=8 Hz, H-3, 5), 7.5 (1H, d, J=15 Hz, H-α), 7.7 (2H, d, J=8 Hz, H-2, 6), 7.8 (1H, d, J=15 Hz, H-β) and 8.1 (1H, d, J=8 Hz, H-6').

Isoliquiritigenin [10]: White crystals, Rf=0.55 (S3). Positive ESI-MS: m/z 257$[M+H]^+$. ^{1}H NMR (500 MHz, DMSO-d6): δ 6.2 (1H, d, J=2 Hz, H-3'), 6.5 (1H, dd, J= 8, 2 Hz, H-5'), 6.9 (2H, d, J=8 Hz, H-3, 5), 7.6 (1H, d, J=15 Hz, H-α), 7.7 (2H, d, J=8 Hz, H-2, 6), 7.8 (1H, d, J=15 Hz, H-β) and 8.0 (1H, d, J=8 Hz, H-6'). ^{13}C NMR spectrum (table 3).

Discussion

Much of recent research on licorice constituents

has indicated that, the pharmacological importance of phenolic compounds, together with saponins in the main reason of medical importance of licorice. Phytochemical investigations of the dry powdered licorice roots have led to isolation of three new compounds: 2-(3',5'-dihydroxyphenyl-4'-methoxy)-7-O-β-D-xylopyranosyl-6-methoxybenzofuran [1], 5,2',4'-trihydroxy,8,3'-dimethoxy-2''-hydroxyl isopropyl dihydrofurano [4,5:5',6'] flavanol [3] and 7-O-β-D-xylopyranosyl-(1—>4)-β-D-glucopyranosyl-6-O-isobutyl isoflavone [5], along with seven known metabolites: ergosta-7,22-dien-3β,5α,6β,9α-tetraol [2] which was previously isolated from *Rhizopus oryzae* (18), isolicoflavanol [4] (19), licorice saponin L 3 [6] (20), lupinifolin [7] was isolated from *Erythrina fusca* and *Derris reticulate* (21,22), licochalcone B [8] (19,23), isoliquirtin [9] (20), isoliquiritigenin [10] (20), the structures were determined by spectroscopic methods (ESI-MS, ^{1}H NMR and ^{13}C NMR).

Compound **1** showed phenolic properties under UV. Acid hydrolysis of the compound produced the aglycone and xylose. ^{1}H NMR spectrum showed three singlet signals in aromatic region, one proton of furan nucleus signal at δ 6.95 (1H, s, H-3), two protons δ 7.16 (2H, br s, H-5,8) and two protons (B ring) at δ 7.06 (2H, br s, H-2',6') [24]. It also showed two singlet of two methoxy groups at δ 3.57 and δ 3.33 (6H, s, 2 x OMe). It showed anomeric doublet at δ 4.09 (1H, d, J=7.0 Hz, H-1 xyl). ^{13}C NMR (table1) showed 21 signals, 5 for xylose sugar moiety and 16 for the aglycone. It showed five oxygenated aromatic carbons at δ 143.1, 145.3, 148.1, 151.4 and 146.9. The ^{13}C NMR of aglycone signals similar to the previously isolated compound 2-(3,5-dihydroxyphenyl)-6-methoxy-7-hydroxybenzofuran (24), but with addition of extra methoxy group signal at δ 151.4 which suggested to be at C-4' according to the signal value at δ 55.6 and the shifted value at C-3', 5'. Positive ESI-MS give a molecular ion fragment at m/z 434.07 $[M]^{+}$ and at m/z 301.53 [M– xylose]. According to these data compound **1** identified as 2-(3',5'-dihydroxyphenyl-4'-methoxy)-7-O-β-D-xylopyranosyl-6-methoxybenzofuran.

Compound **3** showed phenolic properties, it showed yellow color under UV don't change after spraying with ALCl3 which means presence of free hydroxyl group at C-3. ^{1}H NMR spectrum give only two doublet signals in aromatic region at δ 6.91 (1H, d, J=8.4 Hz, H-6) and 6.58 (1H, d, J=8.4 Hz, H-7) indicating presence of two neighbor protons in A-ring and suggest that ring B is full substituted. A sharp singlet signal at δ 8.4 suggested presence of 4'-OH group and the chelated hydroxyl group at δ 12.4 was consistent with a 5-Hydroxy flavone (25). Two methoxy groups showed two singlets at δ 3.23 and 3.33. ^{1}H NMR also showed signals at δ 3.1 (t, H-2''), 4.3 (1H, d, J=7.5 Hz, H-3''), two broad singlet at δ 1.19 and 1.6 (2Me, s, 4'', 5'') which deduced to be isopropyl dihydrofuran group ($-CH_2CH(O-)C(CH3)_2(OH)$) (26). ^{13}C NMR spectrum showed 22 signals, 15 of flavonoid skeleton, two methoxyls and five of the remaining group, ^{13}C NMR spectrum (Table 1) suggest the substitution of attached group. Positive ESI-MS showed a molecular ion at m/z 448 $[M+2H]^{+}$ corresponding to molecular formula $C_{22}H_{22}O_{10}$. According to the above data the structure of compound **3** was 5,2',4'-trihydroxy,8,3'-dimethoxy-2''-hydroxyisopropyldihydrofurano[4,5:5',6']-flavanol. Compound **5** was isolated from the butanol extract; it was consistent with being a flavonoid glycoside from an initial color reaction [blue changed to yellow after spraying with ALCl3]. Acid hydrolysis with 2N HCl gives the aglycone, glucose and xylose which identified by comparison with authentic samples on PC chromatogram. ESI-MS spectrum showed molecular ion fragment at m/z 617.87 $[M+H]^{+}$, 486.40 [M +H –xylose]$^{+}$ and m/z 324.33 [M+H –(xylose + glucose)]$^{+}$.

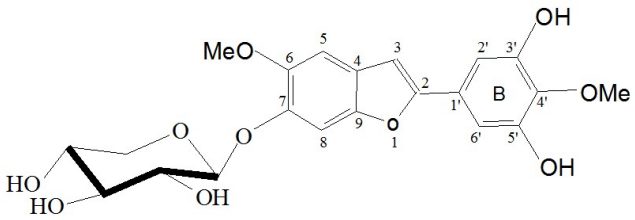
^{1}H NMR spectrum showed a singlet signal at δ 8.25 (1H, s, H-2) characteristic for a proton H-2 in isoflavones (27). It also showed a singlet signal at δ 7.86 (1H, s, H-8) and 7.94 (1H, s, H-5) (28). A broad multiplet signal at δ 6.9-7.3 (5H, m, H-2'-6') (29). A signal at δ 5.3 (1H, d, J=7.6 Hz, H''-glu) was assigned to glucosyl anomeric proton, and suggested that the glycosidic bond had a β-linkage (29). A signal at δ 3.78 (2H, dd, J=14.5, 2 Hz, 2H-6'' glu). A doublet at

δ 4.02 (1H, d, J=4.6 Hz, H-1''' xyl), at δ 3.73 and 3.75 (2H, dd, 12.2, 4 Hz, 2H-5''' xyl) (30). One prenyl oxy moiety at δ 4.48 (2H, d, J=7.5 Hz, 2H-1''''), 5.58 (1H, t, J=7.2 Hz, H-2''''), 1.19 (s, 3H-4'''') and 1.23 (s, 3 H-5'''') (25). These data with ^{13}C NMR (table 1) and those of literature values suggested that compound **5** was identified as 7-O-β-D-xylopyranosyl-(1—>4)-β-D-glucopyranosyl-6-O-isobutyl isoflavone.

As a part of a search for compounds derived from plants used as modulators of inflammation, antiviral and anticancer agents, we have tested effects of the eight isolated compounds from *Glycyrrhiza glabra* L for their anticancer and antiviral effects on three cytokines of the human ascites fluid, TNF-alpha, Interferons-gamma and NO. TNF-α is produced by activated macrophages, fibroblast, and many different cells and well characterized as one of the important defense molecules of body with potent pro-inflammatory effects (31).

The immunological parameters after treatment with compounds were significantly decreased as compared with control ($p<0.01$) after 24 and 48 hours [Tables 4-6]. So, IFN-γ, TNF-α and NO, which indicates that the inflammatory effect of those cytokines was diminished due to the high effect of the isolated compounds used in this study. The explanation of those decreased results was due to the regulation of induced apoptotic cell death and inhibition of inflammation and tumorigenesis and viral replication (32). Tables [4-6] showed (mean + SD) the results of the effect of the isolated compounds [1-8] on the three cytokine in the same case after 24 and 48 hours. All the isolated compounds showed significant decrease as compared with control ($p<0.01$). These differences were statistically significant by ANOVA test ($F=0.003$), but only control cases as significantly different increase from the other compounds in inter groups analysis using scheffe's test ($p<0.05$). The comparison between compound by groups and the significant difference decrease IFN-γ in HCV case at 24 and 48 hours and controls are illustrated in figure (1). The mean level of serum TNF- α was significantly higher in control with active disease when compared with those with different compound 8 (33.6±1.5 ρg/ml versus 18.14±0.48 ρg/ml ($p<0.01$)). It was also significantly higher in compound 3 (33.6±1.5 ρg/ml versus 19.0±0.5) (table 5). NO+HCV 24 hours and NO+HCV 48 hours were lower in compound 8 and compound 3 respectively (26.6±0.27, 27.6±0.48) than those in the control group (42.5±2.5) ($p<0.01$) (table 6).

Compound 8 (licochalcone B) showed significant decrease (higher effect) compared with control ($p<0.01$) in both HCV and malignant HCV cases in the three cytokines of the human ascites fluid after 24 and 48 hours followed by compound 3 (5,2',4'-trihydroxy,8,3'-dimethoxy-2''-hydroxyisopropyldihydrofurano [4,5:5',6']-flavanol). These results may be due to excessive production of NO in malignant HCV cases, since NO is induced in macrophages that have antitumor activity through the inhibition of DNA synthesis in tumor cells, which may also induce apoptosis (33).



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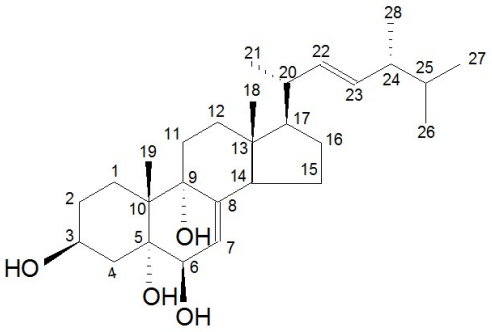

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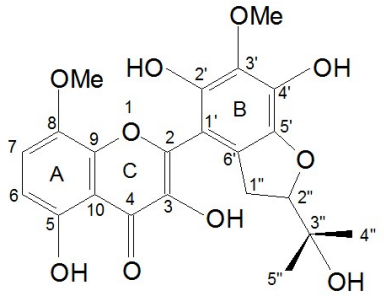

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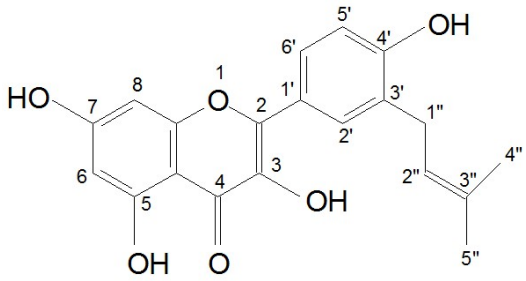

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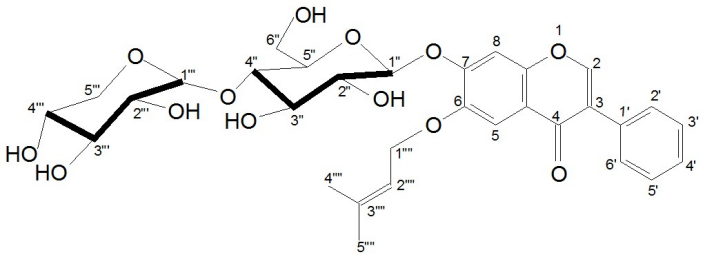

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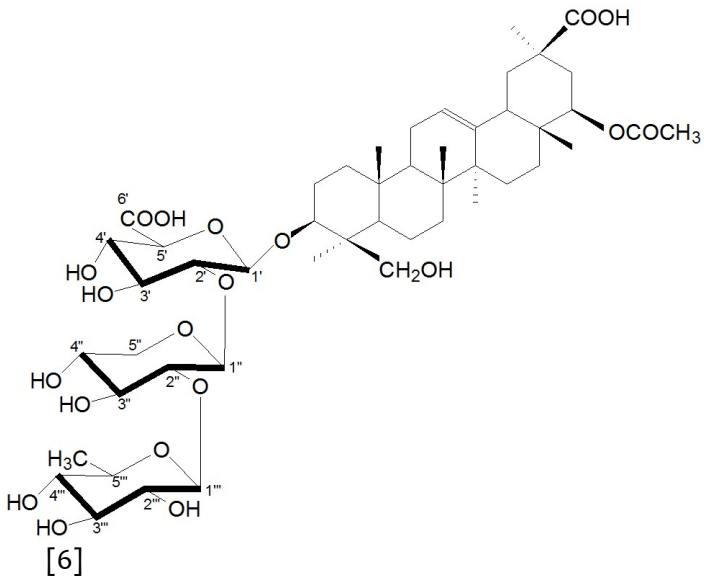

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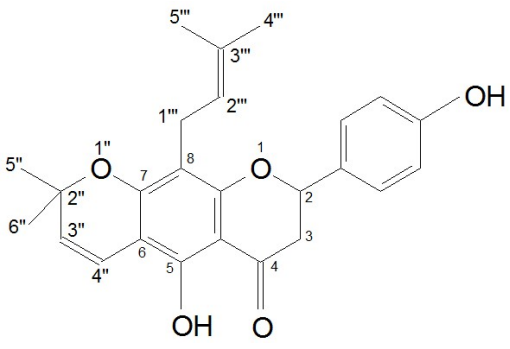

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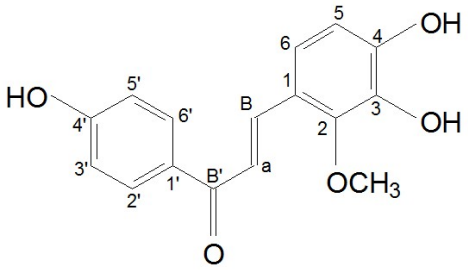

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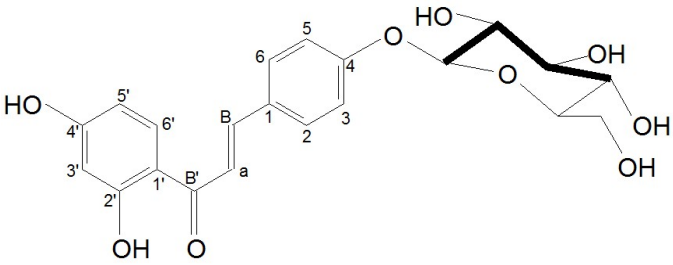

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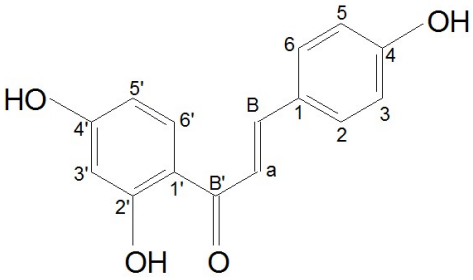

[10]

Conclusion

Licorice is well known medicinal plant for a long time; experimentally it was showed a wide variety of biological activity. Searching for more new secondary metabolites and biological activity, Ten compounds were isolated from the methanol extract of *Glycyrrhiza glabra* L., only three compounds identified as 2-(3',5'-dihydroxyphenyl-4'-methoxy)-7-O-β-D-xylopyranosyl-6-methoxybenzofuran [1], 5,2',4'-trihydroxy,8,3'-dimethoxy-2"-hydroxyisopropyldihydrofurano[4,5:5',6']flavanol [3] and 7-O-β-D-xylopyranosyl-(1—>4)-β-D-glucopyranosyl-6-O-isobutyl isoflavone [5] are new compounds. Eight compounds were evaluated for anticancer and antiviral activities; they were tested on three cytokines of the human ascites fluid, TNF-alpha, Interferons-gamma and NO. All tested compounds

showed a significant effect compared to control. Compound 3 and 8 showed significant decrease compared with control ($p<0.01$) in both HCV and malignant HCV cases in the three cytokines of the human ascites fluid after 24 and 48 hours.

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References

1. Fanali S, Aturkiz, D'orazio G, *et al.* Use of nano-liquid Chromatography for the analysis of glycyrrhizin and glycyrrhetic acid in Licorice roots and candies. J. Sep. Sci. 2005; 28: 982-986.
2. Aly AM, AL-Alousi L, Salem HA. Licorice: a possible antiinflammatory and anti-ulcer drug AAPS. PhramSci.Tech. 2005; 20: 6 (1): E74-82.
3. Kang S, Choi Y, Bae J L J, *et al.* Licorice isoliquiritigenin dampens angiogenic activity via inhibition of MaPk vesposive singaling pathways leading to induction of matrix metallo proteiases. J. Nutritional Biochem. 2010; 21: 55-65.
4. Hatano T, Shintani Y, Aga Y, *et al.* Phenolic Constituents of Licorice. VIII. Structure of Glicophenone and Glicoisoflavanone, and Effects of Licorice phenolics on Methicilline-Resistant Staphylococcus aureus. Chem. Pharm. Bul. 2000; 48(9): 1286-1292.
5. Haraguchi H, Tanimoto k, Tamura Y, Mizutani K, Kinoshita T. Mode of antibacterial action of retrochalconeos from *Glycyrrhiza inflata*. Phytochemistry, 1998; 48:125-129.
6. Agarwal A, Gupta D, Yadav G, Singh Pk, Singh U. An evaluation of efficacy of Licorice gargle for attenuating postoperative sore throat a prospective randomized, single-blind study. Anesth. Anagl. 2009; 109(1):77-81.
7. Ma J, Fu NY, Pang DB, Wu WY, Xu AL. Apoptosis induced by isoliquiritigenin in human gastric cancer MGC-803 Cells. Planta Med. 2001; 67(8): 754-7.
8. Aai H, Li W, Koike K, *et al.* Anovel Biflavoniod from roots of *Glycyrrhiza uralensis* cultivated in China. Chem. Pharm. Bull. 2003; 51(9): 1095-1097.
9. Kitagawa I, Hori K, Taniyama T, Zhou J, Yoshikawa M. Saponin and sapogenol of the roots of *Glycyrrhiza uralensis* fischer from Northeastern China (1). Licorice- Saponins A3, B2 and C2. Chem. Pharm. Bull. 1993; 41(1): 43-49.
10. Kimura Y, Okuda T, Okuda H. Effects of flavonoids from Licorice root (*Glycyrrhiza inflate* Bat.) on arachidonic acid metabolism and aggregation in human platelets. Phitothar Res. 1993; 7: 341-347.
11. Kiso Y, Tohkin M, Hikino H, *et al.* Mechanism of anti-hepatotoxic activity of Glycyrrhizin. I: Effect on free radical generation and lipid peroxidation". Planta Med. 1984; 50: 298-302.
12. Tamir S, Eizenberg M, Somjan D, *et al.* Antiproliferative properties of Glabridin from Licorice in Human Breast Cancer. J. Estrogenic and Cancer Res. 2000; 60: 5704-5709.
13. Chin YW, Jung HA, Liu Y, *et al.* Antioxidant constituenta of the roots and stolons of Licorice (*Glycyrrhiza glabra*). J. Agric. Food Chem. 2007; 55(12): 4691-7.
14. Köhler G, Milstein, C. Continuous cultures of fused cell secreting antibody of predefined specificity. Nature 1975; 256: 495-7.
15. Tracey WR, Tse J, Carter G. Lipopolysaccharide-induced changes in plasma nitrite and nitrate concentrations in rats and mice: pharmacological evaluation of nitric oxide synthase inhibitors. J. Pharmacol. Exp. Ther. 1995; 272: 1011-5.
16. De Myer E, De Mayer GJ. Interferom g Curr Opinion Immunol. 1992; 4: 321- 332.
17. Damas P., Peuter A, Gysen P, Demonty J, Lamy M, Franchimont P. Tumor Necrosis Factor and interleukin 1 serum levels during severe sepsis in humans. Crit. Care Med. 1989; 17: 975-983.
18. Young D, Lee S, Kwak H, *et al.* Sterols isolated from Nuruk (*Rhizopus Oryzae* KSD-815) Inhibit the migratich of cancer cells. J. Microbiol. Biotechnol. 2009; 19(11): 1328-1332.
19. Hatano T, Kagawa H, Yasuhara T, Okuda T. Two new flavonoids and other constituents in Licorice root: Their Relative Astringency and radical Scavenging effect. Chem. Pharm. Bull. 1988; 36(6): 2090-2097
20. Kitagawa I, Hori k, Uchida E, *et al.* Saponin and Sapogenol L. On the constituents of the roots of *Glycyrrhiza uralensis* fischer from Xinjiong, China Chemical Structures of Licorice – Saponin L 3 and Isoliquritin Apioside. Chem. Pharm. Bull. 1993; 41(9): 1567-1572.
21. Mahidol G, Prawat H, Ruchirawat S, *et al.* Prenylated flavanones from Derris reticulata. Phytochemistry, 1979; 45(4): 825-829.
22. Innok P, Ruckachaisirikul T, Suksamrarn A. Flavanoids and Petrocarpans from the Bark of *Erythrina fusca*. Chem. Pharm. Bull. 2009; 57(9): 993-996.
23. Saiton T, Shibata S. New type chalcones from Licorice root. Tetrahedron lett. 1975; (16): 4461.
24. Tan Y, Yang Y, Zang T, Chen R, Yu D. Bioactive 2-arylbenzofuron derivatives from *Morus wittiorum*. Fitoter. 2010; 81(7): 742-746.
25. Amaral DF, Arruda MSP, Arruda AC, *et al.* Flavones from the leaves of *Ficus gomelleria*. J. Praz. Chem. Soc. 2001; 12(4): 538-541.
26. Chen S, Chen J, Gao K. Prenylisoflavone derivatives from the roots of *Hedysarum scoparium*. Chem. Pharm. Bull. 2007; 55(8): 1181-1184.
27. Maver M, Oueiroz EF, Wolfender J, Hosttman N. Flavonoids from the stem of *Eriophorum Scheuchzeri*. J. Nat. Prod. 2005; 68:1094-1098.
28. Lee H, Lee O, Kown Y, *et al.* Isoflavone glycosides from the Bark of *Amorpha fruticosa*. Chem. of Nat. Comp.2006; 42(4): 514.
29. Choi Y, Lim Y, Yeo H, Kim J. A flavonoid diglycoside from *Lepisorus ussuriensis*. Phytochemistry, 1996; 43(5): 1111-1113.
30. Lavaud C, Voutquenne L, Le Men-Olivier L, *et al.* Saponins from the stem Bark of *Filicium decipiens*. Phytochemistry, 1998; 47(3): 441-449.
31. Rojas V, Morales-Lange B, Guzmαn F, Gallardo JA, Mercado L. Immunological strategy for detecting the proinflammatory cytokine TNF-alpha in salmonids. Electronic J. Biotech. 2012; 15: 1-7.
32. Wang Y, Lo G, Lai K, *et al.* Increased Serum Concentrations of Tumor Necrosis Factor-alpha are Associated with Disease Progression and Malnutrition in Hepatocellular Carcinoma. J. Chin. Med. Assoc. 2003; 66: 592-597.
33. Wolf H, Haeckel C, Roessner A. Inducible nitric oxide synthase expression in human urinary bladder cancer. Virchows Arch. 2000; 437: 662-666.

No	Compound 1 δc	Compound 3 δc	Compound 5 δc	Compound 7 δc
1	-	-	-	-
2	154.5	156.4	151.96	77.8
3	102.8	133.7	124.45	44.1
4	120.9	179.4	175.38	195.7
5	104.7	161.6	104.90	156.9
6	145.3	104.2	147.74	103.6
7	143.1	98.8	154.41	160.5
8	94.2	136.4	99.5	109.4
9	148.8	154.5	152.23	159.3
10	-	105.9	117.81	102.9
1'	132.4	110.9	128.2	130.1
2'	106.5	158.5	127.6	127.6
3'	148.1	144.6	128.7	115.3
4'	151.4	147.8	130.4	155.7
5'	146.9	146.1	128.7	116.5
6'	107.3	158.5	127.6	128.6
OMe	52.5, 55.6	52.3, 55.5		-
		Isoprop.dih-		-
Glu		ydrofuran		
1"		27.1	97.3	
2"		91.3	80.9	
3"		72.4	75.6	
4"		26.2	81.5	
5"		25.1	78.0	
6"		-	62.5	
Xyl				
1"	103.5		102.9	-
2"	76.1		75.5	83.2
3"	77.8		78.1	122.3
4"	70.3		70.6	119.0
5"	67.5		67.2	68.5
6"	-		-	23.3
1'"				22.0
2'"				123.5
3'"				132.2
4'"				16.7
5'"				23.9
2""			120.1	
4""			25.9	
5""			17.8	

Table 1: ^{13}C NMR data of compounds 1, 3, 5 and 7.

No	Compound 2 δc	Compound 6 δc
1	29.0	37.9
2	32..1	25.7
3	65.9	88.5
4	42.1	43.5
5	75.2	55.2
6	73.3	17.9
7	121.8	33.1
8	140.8	40.0
9	75.5	46.6
10	40.2	36.1
11	28.0	23.8
12	36.2	122.7
13	44.5	144.4
14	50.9	40.0
15	22.8	26.3
16	28.0	26.6
17	55.7	37.0
18	12.5	44.4
19	22.7	41.3
20	40.5	28.8
21	20.6	40.9
22	135.9	75.7
23	133.4	22.4
24	43.8	63.5
25	32.7	14.6
26	18.7	16.9
27	20.2	25.9
28	17.8	26.9
29		29.0
30		181.5
$COCH_3$		170.7, 21.3
Glcouronic		
1		104.3
2		78.0
3		74.9
4		72.6
5		77.6
6		175.7
Ara		
1		102.7
2		78.1
3		76.5
4		70.2
5		65.4
Rh		
1		101.5
2		72.4
3		72.9
4		75.0
5		70.0
6		18.1

Table 2: ^{13}C NMR data of compounds 2 and 6

No	Compound 10 δc
1	125.7
2,6	130.6
3,5	115.7
4	158.9
1'	112.8
2'	163.6
3'	102.4
4'	165.5
5'	107.8
6'	132.1
α	117.6
β	143.6
β'	190.9

Table 3: ^{13}C NMR data of compound 10.

	IFN-γ in HCV case 24 h	IFN-γ in HCV case 48h	IFN-γ in malignant HCV case 24h	IFN-γ in malignant HCV case 48h
Control	51.3± 2.6	29.9±1.7	80.7±3.0	44.7±1.9
Comp.1	42.2 ± 0.27*	23.3±0.22*	70.0±0.07*	39.8±0.11*
Comp.2	33.7± 0.11*	19.0±0.19*	49.9±0.07*	32.0±0.07*
Comp.3	25.8± 1.1*	14.0±0.19*	51.7±0.11*	31.2±0.14*
Comp.4	34.6±6.2*	19.5±0.31*	66.7±0.12*	38.7±0.07*
Comp.5	46.75±1.9*	28.2±0.14*	74.6±0.13*	39.8±0.18*
Comp.6	33.0±0.38*	18.4±0.19*	63.7±0.1*	32.6±0.07*
Comp.7	29.0±0.34*	15.2±0.2*	67.0±0.1*	32.5±0.16*
Comp.8	22.8±1.3*	12.5±0.4*	44.8±0.13*	28.8±0.06*

Table 4): Effect of Compounds 1-8 on IFN-γ of human ascites fluid after 24 and 48 hours.

	TNF-α in HCV case 24 h	TNF-α in HCV case 48 h	TNF-α in malignant HCV case 24 h	TNF-α in malignant HCV case 48 h
Control	33.6± 1.5	21.9±0.8	52.1±2.3	35.5±1.3
Comp.1	27.7 ±0.24*	18.1±0.18*	42.5±0.14*	32.0±0.09*
Comp.2	21.9± 0.35*	13.0±0.11*	37.6±0.88*	29.5±0.1*
Comp.3	19.0± 0.5*	11.7±0.26*	30.0±0.07*	21.0±0.1*
Comp.4	21.8±0.97*	14.1±0.37*	38.6±0.12*	25.1±0.1*
Comp.5	27.6 ±1.1*	18.07±0.73*	45.0±0.2*	33.0±0.09*
Comp.6	24.2± 1.0*	18.0±0.32*	34.4±0.08*	28.4±0.11*
Comp.7	20.7± 0.33*	14.8±0.67*	32.6±0.09*	23.4±0.11*
Comp.8	18.14±0.48*	11.65±0.37*	27.6±0.11*	19.6±0.9*

Table 5): Effect of Compounds 1-8 on TNF-α of human ascites fluid after 24 and 48 hours.
*Significant differences decrease as compared with control p<0.01

	NO In HCV case 24 h	NO in HCV case 48 h	NO in malignant HCV case 24h	NO in malignant HCV case 48h
Control	42.5± 2.5	33.5±1.1	45.5±1.3	36.2±1.6
Comp.1	33.5 ± 1.1*	26.0±0.8*	32.5±0.16*	27.0±0.17*
Comp.2	29.8± 0.06*	23.9±0.17*	35.0±0.01*	26.4±0.11*
Comp.3	27.6± 0.48*	24.5±0.22*	30.0±0.07*	24.4±0.12*
Comp.4	30.0±0.74*	25.9±0.18*	35.0±0.13*	26.6±0.01*
Comp.5	37.2±1.5*	29.2±0.8*	43.0±0.2*	30.6±0.09*
Comp.6	30.7±1.9*	25.0±1.1*	28.0±0.16*	24,6±0.13*
Comp.7	27.8±0.06*	23.7±0.2*	28.5±0.07*	27.5±0.15*
Comp.8	26.6±0.27*	23.8±0.11*	28.2±0.2*	25.3±0.08*

Table 6): Effect of Compounds 1-8 on NO of human ascites fluid after 24 and 48 hours.
*Significant differences decrease as compared with control p<0.01

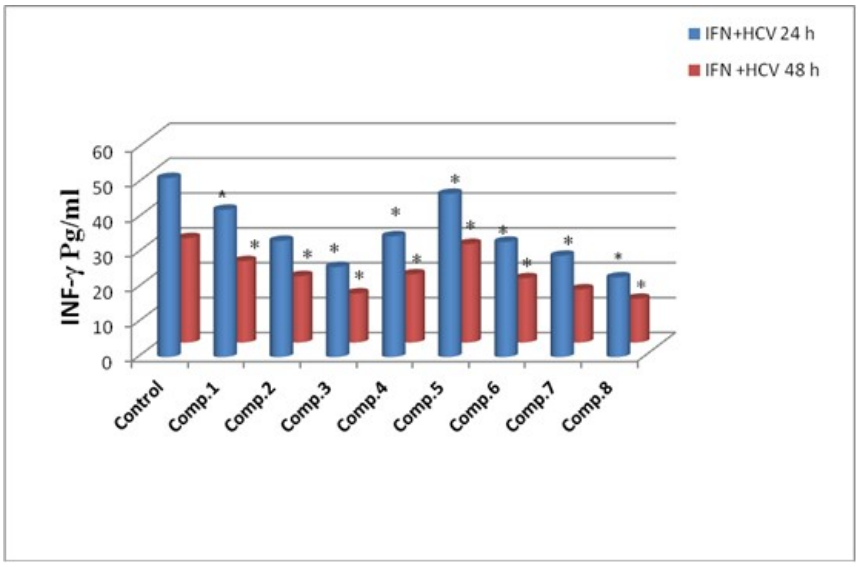


Figure (1)

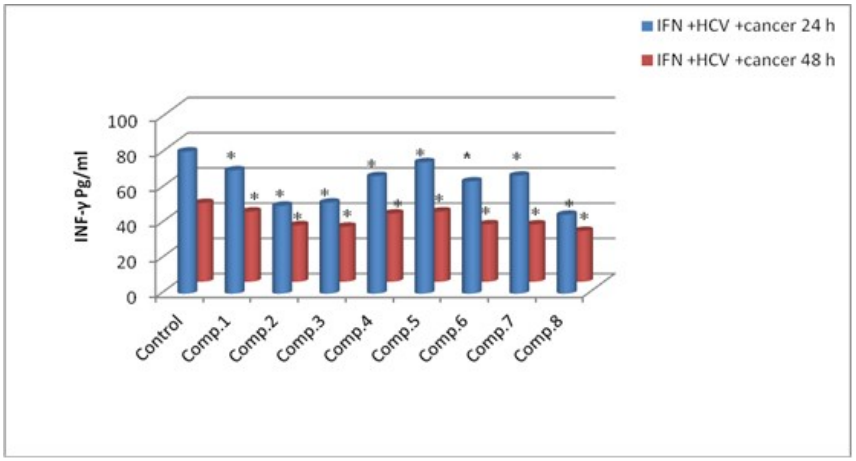
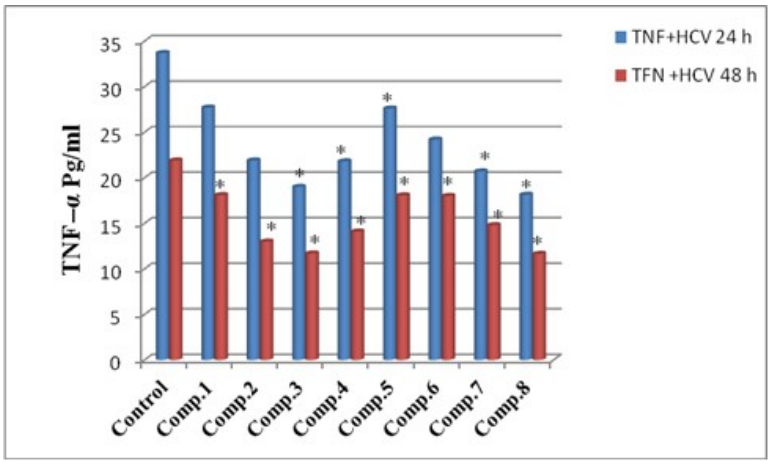


Figure (2)



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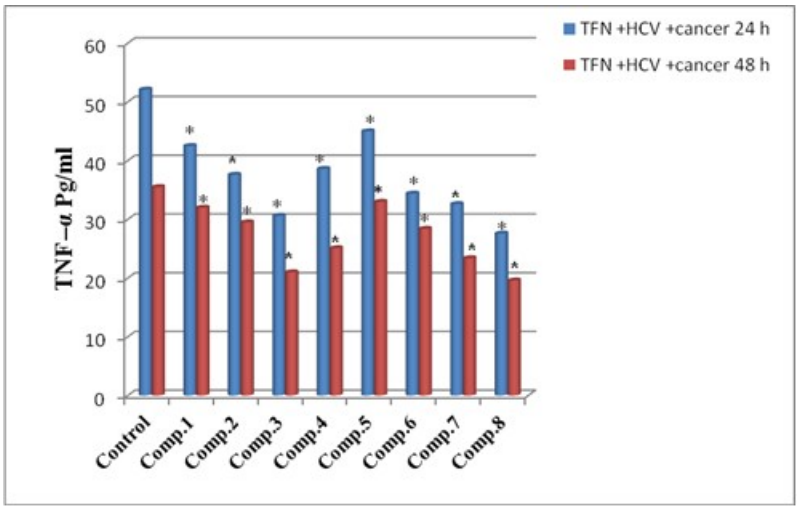

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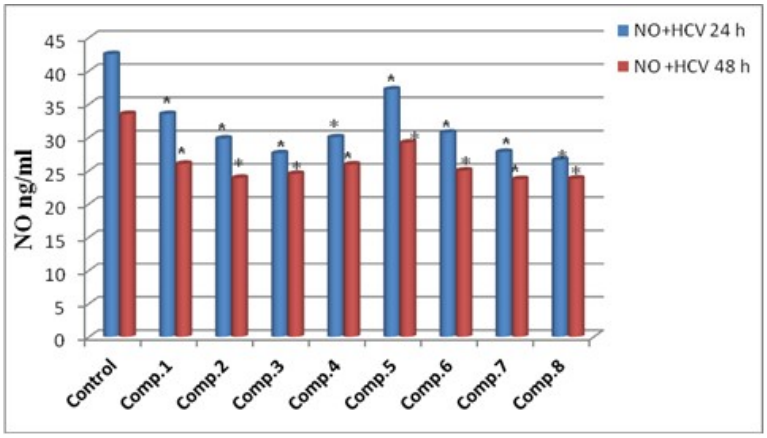

Figure (5)

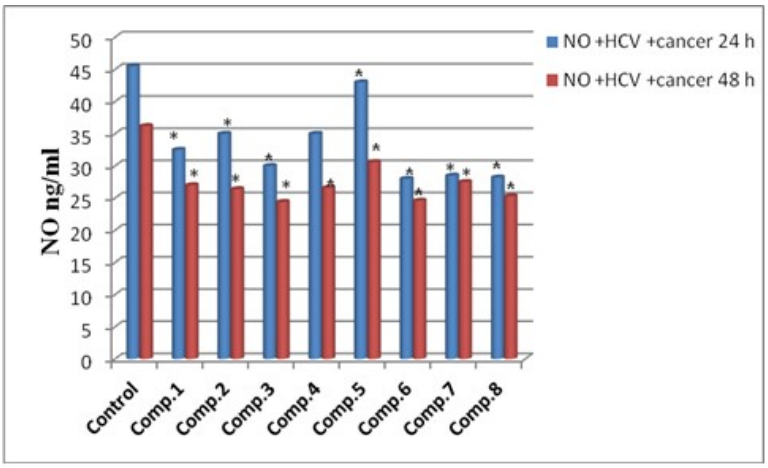

Figure (6)