Original Research Article

Cytotoxicity of N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide and Ethyl 7-hydroxycoumarin-3-yl ester

Abstract:

Background: Coumarins (2H-1-benzopyran-2-one), an important class of heterocyclic compounds, and its derivatives can be found in many natural or synthetic drug molecules and possess versatile bioactivities making them important molecules for medical practitioners and medicinal chemists.

Aims and Objective: Our study aims to evaluate cytotoxicity of new Coumarin derivatives: N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3) and Ethyl 7-hydroxycoumarin-3-yl ester (comp-2) against four human cell lines such as human breast cancer (MCF-7), human liver cancer (HEPG-2), human colon cancer (HCT) and human prostate cancer cell (PC-3).

Methodology: The ethyl-7-hydroxycoumarin-3-ylester (comp-2) was prepared via cyclocondensation of 2, 4-dihydroxybenzaldhyde with diethylmalonate in the presence of piperidine under fusion followed by Amonolyses with 4-chloro-aniline in the presence of acid medium under fusion produced the N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3).

Result: The synthesized compounds have potent cytotoxicity against different tumor cell lines (MCF-7, HEPG-2, HCT, and PC-3).

Discussion: The compound N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3) is better than Ethyl 7-hydroxycoumarin-3-yl ester (comp-2) because of the nature of the halogen atom (a chlorine or a bromine atom) in the 'meta' position of the phenyl ring relative to the ester oxygen atom of 2-oxo-2H-1-benzopyran- 3-carboxylate led to a better anti-tumor effect than that observed in the absence of any substituent.

Keywords: Coumarins, cytotoxicity, tumor cell lines.

1- Introduction:

Cancer is a disease characterized by failure of tissue growth regulation when the genes that regulate cell growth and differentiation are altered. Most cancers have multiple causes, only a small minority of cancer is due to inherited genetic mutations whereas the vast majority is non-hereditary epigenetic mutations that are caused by various agents (environmental factors, physical factors and hormones). Thus, although there are some genetic predispositions in a small

fraction of cancers, the major fraction is due to a set of new genetic mutations (called "epigenetic" mutations) (**Alain, 2017**). Therefore the search for potent, safe and selective anticancer compounds is a crucial aspect of modern cancer research **(Vani *et al*., 2010)**. The side effects of Chemotherapy are usually caused by its effects on healthy cells. Consequently, the principal obstacles to the clinical efficacy of chemotherapy remain their possible toxicity to normal tissues of the body, beside the development of cellular drug resistance especially to conventional anticancer agents **(Sherif, 2010)**.

Natural or synthetic coumarins due to their wide range of biological activities have become an interesting subject of investigation for many researchers. Coumarin scaffold has proven to have an important role in anticancer drug development due to a fact that many of its derivatives have shown an anticancer activity on various cell lines. Action of coumarins on tumor cells is executed by different mechanisms and some of them show very good selectivity towards the cancer cells (**Klenkar *et al*., 2015).**

Coumarins belong to benzopyrone chemical class, more precisely benzo-α-pyrones, where benzene ring is fused to pyrone ring (**Lacy *et al*., 2004)**. In nature, Coumarins are found in higher plants like *Rutaceae* and *Umbelliferae* and some essential oils like Cinnamon barf oil, Cassia leaf oil and Lavender oil are also rich in coumarins. Except from higher plants, coumarins were found in microorganisms as well, like novobiocin and coumermycin from *Streptomyces* and aflatoxins from *Aspergillus* species **(Jain *et al*., 2012).**

Coumarins are proven to possess a wide range of biological activities, anti-influenza **(Yeh *et al*., 2010),** anti-inflammatory **(Lee *et al*., 2011)**,antioxidant **(Kostova *et al*.,2011)**, antitumor **(Huang *et al*., 2011),**antituberculosis**(Manvar *et al*., 2011)**, antimicrobial **(Nitiema *et al*., 2012)**, antinociceptive, anti- Alzheimer **(Anand *et al*., 2012)**, antiasthmatic **(Sanchez-Recillas *et al*., 2014)**, antiviral **(Xu *et al*., 2014),** anti-HIV **(Kudo *et al*., 2015)** , antidepressant **(Sashidhara *et al*., 2015)**, , antihyperlipidemic **(Asif, 2015)**.

Antitumor activity of natural and synthetic coumarin derivatives have been extensively explored by many researchers **(Wang *et al*., 2015)** and it has been proven that coumarins, depending on their structure, can act on various tumor cells by different mechanisms; they inhibit the telomerase enzyme, protein kinase activity and down regulating oncogene expression or induce the caspase-9-mediated apoptosis, suppress cancer cell proliferation by arresting cell cycle in G0/G1 phase, G2/M phase and affecting the p-glycoprotein of the cancer cell **(Amin *et al*., 2013;Nasr *et al*., 2014)**.

Coumarin derivatives can possess not only cytostatic, but cytotoxic properties as well **(Benci *et al*., 2012)**. **(Marshall *et al*., 1991)** showed that coumarin and 7-hydroxyycoumarin can inhibit growth in human cancer cell lines such as A549 (lung),

ACHN (renal), H727 (lung), MCF7 (breast) and HL-60 (leukaemia) and in some clinical trials they exhibited anti-proliferative activity in prostate cancer **(Mohler *et al.*, 1992)**, malignant melanoma **(Thornes *et al.*, 1994).**

Coumarins also exhibited the cytotoxic effect against Hep2 cells (human epithelial type 2) in dose dependent manner and showed some typical characteristics of apoptosis with loss of membrane microvilli, cytoplasmic hyper-vacualization and nuclear fragmentation **(Mirunalini *et al.*, 2014)**.

Our study aims to evaluate the cytotoxicity properties of recently developed synthetic coumarin derivatives: N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3) and Ethyl 7-hydroxycoumarin-3-yl ester (comp-2) against the different tumor cell line Such as MCF-7, HEPG-2, HCT, and PC-3 cell lines.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

2, 4-dihydroxybenzaldehyde, Diethylmalonate, piperidine, ethanol, Hydrochloric acid (2%), p-chloroaniline, acetic acid were obtained from El-Gomhoria Chemical Co. Port-said. All chemicals were used as received without extra purification.

2.1.2. Cell culture

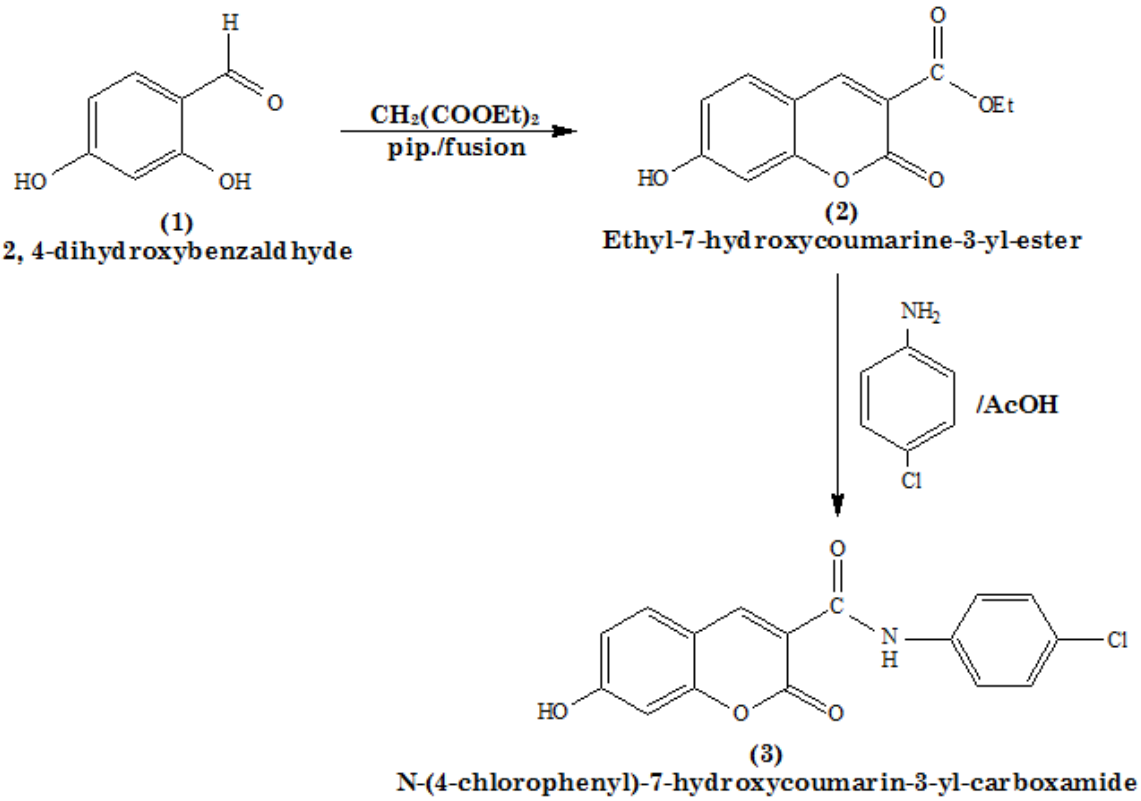
Cancer cells from different cancer cell lines, human breast adenocarcinoma (MCF-7), human hepatocellular carcinoma (HEPG-2), human colon adenocarcinoma (HCT-116) and human prostate cancer cells (PC-3) were purchased from American Type Culture Collection (ATCC, Manassas, USA) and grown on Roswell Park Memorial Institute Medium (PRMI 1640) supplemented with 100mg/ ml of streptomycin, 100 unites / ml of penicillin and 10% of heat-inactivated fetal bovine serum in humidified ,5% (v/v) CO_2 atmosphere at 37 ^{o}C.

2.2. Methods

2.2.1. Chemistry

The ethyl-7-hydroxycoumarin-3-ylester (comp-2) was prepared via cyclocondensation of 2, 4-dihydroxybenzaldhyde (1) with diethylmalonate in the presence of piperidine under fusion according to a literature method (**El-Deen *et al.*, 2004)**.

Amonolyses of ester with 4-chloro-aniline in the presence of acid medium under fusion produced the N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3) [scheme I]



(1)
2, 4-dihydroxybenzaldhyde

(2)
Ethyl-7-hydroxycoumarine-3-yl-ester

(3)
N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl-carboxamide

Scheme (I): Synthesis of ethyl-7-hydroxycoumarin-3-ylester (comp-2) and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3) derivatives.

2.2.2. Synthesis:

All reagents were used as purchased from commercial supplies without further purification.

Melting points were determined by using open capillary tubes and were uncorrected.

The purity of the compound was determined through elemental analysis. Elemental data of C, H and N was found in accordance with ± 0.3% of the theoretical value, respectively as determined by PerkinElmer CHN elemental analyzer.

Using KBr pellets, IR spectrum were obtained with FT-IR spectrometer. ^{1}H-NMR spectra in DMSO-d_6 solutions were respectively recorded at 400 MHz with Bucker 400 ultra – shield TM NMR spectrometer (400 MH_Z) using TMS as internal standard.

Ethyl 7-hydroxycoumarin-3-ylester (1):

A mixture of 2, 4-dihydroxybenzaldehyde (1, 0.01 mole), diethylmalonate (0.01 mole), and piperidine was fused on a hot- plate for 3-4 min, then added ethanol (30 ml).

The reaction mixture was heated under reflux for 2 hour, then cooled and acidified with diluted hydrochloric acid (2%). The solid product was filtered off, dried, and crystallized from ethanol to give 2 as pale yellow crystals, yield 76%, and m.p 165 ^{o}C.

IR (KBr): 3416-2815(br-oH), 1764-1722(C=O of ester and pyranone ring), 1610-1585(C=C), 1125-1095(C-O) cm^{-1}. ^{1}H-NMR(DMSO-d$_6$): δ1.32(m,3H,CH$_3$), 4.41(g,2H,OCH$_2$),7.40-8.01 (m,3H,Ar-H), 8.53(δ,1H,H-4 of pyranone ring) , 10.7(br-S,1H,OH) ppm. Anol.calcd for C $_{12}$H$_{10}$ O$_5$ (234): C, 61.54; H, 4.27. Found: C, 61.52; H, 4.17.

N-(P-chlorophenyl)-7-hydroxycoumarin-3-ylcarboxamide (2):

A mixture of ester (2, 0.01 mole) and p-chloroaniline (0.01 mole) in acetic acid (25ml) was heated under reflux for 4 hour. The reaction mixture was cooled and poured into ice –water with stirring. The resulting solid was collected by filtration ,washed with water, dried and recrystallized from ethanol to give 3 as yellow crystals , yield 71% ,mp.>300 ^{o}C.

IR (KBr): 3430-2981 (br.OH), 3256(NH), 1726-1708(C=O of pyranone and carboxamide), 1615-158(C=C), 1093-1065(C-O) cm^{-1}. ^{1}H-NMR(DMSO-d$_6$):δ 6.76-7.88(m,7H,Ar-H),8.69(S,1H,H-4 of pyranone ring), 8.87 (S,1H,NH),10.71(S,1H,OH)ppm. Anol.calcd for C$_{16}$ H$_{10}$NClO$_4$ (315): C, 60.95; H, 3.17; N4.44.Found: C, 60.73; H3.08; N, 4.11.

2.2.3. Cytotoxicity assay by 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT):

Exponentially growing cells from different cancer cell lines were trypsinized, counted and seeded at the appropriate densities (5000 cells/0.33 cm^2 well) into 96-well microtiter plates. Cells then were incubated in a humidified atmosphere at 37^{o}C for 24 hours. Then, cells were exposed to different concentrations of compounds (0.05, 0.5, 5, 50, and 500µg/ml) for 72 hours as illustrated in table (1). Then the viability of treated cells was determined using MTT technique as follow. Media were removed; cells were incubated with 200µl of 5% MTT solution /well (Sigma Aldrich, MO) and were allowed to metabolize the dye into a colored –insoluble formazan crystal for 2 hours. The remaining MTT solution were discarded from the wells and the formazan crystals were dissolved in 200 µl/well acidified isopropanol for 30 min, covered with aluminum foil with continuous shaking by using a MaxQ 2000 plate shaker (Thermo Fisher Scientific Inc, MI) at room temperature. Absorbance was measured at 570 nm by using a Stat Fax^{R} 4200 plate reader (Awareness Technology, Inc., FL). The cell viability were expressed as percentage of control and the concentration that induces 50% of maximum inhibition of cell proliferation (IC50) were determined using Graph Pad Prism version 5 software (Graph Pad software Inc,CA) **(Mosmann, 1983 and Scudiero *et al*., 1988)**.

3. RESULTS

Fig. (1): IR spectroscopy of Ester (comp-2)

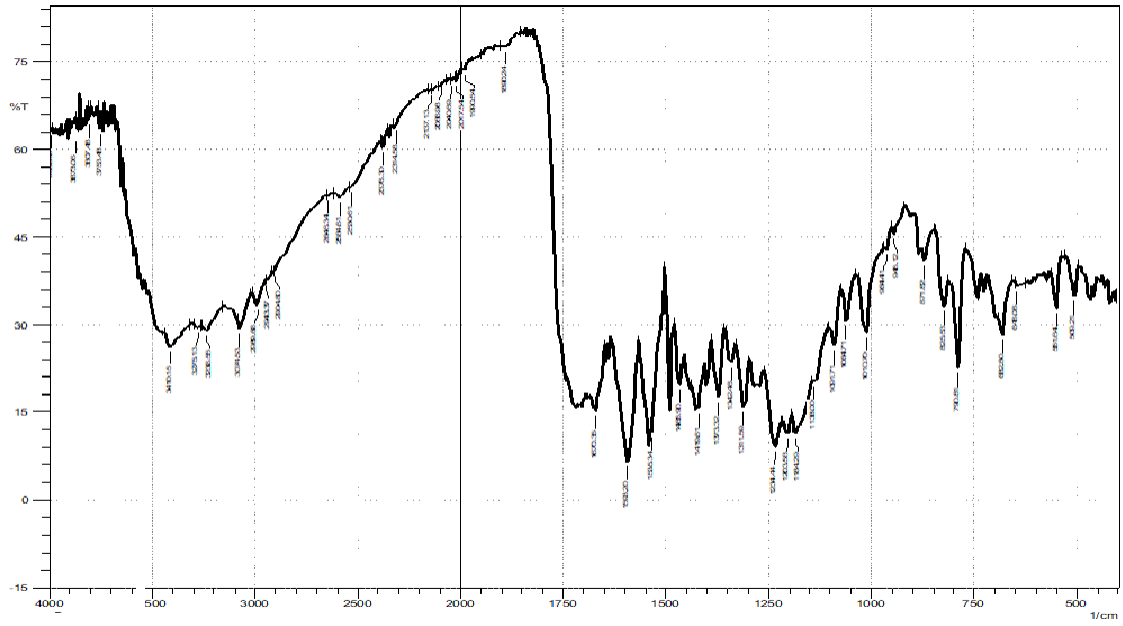


Fig. (2): HNMR spectroscopy for Ester (comp-2)

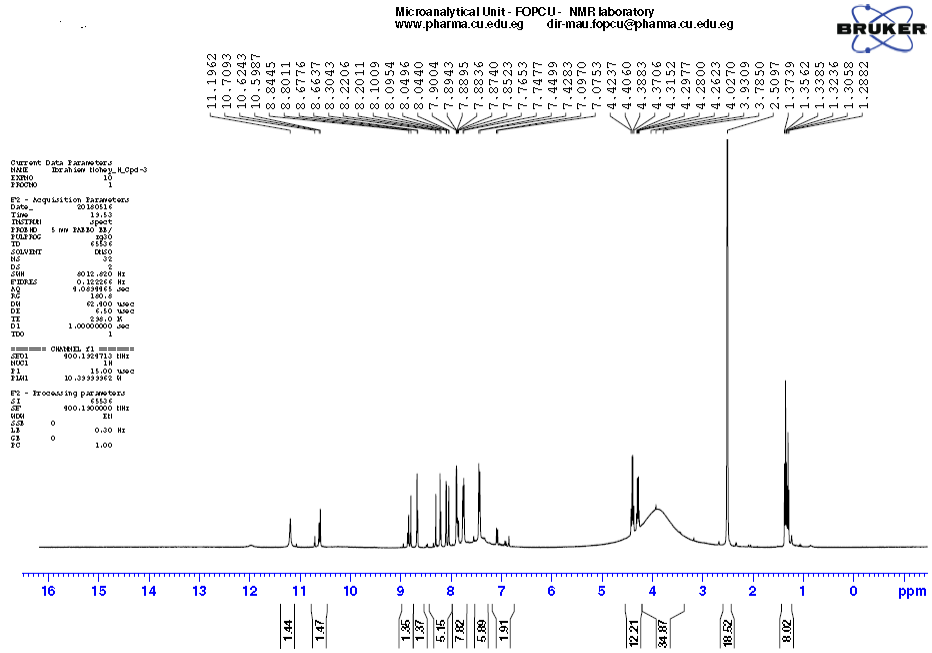


Fig. (3): IR spectroscopy of Carboxamide (comp-3).

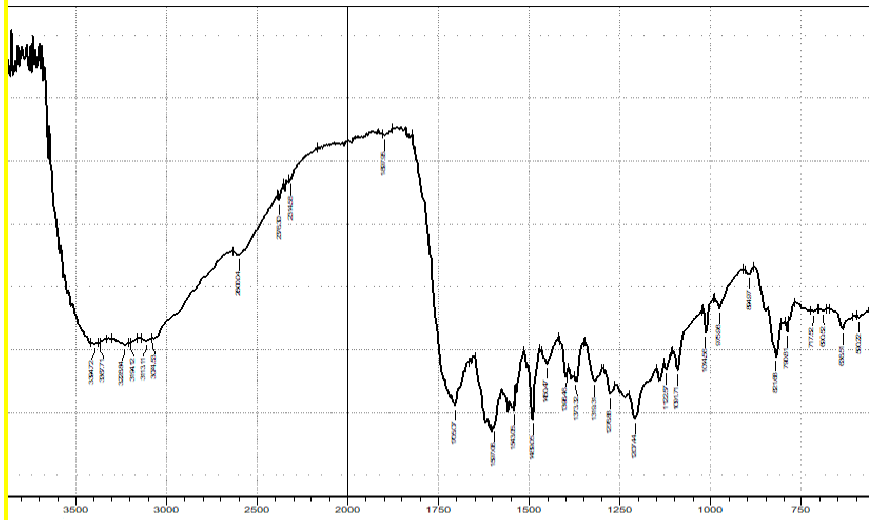
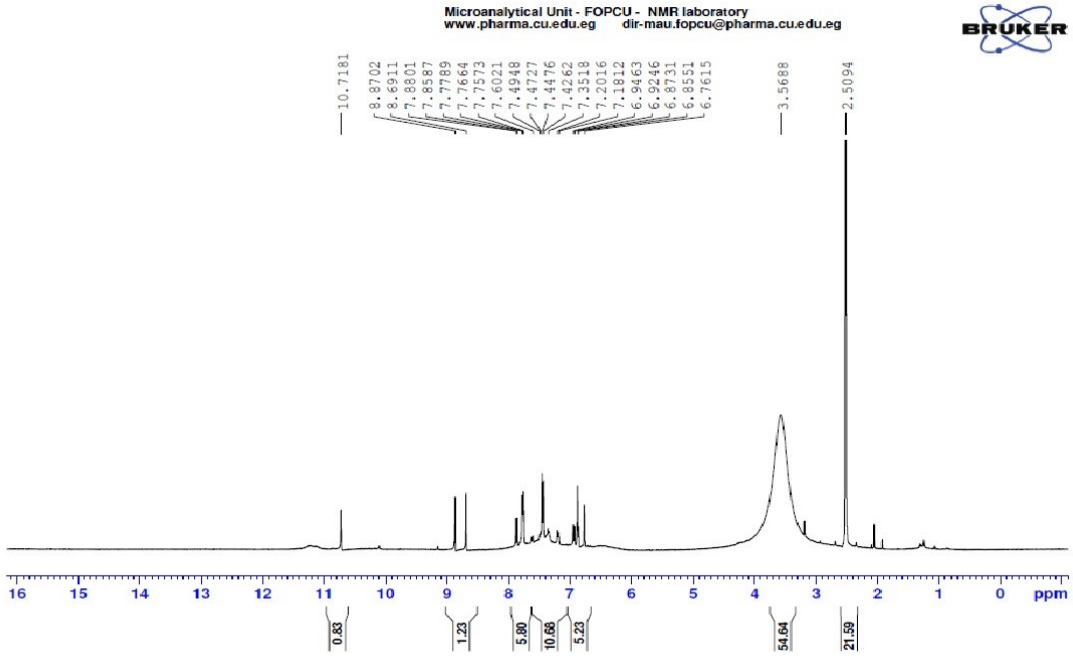


Fig. (4): HNMR spectroscopy of Carboxamide (comp-3).



Cytotoxicity: The in vitro cytotoxic activities of compounds:

N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3) and Ethyl 7-hydroxycoumarin-3-yl ester (comp-2) were showed in table (1) and figures (5-8).

Minimum inhibitory concentrations of synthesized compound N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3) were found to be 12µg/ml, 9.7µg/ml, 18µg/ml and 14.4µg/ml against MCF-7, HEPG-2, HCT, PC-3 cell lines, respectively.

While, Minimum inhibitory concentrations of synthesized compound Ethyl 7-hydroxycoumarin-3-yl ester (comp-2) were found to be 67.5µg/ml, 87µg/ml, 218µg/ml and 91µg/ml against MCF-7, HEPG-2, HCT, PC-3 cell lines, respectively.

Table (1): Minimum inhibitory concentrations of synthesized compounds (comp-2 and comp-3) against MCF-7, HEPG-2, HCT and PC-3 cell line.

Variable	MCF-7 µg/ml	HEPG-2 µg/ml	HCT µg/ml	PC-3 µg/ml
Comp-3	12	9.7	18	14.4
Comp-2	67.5	87	218	91
doxorubicin	56	78	160	80

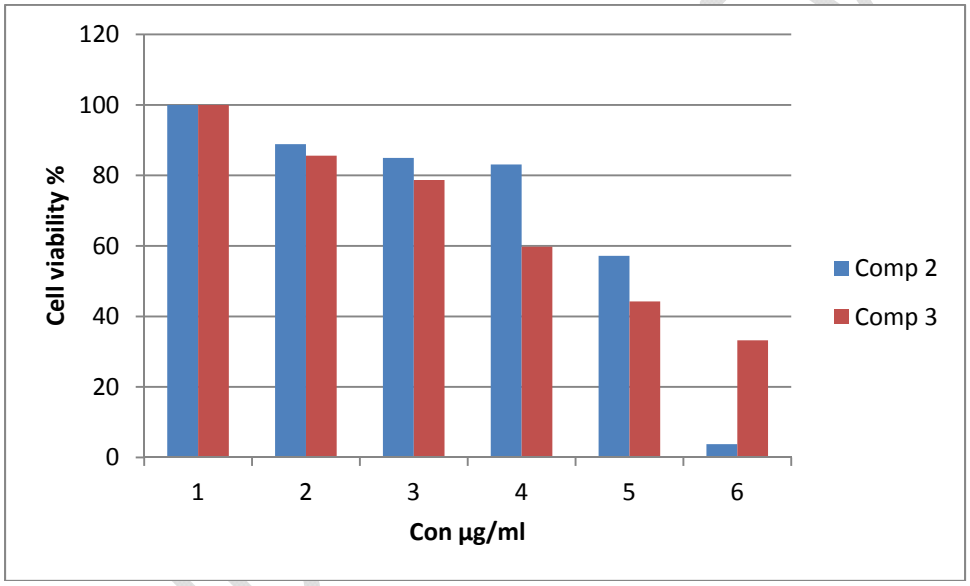


Fig. (5): Minimum inhibitory concentration of comp-2 and comp-3 against MCF-7 cell line

*IC50 of comp-3 against MCF-7 is 12µg/ml while comp-2 is 67.5µg/ml

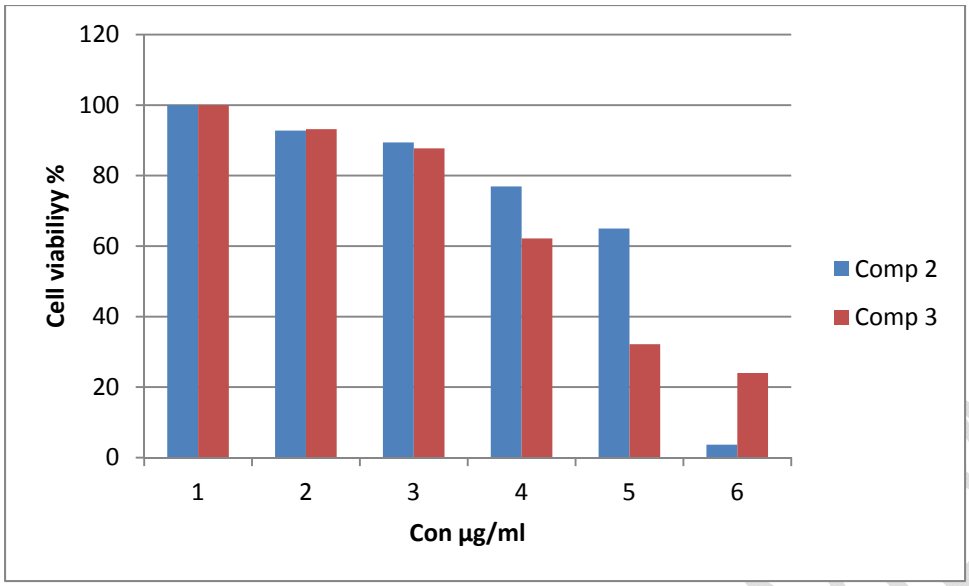


Fig. (6): Minimum inhibitory concentration of comp-2 and comp-3 against HEPG-2 cell line

*IC50 of comp-3 against HEPG-2 is 9.7µg/ml while comp-2 is 87µg/ml

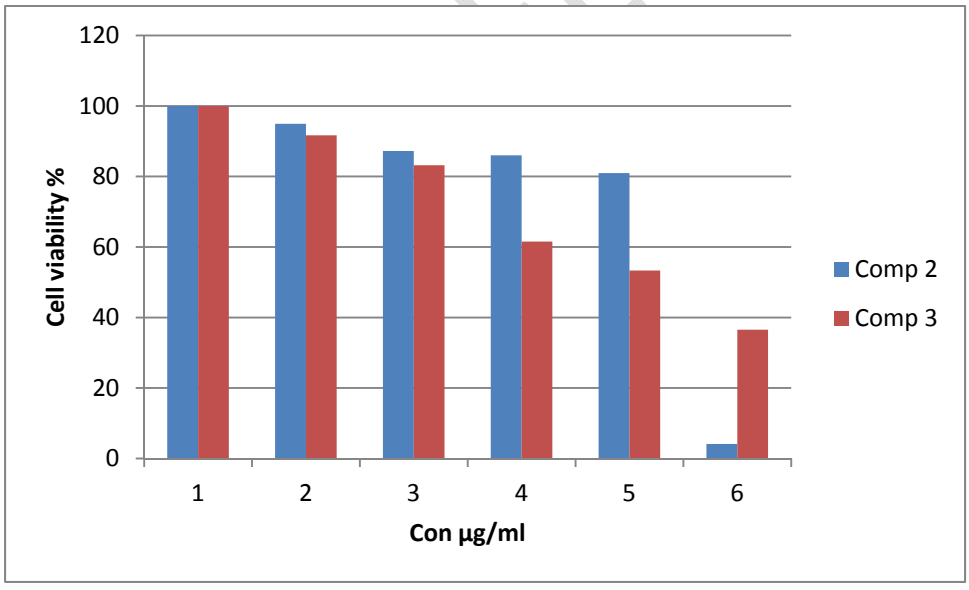


Fig. (7): Minimum inhibitory concentration of comp-2 and comp-3 against HCT cell line.

*IC50 of comp-3 against HCT is 18µg/ml while comp-2 is 218µg/ml

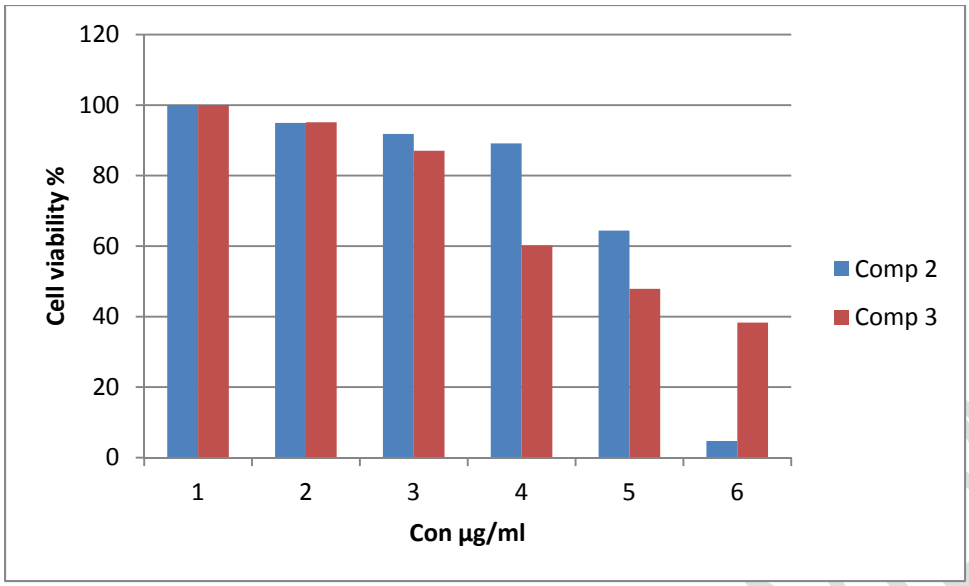


Fig. (8): Minimum inhibitory concentration of comp-2 and comp-3 against PC-3 cell line

*IC50 of comp-3 against HCT is 14.4µg/ml while comp-2 is 91µg/ml

4. DISCUSSION

Cancer is now one of the world's most pressing health challenges. Research continues to deliver new and improved treatment options for thousands of people living with cancer **(ASC, 2016)**. Cancer has not been cured yet. It is estimated that by 2020 there will be 16 million new cancer cases every year (**Lingwood *et al.*, 2008)**. The chemistry of heterocyclic compounds continues to be an explore field in the organic or Pharmaceutical chemistry. The Coumarin (benzopyran-2 one, or chromen-2-one) ring display interesting pharmacological properties has intrigued chemists and medicinal chemists for decades to explore the natural Coumarins or synthetic analogs for their applicability as drugs. Some new derivatives bearing coumarin ring including the furanocomarins (e.g., Imperatorin), pyranocoumarins (e.g., Seselin), and coumarin sulfamates (Coumates), have been found to be useful in photo-chemotherapy, antitumor and anti-HIV therapy (**Kostova *et al.*, 2006)**. All these findings encouraged us to explore the synthesis of coumarin derivatives and examine their activities as in vitro anti-cancer against some different cell lines such as [MCF-7(human breast cancer), HePG2 (Hepatocellular carcinoma), HCT (human colon cancer), PC3 (human prostate cancer)] to assess their cytotoxicity effects. The results indicated that N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3) has cytotoxicity potency. N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (comp-3) showed a very potent activity against MCF-7, HePG2, HCT, and PC3 with minimum inhibitory concentration [12, 9.7, 18, and 14.4 µg/ml, respectively] but Ethyl 7-hydroxycoumarin-3-yl ester (comp-2) showed low activity against MCF-7, HePG2, HCT, and PC3 than comp-3 with minimum inhibitory concentration [67.5, 87, 218, 91 µg/ml, respectively] compared with doxorubicin as reference drug. The most intriguing biological activities of Coumarins is the notable effect of, some of the Coumarins against breast cancer, some Coumarins and their active metabolite 7-hydroxycoumarin analogs have shown sulfatase and aromatase inhibitory activities (**Momekov *et al.*, 2006)**.

Coumarin based selective estrogen receptor modulators (SERMs) and Coumarin estrogen conjugates have also been described as potential anti-breast cancer agents according some recently publications (**You *et al*., 2010)**. The natural form of coumarin itself has demonstrated an anti-tumor activity. Coumarin (known as 1, 2-benzopyrone), consisting of fused benzene and α-pyrone ring, is an important group of low molecular weight (**Fylaktakidou *et al*., 2004)**. This effect is probably linked to its metabolites (*e.g.* 7-hydroxycoumarin, 7-HC) transformed by cytochromes P450 **(Pelkonen *et al*., 2000)**. Recently, several groups have attempted to establish a structure activity relationship (SAR) between coumarins and their various anticancer properties **(Bruyere *et al*., 2011)**. The hydroxyl group on position C-7 seems to be pivotal for the anticancer activity **(Wu *et al*., 2009)**. Moreover, 7-HC and several of its derivatives inhibit proteins implicated in the cell cycle and overexpressed in many types of cancers, such as Cyclin D1 and Cdc25 **(Jimenez-Orozco *et al*., 2001) (Valente *et al*., 2001)**. Our results agreed with **Stanway *et al*., (2006)**, who studied the growth-inhibitory cytostatic activity in human cancer cell line: MCF-7 breast carcinoma cells. They reported that, osthole "Coumarin derivatives" demonstrated some estrogenic activity by preventing the synthesis and action of estrogens (ER antagonists), and this indicated that, osthole has the potential to be a breast cancer treatment reagent. As **Kempen *et al***., who stated that, the inhibition capacity varied according to the substituent present in the 6-position of the coumarin, and according to the nature of the halogen atom in the 3-position of the phenyl ring. In general, (substitution by a halogen atom particularly, a chlorine or a bromine atom) in the 'meta' position of the phenyl ring relative to the ester oxygen atom of 2-oxo-2H-1-benzopyran- 3-carboxylate led to a better anti-tumor effect than that observed in the absence of any substituent **(Kempen *et al*., 2003) (El-behary *et al*., 2013) .** Our results agreed with **El-behary *et al*., 2013,** who studied the cytotoxicity of new coumarin derivatives: Potassium salt of 2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine and 9-bromo-2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine against some different cell lines such as [MCF-7(human breast cancer), HePG2 (Hepatocellular carcinoma), HCT (human colon cancer), PC3 (human prostate cancer)].

5. CONCLUSIONS

The in vitro cytotoxic activity for the compounds: N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide and Ethyl 7-hydroxycoumarin-3-yl ester (comp-2 & comd-3) against the human breast tumor cells (MCF-7), human hepatocellular cancer cells (HePG2), HCT16 (colon cancer), and PC3 (prostate cancer). Comp-3 exhibits minimum inhibitory concentration against all cell lines at higher doses than comp-2. On the basis of these results, comp-3 may be considered as attractive leads in the future development of potential anticancer agent more than comp-2.

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