Abstract: The defatted 85 % methanolic extract from the leaves of *Cassia glauca* Lan. (Family Leguminaceae) showed high cytotoxic effect against liver carcinoma cell line (HepG2) (IC$_{50}$ = 17 μg/ml). Therefore this extract was fractionated using different organic solvents; chloroform, ethyl acetate and n-butanol. Each fraction was submitted to chromatographic separation and the structure of the isolated compounds were elucidated using physical properties and certain spectroscopic analysis. From the chloroform fraction, three compounds; Di-(2-ethylhexyl) phthalate (DEHP) (1), apigenin (2) and luteolin (3) were isolated. Quercetin (4), quercetin-3-O-β-D-glucopyranoside (5) and kaempferol-3-O-rutinoside (6) were isolated and identified from ethyl acetate fraction whereas from the n-butanol fraction three compounds were identified as D (+)-pinitol (7), quercetin-3-O-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (Rutin) (8) and quercetin-3-O-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→6)]-β-D-glucopyranoside (9). The cytotoxicity of the isolated compounds 5, 6 and 9 have promising cytotoxicity with IC$_{50}$ = 16.1, 14.3 and 15.2 μg/ml respectively. From the above results and our previous reports, the methanol extract of the leaves of *Cassia glauca* could be used as promising antioxidant and anticancer agent after more in vitro and in vivo studies.

1. Introduction
Cancer is one of the most dangerous diseases in humans and presently there is a considerable scientific discovery of new anticancer agents. One possible solution is to explore the potential antioxidant and anticancer properties of plant extracts or isolated products of plant origin (Namiki, 1990; Qiu et al., 2009; Woraratphoka et al., 2012). It is well known that many polyphenolic compounds, such as phenolic acids, flavonoids, anthocyanidins, and tannins, which possess remarkable antioxidant and anticancer activities, are rich in plant materials (Mohsen & Ammar, 2009; Khan et al., 2012; Saeed et al., 2012). Many studies showed that there is a positive correlation between the total phenolic compounds in the plant material and their antioxidant and anticancer properties (Noyeem & Karnekar 2010; El-Sayed et al., 2011; Saeed et al., 2012; Eman et al., 2013). In our previous studies (El-Hashash et al., 2010; El-Sayed et al., 2011) it has been found the methanolic extract of *Cassia glauca* Lan. (Family Leguminaceae) and certain fractions derived from it have high total phenolic and flavonoid contents as well as they showed high antioxidant activity. To our best knowledge, no previous investigation has been done on the isolation of chemical constituents and cytotoxic activity of the compounds isolated from this plant. Therefore, the aim of this study was to chromatographic separation and investigation the cytotoxicity effect of the crude extract and some isolated flavonoids from *C. glauca*.

2. Materials and Methods
2.1. General experimental procedures
Melting points were determined on an electrothermal apparatus (Electrotherm 9200). The nuclear magnetic resonance as 1H-NMR and 13C-NMR spectra were recorded on a Brucker Avance-500 and JEOL GX-spectrometer (500 MHz for 1H and 125 MHz for 13C). The chemical shifts were expressed in δ (ppm) with TMS as reference and coupling constant (J) in Hertz. UV spectra (λ$_{max}$) of the isolated compounds were determined in methanol before and after addition of different reagents on Perkin Elmer UV/Vis spectrophotometer. Mass spectra were measured using Micromass Q-TOF Micro instrument. Infra red analysis was performed using IR spectrometer JASCO, FT/IR-6100 LE, USA. Silica gel 60 (70-230 mesh, Merck) and Sephadex LH-20 (25-100 μm, Sigma) were used for column chromatography. Analytical and preparative thin layer chromatographies (TLC) were performed on silica gel GF$_{254}$ pre-coated plates (Merck).
The leaves of *Cassia glauca* (Syn. *Senna surattensis*) were collected from Garden of Faculty of Agriculture, Cairo University, Giza, Egypt. They were authenticated by Mrs. Treaza Labib, high specialist of plant taxonomy, Department of flora and taxonomy, Orman Botanical Garden Giza, Egypt. Voucher specimen of the plant was deposited at laboratory of medicinal chemistry, Theodor Bilharz Research Institute, Giza, Egypt. The collected plant leaves were dried in shad and finally powdered by electric mill to give 1.6 Kg.

### 2.2. Plant Materials

The leaves of *Cassia glauca* were subjected to column chromatography (80 × 4 cm) packed with silica gel 60 (70-230 mesh, Merck) as stationary phase. Elution started with pure chloroform followed by chloroform/methanol gradient. Fractions 250 ml each were collected, concentrated and examined by TLC (silica gel, solvent system n-ButOH:AcOH:H₂O; 4:1:5) and PC (n-ButOH:AcOH:H₂O; 7:3:0.5) and PC using solvent system (n-ButOH:AcOH:H₂O; 4:1:5). Fractions eluted with CHCl₃:MeOH (90:10) were evaporated and purified on preparative TLC with solvent system (Toluene:CHCl₃; 7:3) to give compound 1 as a colorless oily liquid. Fractions eluted with CHCl₃:MeOH (85:15) were concentrated and subjected to re-chromatography on a Sephadex LH-20 column with methanol as the eluting solvent to obtain compounds 2 and 3.

**Compound 1** was obtained as a colorless oily liquid. It is soluble in chloroform, ethyl acetate and ethanol but insoluble in water. It appeared as a single spot on TLC with *R*ₖ value = 0.6 (solvent system: Toluene:CHCl₃; 7:3). IR νₘₐₓ (KBr): 3430.42, 2930.31, 2866.67, 2734.57, 1944.88, 1729.83, 1481.78, 1381.76 1279.64, 1126.22, 1072.23, 956.62 and 743.42 cm⁻¹. ¹H-NMR δ: 7.67 (2H, dd, J = 7.5 Hz, 1.55 Hz), 7.48 (2H, dd, J = 7.5 Hz, 1.66 Hz), 4.21 (4H, d, J = 6.1 Hz), 1.66 (2H, m), 1.32 (2H, m), 1.29 (2H, m), 0.89 (3H, t) and 0.89 (3H, t). ¹³C-NMR δ: 132.53 (C-1, C-2), 128.85 (C-3), 130.94 (C-4), 128.85 (C-6), 167.78 (C-7, C-8), 68.17 (C-1′), 38.81 (C-3′), 30.44 (C-2′), 28.99 (C-4′), 23.05 (C-5′), 23.82 (C-7′), 14.10 (C-6′) and 11.02 (C-8′).

**Compound 2** was obtained as pale yellow, its m.p. 348-350 °C, *R*ₖ values = 0.17 (15% Acetic acid, PC), 0.8 (n-ButOH:AcOH:H₂O; 4:1:5, PC). UV *λ*ₘₐₓ (MeOH) 268, 295, 339; (MeOH+NaOMe) 274, 330, 391; (MeOH+AlCl₃) 275, 298, 386; (MeOH+AlCl₃+HCl) 276, 343, 385; (MeOH+NaOAc) 275, 321, 393; (Me+NaOAc +H₂BO₃) 270, 299, 357.

**Compound 3** was isolated as yellow powder, m.p. 328-330 °C, *R*ₖ values = 0.09 (15% AcOH, PC), 0.76 (n-ButOH:AcOH:H₂O; 4:1:5, PC) and 0.26 (CHCl₃:MeOH:H₂O; 7:3:0.5, TLC). UV *λ*ₘₐₓ(MeOH) 267, 296, 355; (MeOH+NaOMe) 270, 347, 409; (MeOH+AlCl₃) 272, 314, 418; (MeOH+AlCl₃+HCl) 275, 324, 382; (MeOH+NaOAc) 273, 321, 395; (Me+NaOAc +H₂BO₃) 268, 306, 370.

### 2.3. Extraction and Fractionation

A total of 1.6 kg of air-dried powder of the leaves of *Cassia glauca* was extracted by 85% aqueous methanol on cold till exhaustion. The solvent was distilled using rotatory evaporator under vacuum till dryness. The dried extract (450 g) was defatted with petroleum ether (60-80 °C) and the aqueous defatted methanolic extract (400 g) was successively partitioned with chloroform, ethyl acetate and n-butanol. The obtained extracts were evaporated under reduced pressure to dryness. The chloroform, ethyl acetate and butanolic extracts (23, 32 and 38 g) were kept for chromatographic separation process.

#### 2.3.1. Chromatographic Separation of Chloroform Extract

The chloroform extract (20 g) was submitted to column chromatography (80 × 4 cm) packed with silica gel 60 (70-230 mesh, Merck) as stationary phase. Elution started with pure chloroform followed by chloroform/methanol gradient. Fractions 150 ml each were collected, concentrated and examined by TLC (Silica gel, solvent system CHCl₃:MeOH:H₂O; 7:3:0.5) and PC using solvent system (n-ButOH:AcOH:H₂O; 4:1:5). Fractions eluted with CHCl₃:MeOH (90:10) were evaporated and purified on preparative TLC with solvent system (Toluene:CHCl₃; 7:3) to give compound 1 as a colorless oily liquid. Fractions eluted with CHCl₃:MeOH (85:15) were concentrated and subjected to re-chromatography on a Sephadex LH-20 column with methanol as the eluting solvent to obtain compounds 2 and 3.

**Compound 1** was obtained as a colorless oily liquid. It is soluble in chloroform, ethyl acetate and ethanol but insoluble in water. It appeared as a single spot on TLC with *R*ₖ value = 0.6 (solvent system: Toluene:CHCl₃; 7:3). IR νₘₐₓ (KBr): 3430.42, 2930.31, 2866.67, 2734.57, 1944.88, 1729.83, 1481.78, 1381.76 1279.64, 1126.22, 1072.23, 956.62 and 743.42 cm⁻¹. ¹H-NMR δ: 7.67 (2H, dd, J = 7.5 Hz, 1.55 Hz), 7.48 (2H, dd, J = 7.5 Hz, 1.66 Hz), 4.21 (4H, d, J = 6.1 Hz), 1.66 (2H, m), 1.32 (2H, m), 1.29 (2H, m), 0.89 (3H, t) and 0.89 (3H, t). ¹³C-NMR δ: 132.53 (C-1, C-2), 128.85 (C-3), 130.94 (C-4), 128.85 (C-6), 167.78 (C-7, C-8), 68.17 (C-1′), 38.81 (C-3′), 30.44 (C-2′), 28.99 (C-4′), 23.05 (C-5′), 23.82 (C-7′), 14.10 (C-6′) and 11.02 (C-8′).
Compound 5 was obtained as yellow powder, m.p. 235-238 °C, Rf values = 0.56 (15% AcOH, PC), 0.47 (n-BuOH:AcOH:H2O; 4:1:5, PC) and 0.5 (CHCl3; MeOH; 7:3:0.5, TLC). UV \( \lambda_{\text{max}} \) (MeOH) 256, 292, 360; (MeOH+NaOMe) 274, 327, 405; (MeOH+AlCl3) 272, 305, 420; (MeOH+AlCl3+HCl) 269, 361, 385; (MeOH+NaOAc) 270, 328, 395; (Me+NaOAc+H3BO3) 262, 298, 385. \(^1\)H-NMR δ: 12.60, 7.53 (2H, dd, J = 2.1, 8.4 Hz, H-2', H-6'). 6.81 (1H, d, J = 8.5 Hz, H-5'), 6.36 (1H, d, J = 1.75 Hz, H-8'), 6.15 (1H, J = 1.9 Hz, H-6) and 5.42 (1H, d, J = 7.5, H-1').

Compound 6 was isolated as yellow amorphous powder m.p. (163-164 °C), Rf value = 0.66 (15% AcOH) and 0.35 (n-BuOH:AcOH:H2O; 4:1:5). UV \( \lambda_{\text{max}} \) (MeOH) 258, 321, 357; (MeOH+NaOMe) 273, 345, 410; (MeOH+AlCl3) 274, 328, 405; (MeOH+AlCl3+HCl) 270, 303, 400; (MeOH+NaOAc) 273, 310, 392; (Me+NaOAc+H3BO3) 262, 307, 376. \(^1\)H-NMR δ: 12.55 (1H, s, 5-OH), 6.22 (1H, d, J = 2.1 Hz, H-6), 6.43 (1H, d, J = 2.0 Hz, H-8), 7.99 (2H, d, J = 8.75 Hz, H-2', H-6'). 6.88 (2H, d, J = 8.9 Hz, H-3', H-5'), 5.32 (1H, d, J = 6.90 Hz, H-1''), (Glc), 4.30 (1H, d, J = 1.24 Hz, H-1'''), Rha) and 1.03 (3H, d, J = 6.25 Hz, Rha-6'''). \(^1\)C-NMR δ: 177.75 (C-4'), 164.53 (C-7'), 161.54 (C-5'), 160.28 (C-8'), 157.20 (C-9'), 156.84 (C-2'), 133.59 (C-3'), 131.21 (C-2', C-6'), 121.23 (C-1'), 115.46 (C-3', C-5'), 104.34 (C-10), 101.70 (C-1''), 101.12 (C-1'''), 99.09 (C-6'), 94.11 (C-8'), 74.54 (C-2''), 76.11 (C-3''), 72.20 (C-4''), 76.75 (C-5''), 67.25 (C-6''), 70.70 (C-2'), 70.30 (C-3''), 70.98 (C-4''), 68.59 (C-5''), 18.07 (C-6'').

2.3.3. Chromatographic Isolation of Butanolic Extract

Butanolic extract of C. glauca (35g) was chromatographed on Silica gel 60 (70-230 mesh, Merck) column (6×120 cm). Elution was started with CHCl3 and then with CHCl3/MeOH gradient. Fractions 250 ml each were collected, concentrated and examined by PC (Whatmann No. 1 and 3) with solvent systems (n-BuOH:AcOH:H2O; 4:1:5 and 15% AcOH). Chromatograms were visualized under UV before and after exposure to NH3 and spraying AlCl3. Fractions eluted with pure CHCl3 were purified on preparative silica gel TLC using solvent system (CHCl3;MeOH; 7:3) to give compound 7. Fractions eluted with CHCl3;MeOH (95:5) were rechromatographed on silica gel column. Elution started with CHCl3, followed by CHCl3;MeOH mixtures and finished with pure MeOH. Two major fractions I (CHCl3;MeOH 70: 30) and II (CHCl3;MeOH; 60:40) were obtained. Fraction I was subjected on column Sephadex LH-20 and eluting with aqueous methanol to give compound 8 whereas fraction II was subjected to preparative PC using solvent system (n-BuOH: AcOH:H2O; 4:1:5 and 15 % AcOH) to give compound 9.

Compound (7) was obtained as a white crystal with m.p. 185-186 °C, Rf value = 0.42 (CHCl3; MeOH; 7:3, TLC) and 0.61 (n-BuOH:AcOH:H2O; 4:1:5, PC). IR \( \nu_{\text{max}} \) (KBr): 3405.07, 2907.10, 1511.92, 1465.03, 1382.71, 1343.19, 1280.50, 1132.97, 750.17, 572.75 cm\(^{-1}\). \(^1\)H-NMR δ: 4.69 (1H, m), 4.59 (1H, m, H-2'), 4.47 (1H, t, J = 9.3 Hz, H-4'), 4.43 (1H, t, J = 9 Hz, H-3') and 3.57 (3H, s, OCH3). \(^1\)C-NMR δ: 84.28 (C-3), 73.09 (C-1), 72.91 (C-5), 72.45 (C-6), 71.42 (C-4, C-2'). 60.90 (O-CH3).

Compound (8) was obtained as yellow powder, m.p. 198-200 °C, Rf values = 0.68 (15% ACOH, PC), 0.32 (n-BuOH:AcOH:H2O; 4:1:5, PC) and 0.38 (n-BuOH:AcOH:H2O; 4:1:1, TLC). UV \( \lambda_{\text{max}} \) (MeOH) 254, 259, 356; (MeOH+NaOMe) 268, 334, 410; (MeOH+AlCl3) 270, 312, 420; (MeOH+AlCl3+HCl) 269, 352, 409; (MeOH+NaOAc) 271, 312, 399; (Me+NaOAc+H3BO3) 265, 296, 380. \(^1\)H-NMR δ: 12.55 (1H, s, 5-OH), 7.51 (1H, d, J = 2.0 Hz, H-2'), 7.49 (1H, dd, J = 8.4, 2.1 Hz, H-6), 6.81 (1H, d, J = 8.4 Hz, H-5'), 6.35 (1H, d, J = 2.1 Hz, H-8), 6.16 (1H, d, J = 2.1 Hz, H-5'), 5.31 (1H, d, J = 6.85 Hz, H-1'''), Glae), 4.34 (1H, d, J = 1.25 Hz, H-1'''), Rha), 0.95 (3H, d, J = 6.1 Hz, Rha-6'''). \(^1\)C-NMR δ: 177.88 (C-4'), 164.61 (C-7'), 157.14 (C-5'), 156.94 (C-9, C-2'), 148.92 (C-4'), 145.25 (C-3'), 122.11 (C-1'), 121.69 (C-6'), 116.77 (C-5'), 115.74 (C-2'), 104.47 (C-10), 101.67 (C-1''), 99.21 (C-6), 94.13 (C-8), 76.53 (C-5''), 76.39 (C-3''), 74.58 (C-2''), 72.35 (C-4''), 71.06 (C-4''), 70.88 (C-2''), 70.50 (C-3''), 68.76 (C-5''), 67.85 (C-6''), 18.24 (C-6'').
2.4. Cytotoxic Assay

The cytotoxic activity of the defatted methanolic extract of C. glauca and three isolated compounds toward human liver carcinoma cell lines (HepG2) were carried out according to the reported method by Skehan et al. (1990) at National Cancer Institute, Cairo University, Egypt. The cells were cultured in RPMI-160 media supplemented with 10% heat inactivated fetal bovine serum (FBS), 10% (v/v) Penicillin/ Streptomycin and 1 % glutamine at 37 °C under 5 % CO₂ in air in a humidified incubator at 37 °C with 5% CO₂. Briefly, cells were seeded at a density of 5×10⁴ cells/well in 96-well plates. After 24 h, serial dilutions of samples and standard drug (Methotrexate) solutions were added for different concentrations (0, 10, 25, 50 and 100 μg/ml) and incubation of the plate was continued for 24, 48 and 72 hours left without treatment. For cell fixation, the culture medium was removed and trichloroacetic acid (50%, 50 μL) was added in each plate. Then the plates were air-dried and 0.4% SRB (Sigma) in 1% acetic acid was added for 30 min and unbound dye was washed out with 1% acetic acid. After air-drying, SRB dye within cells were dissolved with 100 μL solution of tris-base 10mM (pH 10.5). The optical density of the extracted SRB dye was measured with a microplate reader at 564nm with an ELIZA microplate reader. The experiment was repeated three times for each cell line. The 50% inhibitory concentration (IC₅₀) of the test samples was calculated from the prism program obtained by plotting the percentage of surviving cells versus the concentrations interpolated by cubic spine.

3. Results and Discussion

According to the American National Cancer Institute (NCI) guidelines, an extract with IC₅₀ values less than 20 μg/ml is considered active (Boyed, 1997). In the present study, the 85% methanol extract of the leaves of Cassia glauca exhibited high cytotoxic potential with IC₅₀ value of 17 μg/ml against HepG2 using SRB assay. This result fall within the NCI criteria, thus this extract is considered as of promising anticancer agent. Thus, fractionation of the methanolic extract of C. glauca with chloroform, ethyl acetate and n-butanol was carried out. Each of these fractions was submitted to chromatographic isolation. Three compounds 1-3 were isolated from the chloroform fraction, and compounds 4-6 were isolated from the ethyl acetate fraction whereas compounds 7-9 were isolated from the butanolic fraction. The chemical structures of the isolated compounds (Figure 1) were elucidated as follows:

**Compound (1)** was obtained as a colorless oily liquid. It is soluble in chloroform, ethyl acetate and ethanol but in soluble in water. The IR spectrum of this compound showed a characteristic peak of the carbonyl group (C=O) at 1729.83 cm⁻¹. Other peaks appeared at 2930.31 cm⁻¹ (-CH₂ stretch), 2866.67 cm⁻¹ (-CH stretch), 1461.78 and 743.42 cm⁻¹ (methylene C-H bend and (CH₃)-rocking) respectively and 1126.22 cm⁻¹ (C-O stretch) (Rao et al., 2000; Lyuts-Kanova et al., 2009). The ¹H-NMR spectrum of the compound showed the aromatic protons at δ: 7.67 and 7.48, two protons at δ: 4.21, one proton at δ: 1.66 and two methyl resonances at δ: 0.89 (Rao et al., 2000; Al-Bon et al., 2006). Also the characteristic signals of the methylene groups, the existence of only two aromatic protons in the molecule were showed in the ¹H-NMR spectrum. This suggested that the compound must has an ortho-disubstituted benzene ring bearing the same substituted in both protons (Al-Bon et al., 2006). The ¹³C-NMR spectrum of compound 1 confirmed the symmetry of the molecule and exhibited the expected 12 carbon resonances including two quaternary, three methine and five methylene carbons with two methyl groups (Rao et al., 2000). ESI mass spectrum of compound 1 displayed pseudo-molecular ions at m/z 413.28 [M+Na]+ and 803.54 [2M+Na]+. ESI-HRMS due to m/z 391.28 (Rao et al., 2000). By comparison of ¹H-NMR and ¹³C-NMR data to those published in literatures (Al-Bon et al., 2006; Lyuts-Kanova et al., 2009) compound 1 was identified as Di-(2-ethylhexyl) phthalate (DEHP).

**Compound (2)** was obtained as pale yellow. UV spectrum of methanolic solution of the compound 2 showed characteristic bands of flavone structure at 268 nm and 339 nm. Bathochromic shift of band I (52 nm) in presence of NaOMe indicated to the presence of free OH at 4. Also, a bathochromic shift of band II (7 nm) after addition of NaOAc indicated the presence of OH group in position 7. Bathochromic shift of band II with AlCl₃ (7 nm) reflected the presence of free OH at position 5. Addition of NaOAc+H₂BO₃ led to shift of band I (18 nm) indicating the absence of OH group in position 3. Its UV data was in good agreement with that of apigenin (Selda et al., 1990; Fathiazl et al., 2006; Moussaui et al., 2010). This compound was identified as apigenin by direct comparing its m.p. and Co-PC behavior with authentic apigenin.

**UV spectrum of Compound 3** showed two characteristic bands at 267 nm and 355 nm reflecting the compound belongs to flavonoid group (Moussaui et al., 2010). Bathochromic shift in band I by addition of AlCl₃ (63 nm) indicated the presence of free ortho-dihydroxy groups at position 3’,4’. And a bathochromic shift in band II (5 nm) indicated to presence of free OH group in position 5. By addition NaOAc, a bathochromic shift observed with band II at (6 nm) reflecting the presence of OH group in position 7. Bathochromic shift in band in band I (15 nm) by addition of NaOAc+H₂BO₃ indicated the presence of OH groups in position 3’ and 4’ and absence of OH
group in position 3 (Moussaui et al., 2010). This compound was identified as luteolin by direct comparison of m.p. Co-PC with authentic luteolin.

**UV spectrum of the compound 4** in methanolic solution showed two major absorption bands at 371 nm and 256 nm which confirmed the flavonol structure. Observation of bathochromic shift (17 nm) of band II with NaOMe indicated the presence of free 7-OH and 5-OH groups. Bathochromic shift in band I with AlCl₃ (85 nm) reflected the presence of O-dihydroxy group in B-ring at 3' and 4' positions and band II with (18 nm) indicated of free 5-OH group. Hypsochromic shift in band I with AlCl₃/HCl (30 nm) indicated the presence of 3', 4'-OH groups. Bathochromic shift (16 nm) in band I with NaOAc/H₂BO₃ also indicated the presence of O-dihydroxy groups in B-ring at 3' and 4' positions (Lee et al., 2004; Awaad et al., 2008). From this data of compound 4 and by direct comparing its m.p. and Co-PC behavior with authentic quercetin compound 4 was elucidated as quercetin.

**UV spectra of compound 5** showed a characteristic two major absorptions bands of flavonoid compounds at 256 nm and 360 nm. Bathochromic shift in band I (45 nm) was appeared by addition of NaOMe reflected the presence of OH group at position 3', 4'. Bathochromic shift in band I (25 nm) was appeared by addition NaOAc:H₂BO₃ reflecting the presence of free OH group at position 3' and 4'. By addition of NaOAc, a bathochromic shift (14 nm) in band II was clear due to presence of OH group at position 7. Bathochromic shift (60 nm) in band I upon the addition of AlCl₃ indicated the free ortho dihydroxy groups at position 3' and 4'. (Sikorska & Matlawksa 2000; Awaad et al. 2008; El-Sawi & Sleem 2010).

**1H-NMR spectrum of compound 5** showed a characteristic signals of quercetin skeleton at δ: 12.60 (s, 5-OH), 7.53 (2 H, dd, J = 2.1, 8.4 Hz, H₂; H-6), 6.81 (1 H, d, J = 8.5 Hz, H-5'), 6.36 (1 H, d, J = 1.75 Hz, H-8), 6.15 (1 H, d, J = 1.9 Hz, H6) beside the anomeric proton signal of glucose unit at δ: 5.42 (1 H, d, J = 7.5, H-1'). Acid hydrolysis of compound 5 gave quercetin as aglycone and glucose as sugar moiety. Both the aglycone and glucose was identified by comparison with authentic quercetin and glucose. Therefore, the structure of compound 5 was characterized as quercetin-3-O-β-D-glucopyranoside.

**Compound (6)** UV absorption spectrum of this compound in methanol exhibited two absorption maxima at 357 nm and 258 nm indicating it's a flavonol structure. A bathochromic shift of band I (53 nm) with addition NaOMe indicated the presence of 3', 4'-dihydroxyflavone and a bathochromic shift of band II (15 nm) indicated the presence of free 7-hydroxy group. A bathochromic shift (48 nm) in band I with addition AlCl₃ indicated the existence of 5-hydroxy group (Han et al., 2004; Hashimoto et al., 2008). The 1H-NMR spectrum of the compound showed the expected signals of 4-disubstituted B-ring protons and two ortho-coupled resonances at δ: 7.99 (2H, d, J = 8.9 Hz) and δ: 6.88 (2H, d, J = 8.7 Hz) for H-2', H-6', H-3' and H-5', respectively (Song et al., 2007; Mu et al., 2009). The presence of a 5, 7-dihydroxy A-ring was deduced from the typical metal-coupled resonances of H-8 and H-6 at δ: 6.43 (1H, d, J = 2.0 Hz) and δ: 6.22 (1H, d, J = 2.1 Hz) respectively. The presence of a β-glucopyranosyl moiety directly attached to the aglycone was detected from the relatively downfield β-anomeric proton H-1' resonance at δ: 5.32 (1H, d, J = 6.90 Hz). The terminal attachment of α-rhamno-pyranosyl moiety to C-6' of the inner glucose was evidenced from the resonance of the anomeric proton H-1₆' at δ: 4.30 (1H, J = 1.24 Hz) and CH₃-resonance at δ: 1.03 (3H, d, J = 6.25 Hz, Rha-6₃) (Moustafa et al., 2009; Mu et al., 2009). Comparison of the 13C-NMR spectral data of compound 6 with its aglycone spectrum of kaempferol showed that there is a down field of 2.6 ppm for C-3 signal which confirm the glycosylation at position C-3 (Song et al., 2007; Mu et al., 2009). The (1°→6°)-O-glycosidic linkage of the rhamnopyranosyl on the glucoside moiety was evidenced from the fact that the C-6' signal at 67.25 ppm was shifted downfield (6.65 ppm) (Song et al., 2007; Mu et al., 2009). This related to the 67.25 ppm chemical shift of the corresponding carbon (C-6') of the inner glucose. Two anomeric carbon signals were observed at δ: 101.70 and 101.12 ppm in 13C-NMR spectrum (Moustafa et al., 2009; Mu et al., 2009). ESI-Ms exhibited a molecular ion peak at m/z 617.14 and fragment at m/z 287.05 which corresponding to [M+Na]+ and [M-Na-146-163] suggesting the presence of two glycosyl moieties; hexose and deoxyhexose (glucose and rhamnose). Acid hydrolysis of compound 6 gave kaempferol as aglycone and D-glucose and L-rhamnose as sugar moiety. Both of aglycone and sugar was identified by comparison with authentic samples. Therefore, compound 6 was concluded as kaempferol-3-O-rutinoside.

**Compound (7)** was obtained as a white crystal. Its IR spectrum showed characteristic bands of hydroxyl groups at 3405 cm⁻¹ and other bands at 2907, 1455, 1132 and 1061 cm⁻¹ (Chaubal et al., 2005; Sureshan et al., 2009). 1H-NMR spectrum of the compound showed a multiplet at δ: 4.69 for H-2 and H-4, a multiplet at δ: 4.59 for H-1 and H-5, a triplet at δ: 4.47, a triplet at δ: 4.43 for H-6 and H3 and a singlet at δ: 3.57 for OMe, 3H atoms (Abdoulaye et al., 2004; Jain et al., 2007). The 13C-NMR spectrum of compound 7 showed a characteristic signals of C-3 attached to OMe group appeared at δ: 84.28, a signals of C-2 and C-4 at δ: 71.42, a signal of C-1 at 73.09, a
signal of C-5 at 72.91, a signal of C-6 at 72.45, and OMe appeared at δ: 60.09 (Narayanan et al., 1987; Boven et al., 2001; Abreu & Relva 2002; Yu et al., 2005). ESI-MS showed two peaks at 217.06 and 411.14 corresponding to [M+Na]+ and [2M+Na]+. From the above data, compound 7 was elucidated as D(+)--pinitol. This compound had been isolated from the leaves of bongainvillea spectabilis, Lespedeza cuneata and the root bark of Tamarindus indica (Narayanan et al., 1987; Jain et al., 2007). Pinitol is known for its antidiabetic, anti-inflammatory, and feeding stimulating activities (Narayanan et al., 1987). This is the first time for the isolation of this compound from C. glauca.

Figure 1: Chemical structures of compounds 1-9 isolated from the leaves of Cassia glauca.
Compound (8) was isolated as yellow powder. Its UV spectrum exhibited characteristic absorption bands of flavonoid structure at 254 and 356 nm (El-Sawi & Sleem 2010; Noyeem & Karnekar 2010). Bathochromic shift in band II (14 nm) was clear by addition NaOAc reflected the presence of free OH at C-7. Also, shifts was obtained by band I (43nm) with NaOAc indicate the presence of OH groups at position 3` and 4`. Bathochromic shift (64 nm) in band I by addition of AlCl3 reflecting the presence of free OH groups at position 3` and 4`. Bathochromic shift band I (24 nm) with NaOAc+H3BO3 indicating the presence of OH group at position 3 and 4 (Noyeem & Karnekar 2010). In its 1H-NMR spectrum a sharp singlet was appeared at δ: 12.55 for 5-OH group. Other five protons of the aglycone moiety was appeared at δ: 7.51 (1H, d, J = 2.0 Hz), 7.49 (1H, dd, J = 8.4 Hz and 2.1 Hz), 6.81 (1H, d, J = 8.4 Hz), 6.35 (1H, d, J = 2.1 Hz) and 6.16 (1H, d, J = 2.1 Hz) corresponding to H-2’, H-6’, H-5’ and H-8 and H-6 respectively. Also two anomeric proton signals were appeared at δ: 5.31 (1H, d, J = 6.85 Hz, H-1’ Glc) and δ: 4.34 (1H, d, J = 12.5 Hz, H-1/Rha), and methyl group signal at δ: 0.95 (Huelin & Tzelin 1999; Sikorska & Matlaw ska, 2000; El-Sawi & Sleem 2010). This confirmed by the presence of twenty-seven carbon signals in 13C-NMR of compound 8. Fifteen of them assigned to the carbon signals the aglycone part where as the remaining carbon signals for the two sugar moiety; D-glucose and L-rhamnose were obtained by complete acid hydrolysis of compound 8. Both of the aglycone part and of sugar part was identified by comparison with authentic sample. Therefore, compound 8 was determined as quercetin-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (Rutin).

Compound (9) was obtained as yellow amorphous powder. Its UV spectra of this compound in methanol as shown in gave two major spectral peaks at 258 nm (Band II) and 360 nm (Band I). The bathochromic and hypsochromic shifts of the compound in the usual shifts reagents were in a good agreement with quercetin 3-O-glycoside structure (Beck & Haberlein 1999). Free 4′-OH group was clear from the bathochromic shifts in band I (48 nm) with the increase of its intensity upon addition of NaOMe. Also a bathochromic shift (16 nm) in band II was clear by addition NaOAc reflected presence of free 7-OH. The bathochromic shift (68 nm) in Band I upon the addition AlCl3 indicated to free ortho dihydroxy in position 3 and 4′. The presence of ortho-dihydroxy groups in B-ring was deduced from the hypsochromic shift in band I in AlCl3/HCl spectrum (39 nm) relative to that in case of addition of AlCl3 (Beck & Haberlein 1999; Hossain et al., 2006). The 1H-NMR spectrum indicated a 5,7-dihydroxylated pattern for ring-A (two signals at δ: 6.19 and 6.39 (J = 2.1 Hz, H-6 and H-8). For ring-B signals at δ: 6.85 (1H, d, J = 8.5 Hz), δ: 7.49 (1H, dd, J = 8.4, 2.2 Hz) and δ: 7.55 (1H, d, J = 2.3 Hz) for H-5′, H-6′ and H-2′ respectively, allowing the aglycone to be recognized as quercetin (Han et al., 2001; Manguro et al., 2004; El-Sawi & Sleem 2010). Three anomic signals of anomic protons were cleared in the 1H-NMR spectrum at δ: 5.54, 5.14, and 4.37 suggested the presence of a trisaccharide.

Flavonoids and polyphenols have previously been anticancer. So, flavonoid can be considered as therapeutic agents against cancer (Moussaui et al., 2010; El-Sawi & Sleem 2010). Acid hydrolysis of compound 9 gave Quercetin as aglycone and rhamnose and glucose as sugar. Each of aglycone and sugar were identified by comparison with authentic samples. On these bases, compound 9 was established as quercetin-3-O-[α-L-rhamnopropyrosyl-(1→2)-α-L-rhamnopropynosyl-(1→6)]-β-D-glucopyranoside.

It has been reported that, the anticancer activity of the plant extracts is associated with a variety of classes, such as polyphenols, flavonoids and catechins (Abdel-Hady et al., 2011; Ghasemzadeh & Neda 2011). Flavonoids are non toxic polyphenolic compounds display a remarkable spectrum of biological activities including antioxidant and anticancer. So, flavonoid can be considered as possible chemopreventive and therapeutic agents against cancer (Wang, 2000; Mavundza et al., 2010; Ghasemzadeh & Neda 2011). A number of flavonoids and polyphenols have previously been isolated from different parts of plant extracts which may be involved in their reported antioxidant and cytotoxic properties (Moussaui et al., 2010; Saeed et al., 2012; Eman et al., 2013). In this study, the three flavonoids compounds 5, 6 and 9 showed high cytotoxic agent against human liver carcinoma cell lines HepG2 (IC50 = 14.3, 9 and 15.2 μg/ml) respectively.

The results of cytotoxicity of the three isolated compounds revealed that compound 6 (kaempferol-3-O-rutinoside) has higher activity than the other two compounds; 5 (quercetin-3-O-β-D-glucopyranoside).
and 9 [quercetin-3-O-([α-L-rhamnopyrinosyl-(1→2)-
α-L-rhamnopyrinosyl-(1→6)]-β-D-glucopyrano-side)], then the kind of flavonoid aglycon play important role in the activity. The presence of two hydroxyl groups of the B-ring (quercetin aglycon) decrease the cytotoxic activity rather than the presence of one hydroxyl group in B-ring of the flavonoid (kaempferol aglycon). This result was previously reported by Kathrin et al., (2007) who stated that there is inverse correlation between the cytotoxicity and the number of hydroxyl phenolic groups of flavonoids. Also, it is noticed that the number of sugar units play a role in cytotoxic activity as shown between the two compounds 5 and 9 which have the same quercetin aglycon. On the other hand the antioxidant properties of the flavonoid compounds increased by increasing the number of free phenolic hydroxyl groups (Choi et al., 2002).

Conclusions
The present study revealed that the methanolic extract of Cassia glauca leaves exhibited high in vitro anticancer activity toward hepatocellular carcinoma cell line (HepG2). Nine compounds including eight flavonoid compounds were isolated from the methanolic extract, three of these compounds showed high cytotoxic activity. From the above results and our previous reports, the methanolic extract of Cassia glauca leaves of seven Egyptian Cassia species showed high cytotoxic activity toward hepatocellular carcinoma cell line (HepG2) and has the same quercetin aglycon. On the other hand the antioxidant properties of the flavonoid compounds increased by increasing the number of free phenolic hydroxyl groups.

Conflict of interest
Authors have declared that there is no conflict of interest in this manuscript.

Corresponding author
El-Sayed S, Abdel-Hameed
1Medicinal Chemistry Laboratory, Theodor Bilharz Research Institute, Warrak El-Hader, Giza, Egypt.
2Natural Products Analysis Laboratory, Faculty of Science, Taif University, Kingdom of Saudi Arabia.

References


