

Original Research Article

Biochemical studies on coumarin derivatives as antioxidant and antitumor agents

Abstract:

Background: Coumarin derivatives have attracted intense interest in recent years, because they have anti-tumor, antioxidant activities, and induce apoptosis.

Aims and Objective: Our study aims to evaluate the antitumor and anti-oxidant activities of new Coumarin derivatives: N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide and Ethyl 7-hydroxycoumarin-3-yl ester against *in vivo* tumor model.

Methodology: the toxicity for the synthesized compounds was determined. The anticancer and anti-oxidant activities were studied by evaluation the viability of tumor cells, life span prolongation, and estimation of antioxidants.

Result: Our compounds exhibited significant anti-oxidant activity towards Ehrlich ascites carcinoma (EAC) cells by reduction the MDA and NO concentration ($p < 0.001$) compared to the positive control group. Whereas significantly increase in the G.peroxidase activity ($p < 0.001$) in treated groups compared to the positive control group. Anticancer agent kills tumors by induction of apoptosis that showing significantly increases in Caspase-3 and Bax activity compared to positive control group.

Discussion: The compound N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide is better than Ethyl 7-hydroxycoumarin-3-yl ester compound 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, Ehrlich ascites carcinoma cells, apoptosis.

1- Introduction:

Cancer is one of the leading causes of death in the developed world. Tumor is a group of cells that have undergone un-regulated growth, and will often form a mass or lump, but may be distributed diffusely (National Cancer Institute.,2013). Cancer is characterized by a progression of changes at both, cellular and genetic level, that reprogram a cell to undergo uncontrolled division, thus forming a malignant mass (tumor) that can spread to distant locations (Suarez-Jimenez *et al.*, 2012). Many therapeutic anticancer have been developed which has relied on surgery, chemotherapy, radiotherapy, hormone therapy and immunotherapy (Khorshid. 2011). 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 tumor 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 bark 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 and Lacy *et al.*, 2004).

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), anti-HIV (Kudo *et al.*, 2015) , antiasthmatic (Sanchez-Recillas *et al.*, 2014), antiviral (Xu *et al.*, 2014) 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-gp of the cancer cell (Amin *et al.*, 2013; Nasr *et al.*, 2014).

Our study aims to evaluate the anti-tumor, and the anti-oxidant properties of recently developed synthetic coumarin derivatives: N-(P-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide and Ethyl 7-hydroxycoumarin-3-yl ester against Ehrlich ascites carcinoma "EAC" cells, and study the mechanism of killing cancer cells.

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. Animals:

Female Swiss albino mice of 8 weeks of age, weighed 22 to 25 g body weight were raised at the experimental animal house of the faculty of Science, Port-Said University. The animals were maintained in controlled environment of temperature, humidity and light. They were fed on a commercial standard diet and tap water *ad libitum*.

2.1.3. Tumors:

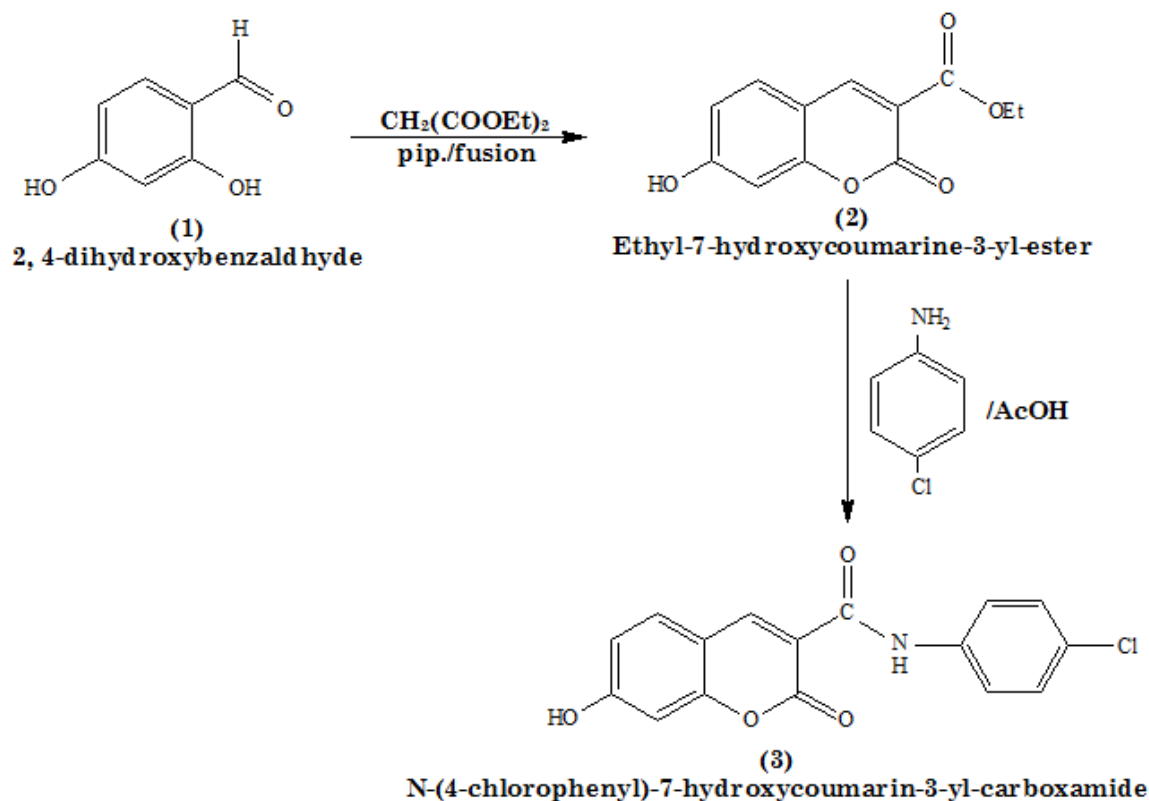
Ehrlich ascites carcinoma (EAC) was initially supplied by the National Cancer Institute, Cairo, Egypt, and maintained in female Swiss albino mice through serial intraperitoneal (I.P) inoculation at 8 or 10 day intervals in our laboratory in an ascites form.

2.2. Methods

2.2.1. Chemistry

The ethyl-7-hydroxycoumarin-3-ylester was prepared via cyclocondensation of 2, 4-dihydroxybenzaldehyde 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 [scheme I]



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

2.2.2. Determination median lethal dose (LD 50) of the synthesized compounds:

Approximate LD₅₀ of our compounds in mice were determined according to the method described by (Meier and Theakston 1986).

2.2.3. Dose response curve:

Dose response curve of our compounds in mice was determined according to the method of (Crump *et al.*, 1976).

2.2.4. Experimental design:

90 female albino Swiss albino mice were divided into 6 groups each one contains of 15 mice: Group I "served as negative control group" injected I.P. with sterile saline for 10 days (day after day); group II "positive control"; injected i.p. with 2.5×10^6 of Ehrlich ascites carcinoma "EAC" cells. Group III " ethyl-7-hydroxycoumarin-3-ylester therapeutic group", injected i.p. with 20 mg/kg one day after EAC injection and repeated dose five times during the experiment; Group IV " N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide therapeutic group", injected i.p. with 20mg/kg one day after EAC injection and repeated dose five times during the experiment. Group V "

ethyl-7-hydroxycoumarin-3-ylester preventive group", injected i.p. with 20 mg/kg one day before EAC injection and repeated dose five times during the experiment; Group VI " N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide preventive group", injected i.p. with 20mg/kg one day before EAC injection and repeated dose five times during the experiment. At the end of the experiment, EAC cells were harvest from each mouse in centrifuge tube containing heparinized saline. Note the volume of ascetic fluid in each mouse in each group. Each sample of cells was undergoing viability test of EAC cells and antioxidants assays were performed.

2.2.5. Viability of EAC cells and life span prolongation

The viability of EAC cells was determined by the Trypan Blue Exclusion Method described by **McLiman et al., (1957)**. Life span was calculated according to the method described by **Mazumdar et al., (1997)**.

2.2.6. Antioxidant assays

Malondialdehyde (MDA), Nitric Oxide (NO) levels, and Glutathione peroxidase (GPx) activity were estimated according to methods of described by **Satoh,(1978)**; **Montgomery &Dymock, (1961)**, and **Paglia& Valentine et al., (1967)**; respectively.

2.2.7. Apoptosis Assays

Caspase-3 activity determination was carried out according to the method of **Casciola-Rosen et al., (1996)**.

2.2.8. Statistical analysis

Statistical analysis was performed using SPSS software II version 14 (**Levesque, 2007**). The effect of each parameter was assessed using the one way analysis of variance. Individual differences between groups were examined using Dunnett's test and those at $p < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Determination of median lethal dose (LD₅₀) of tested compounds

The acute toxicity LD₅₀ was estimated by I.P. injection of compounds ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide; doses up to 1000 mg /kg and 2000 mg /kg were considered safe for compounds; respectively, where no mortality was observed.

3.2. Dose response curve of tested compounds

It was cleared that 20 mg/kg was found to be the most effective dose of compounds **ester** and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide as this dose reduced the number of EAC cells compared to positive control group.

3.3. Effect of tested compounds on volume, EAC cell count

Table (I) summarized the effect of compounds ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide on EAC cells volume and count. The mean volume of EAC in the positive control group was found to be 4.56 (ml). This value was significantly decreased to 2.03, 1.94, 2.25, 1.91 ml by 124.6%, 135.05%, 102.6%, 138.94%, ($p < 0.01$) in compounds ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide therapeutic and preventive groups; respectively.

Also, the mean count of EAC cells in the positive control group was found to be 288×10^6 , which significantly decreased to 181.4, 185.7, 189.3, 179.5×10^6 by 58.76%, 55.08%, 52.13%, 60.44%, ($p < 0.01$); respectively in therapeutic and preventive groups of compounds ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide; respectively, compared to the positive control group.

3.4. Effect of tested compounds on life span in all studied groups

Table (II) illustrated the life span prolongation of the studied tested compounds. The mean life span prolongation in the positive control group (EAC bearing tumor group) was found to be 14 days. Compounds ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide therapeutic and preventive groups showed a significant increase in the life span prolongation to 21 days by 50.0% (T/ C ratio = 150.0%), 21 days by 50.0% (T/ C ratio = 150.0%) , 22 days by 57.14% (T/ C ratio = 157.14%), ,11 days by -21.42% (T/ C ratio = 78.57%); respectively compared to the positive control group.

3.5. Antioxidants Assays

3.5.1. Effect of tested compounds on antioxidants in EAC of all studied groups

Table III illustrated the EAC anti-oxidant activity of the investigated compounds. The mean values of EAC MDA were found to be 4.88 ± 0.18 (nmol /g) in the negative control group. These values were very highly increased significantly in the positive control group to 14.22 ± 0.32 (nmol/g) by ($p < 0.001$). Treated groups showed a reduction in MDA levels to 8.76 ± 0.20 (nmol /g) , 7.99 ± 0.42 (nmol /g) and 5.22 ± 0.12 (nmol /g) , 5.89 ± 0.27 (nmol /g) by 38.39% ,43.81% (nmol /g) and by 63.29% ,58.57% (nmol /g) for compounds ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide therapeutic and preventive groups respectively; compared to positive control group ($p < 0.001$).

Also, there was a very highly significant decrease in EAC NO levels from 90.36 ± 0.36 in positive control group to 46.00 ± 3.109 , 61.58 ± 3.24 and 31.72 ± 1.48 , 40.48 ± 3.20 by

49.09% , 31.85% and by 64.89% ,55.20 % ($P<0.001$) in both therapeutic and preventive groups of ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds respectively; compared to positive control.

Meanwhile; EAC G.peroxidase activities were significantly increased in all treated groups by from 105.0 ± 0.81 (U/gT) by ($p<0.001$) in positive control group to 191.6 ± 2.60 (U/gT) , 580.2 ± 26.60 (U/gT) and 341.2 ± 28.46 (U/gT) , 446.6 ± 10.35 (U/gT) by -82.47% , -166.85% (U/gT) and -224.95% , -325.33% (U/gT) for both therapeutic and preventive groups of ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds, respectively; compared to positive control group ($p<0.001$).

3.5.2. Effect of tested compounds on antioxidants in liver of all studied groups

Table (IV) illustrated the liver anti-oxidant activity of the investigated compounds. The mean values of liver MDA were found to be 4.88 ± 0.18 (nmol /g tissue) in the negative control group. These values were very highly increased significantly in the positive control group to 7.63 ± 0.34 (nmol/g tissue) by ($p<0.001$). Treatments by ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds showed a significant reduction of liver MDA levels to 6.73 ± 0.44 (nmol /g tissue) , 6.80 ± 0.42 (nmol /g tissue) and 4.40 ± 0.46 (nmol /g tissue) , 3.78 ± 0.17 (nmol /g tissue) by 11.79% , 10.87% and by 42.33% , 50.35% (nmol /g tissue) for both therapeutic and preventive groups, respectively; compared to positive control group ($p<0.001$).

Also, there was a very highly significant decrease in liver NO activity to 33.06 ± 2.44 , 35.15 ± 0.89 and 15.10 ± 0.51 , 15.98 ± 1.23 by 23.25% , 18.40 % and by 64.94 % , 62.90% in both therapeutic and preventive groups of ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds ; respectively compared to positive control group ($P<0.001$).

Meanwhile; there was a significant increase of liver G.peroxidase activity to 384.1 ± 25.87 , 491.5 ± 30.45 (U/gT) and 452.6 ± 21.83 , 565.9 ± 23.85 (U/gT) by -322.08% , -440.10% (U/gT) and by -397.36% , -521.86% (U/gT) for both therapeutic and preventive groups of ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds ; respectively compared to positive control group ($p<0.001$).

3.6. Apoptosis Assays

3.6.1. Effect of tested compounds on Caspase-3 and Bax (BCL-2 associated protein) in EAC of all studied groups

Table (V) illustrated the EAC apoptosis assays of the investigated compounds. The mean values of EAC caspase-3 level were found to be 3.07 ± 0.11 (ng/ml) in negative control group. These values were decreased in the positive control group to 0.27 ± 0.0047 (ng/ml). The mean values of EAC caspase-3 increased for both therapeutic and preventive groups treatments with ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds to be 0.78 ± 0.06 (ng/ml) , 0.41 ± 0.12 (ng/ml) and 0.52 ± 0.13 (ng/ml) , 1.006 ± 0.06 (ng/ml) by -189.62% , -54.07% and -95.18% , -272.59% ; respectively compared to the positive control group.

Also, the mean values of EAC Bax level were found to be 17.84 ± 0.20 (ng/ml) in negative control group. These values were decrease in the positive control group to 2.14 ± 0.12 (ng/ml). The mean values of EAC Bax were very highly significantly increased for both therapeutic and preventive groups treatments with ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds to be 5.04 ± 0.31 (ng/ml) , 2.46 ± 0.17 (ng/ml) and 4.17 ± 0.21 (ng/ml), 3.58 ± 0.55 (ng/ml) by -135.83% , -15.14% and -94.91% , -67.41% respectively; compared to the positive control group ($p < 0.001$).

3.6.2. Effect of tested compounds on Caspase-3 and Bax (BCL-2 associated protein) in liver of all studied groups

Table (VI) summarized the results of activity of liver caspase-3 among the different groups of animals. The mean values of liver caspase-3 were found to be 3.07 ± 0.11 (ng/ml) in negative control group. These values were very highly significantly decreased in the positive control group to 1.70 ± 0.12 (ng/ml) by ($p < 0.001$). The mean values of liver caspase-3 increased for both therapeutic and preventive group's treatments of ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds to be 2.81 ± 0.3 (ng/ml), 2.42 ± 0.23 (ng/ml) and 2.92 ± 0.10 (ng/ml), 2.67 ± 0.09 (ng/ml) by 8.48%, 21.29% and 4.87%, 13.19%, respectively; compared to the positive control group.

Also, the mean values of liver Bax level were found to be 17.84 ± 0.20 (ng/ml) in negative control group. These values were decreased in the positive control group to 9.92 ± 0.35 (ng/ml). The mean values of liver Bax were very highly significantly increased for both therapeutic and preventive groups treatments of ethyl-7-hydroxycoumarin-3-ylester and

N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds to be 14.63 ± 0.92 (ng/ml), 10.03 ± 0.14 (ng/ml) and 13.73 ± 0.64 (ng/ml), 11.38 ± 0.45 (ng/ml) by -47.47%, -1.10% and -38.40%, -14.71% respectively; compared to the positive control group ($p < 0.001$).

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). The recent advances in science, cancer have not been cured yet. It is estimated that by 2020 there will be 16 million new cancer cases every year (Lingwood *et al.*, 2008). Our study describes the evaluation of the anti-invasive, anti-oxidant properties of recently developed synthetic coumarin derivatives (ethyl-7-hydroxycoumarin-3-ylester & N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide). Heterocycles such as chromone and coumarin derivatives were investigated for their cytotoxicity against human normal and tumor cells. These compounds induced moderate tumor-specific cytotoxicity, although they have been reported to induce apoptosis-inducing activity (Hiroshi *et al.*, 2008). Our results found that, these doses were significant prolonged the life span to 21 days by 50.0% (T/ C ratio = 150.0%), 21 days by 50.0% (T/ C ratio = 150.0%), 22 days by 57.14% (T/ C ratio = 157.14%), 11 days by -21.42% (T/ C ratio = 78.57%) in therapeutic and preventive groups; respectively compared to the positive control group as showed in table (II). Coumarins are a vast group of natural compounds. As substitutions can occur at any of the six available sites of their basic molecular moiety, leads to compounds displaying multiple biological properties that promote human health and help reducing the risk of diseases (Zhang *et al.*, 2011). Our results found that, MDA and NO levels in EAC cells in both ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide therapeutic and preventive groups showed a significant decrease by 38.39%, 43.81% & 63.29%, 58.57% and by 49.09%, 31.85% & 64.89%, 55.20% ($p < 0.001$) respectively; compared to the positive control group. While, EAC GPx activity showed a significantly increase in ethyl-7-hydroxycoumarin-3-ylester therapeutic and preventive groups by -82.47%, -224.95% and -166.85%, -325.33% in N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide therapeutic and preventive groups, compared to positive control, ($p < 0.001$), Table (III). Also, the therapeutic strategies should aim to reducing free-radical formation and scavenging free radicals (Berg *et al.*, 2004). Coumarins possess, antioxidant activities, probably due to their structural analogy with flavonoids and benzophenones (Farombi *et al.*, 2005). The coumarins having a catechol group showed significant free radical scavenging activity and inhibitory effects on lipid peroxidation, indicating that the catechol group significantly contributed to the antioxidant activities of coumarins. Also, liver MDA and NO levels showed significant reduction in ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide therapeutic and preventive groups by 11.79%, 10.87% & 42.33%, 50.35% and 23.25%, 18.40% & 64.94%, 62.90% ($p < 0.001$) respectively; compared to the positive control group. The α -pyrone rings of coumarins significantly enhanced the capacity of inhibiting oxidative reactions of coumarins (Thuong *et al.*, 2010). The provide information on the mechanisms by which coumarin induces cell cycle arrest and apoptosis in cancer cells (Chuang *et al.*, 2007). The apoptotic effect of ester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compounds was evaluated by measurement Caspase-3 activity and Bax in the EAC and liver cells in all studied groups. Furthermore, the treatments with ethyl-7-hydroxycoumarin-3-ylester and N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide (20 mg/kg, and 20 mg/kg, I.P.) showed a significantly increase in EAC and liver Caspase-3 activity, by -189.62%, -54.07% & -95.18%, -272.59% and 8.48%, 21.29% & 4.87%, 13.19% ($p < 0.001$), respectively in therapeutic

and preventive groups ; compared to the positive control group. And a very highly significantly increase in EAC and liver Bax, by -135.83% , -15.14% & 94.91%, -67.41% and -47.47%, -1.10% & -38.40%, -14.71% ($p < 0.001$), respectively in therapeutic and preventive groups; compared to the positive control group. Programmed cell death, or apoptosis, plays an important role in the development of cancers. It is known that many anticancer agents kill tumors at least partially through induction of apoptosis. Caspase-3 is known to be the key effector caspase that cleaves multiple protein substrates in cells leading to cell death (**Kemnitzner *et al.*, 2009**). Coumarins and coumarin derivatives as well as diallylpolysulfides are well known as anticancer drugs. They reduced cell viability of cancer cells in a time and concentration dependent manner. Cells tested with these coumarin polysulfides accumulate in the G2/M phase of the cell cycle and finally they go into apoptosis. A decrease in bcl-2 level, and increase in the level of Bax, Cytochrome c release into the cytosol, cleavage of caspase 3/7 suggested that coumarin polysulfides induced the intrinsic pathway of apoptosis (**Saidu *et al.*, 2012**). The effects of coumarin on cell viability, cell cycle arrest and induction of apoptosis were investigated in human cervical cancer HeLa cells. Coumarin induced morphological changes, and caused G0/G1 arrest and apoptosis. The decreasing number of viable cells appeared to be due to induction of cell cycle arrest and apoptotic cell death, since coumarin induced morphologically apoptotic changes. Coumarin treatment gradually decreased the expression of G0/G1-associated proteins which may have led to the G0/G1 arrest, and the anti-apoptotic proteins Bcl-2 and Bcl-xL, and increased the expression of the pro-apoptotic protein Bax. Coumarins decreased the mitochondrial membrane potential and promoted the release of cytochrome c and the activation of caspase-3 before leading to apoptosis (**Chuang *et al.*, 2007**). The cytotoxic molecules were evaluated in apoptosis assays and some of them exhibited great apoptosis induction, being able to promote caspase-3 activation and DNA fragmentation (**Cordeu *et al.*, 2007**). In the light of our *in vivo* results, the coumarin derivative appears to be very promising as potential anti-tumoral agent. Although coumarin derivatives might constitute an alternative to matrix metalloproteases (MMPs) inhibitors as anticancer agents, further biological investigations are required before any clinical trial. 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**) .

5. CONCLUSIONS

The *in vivo* effect of ester and carboxamide compounds exhibited significant anticancer and anti-oxidant activities towards EAC cells by induction of apoptosis. On the basis of these results, N-(4-chlorophenyl)-7-hydroxycoumarin-3-yl carboxamide compound may be considered as attractive leads in the future development of potential anticancer and anti-oxidant agent more than **ester** compound because of the presence of the halogen atom in the 3-position of the phenyl ring.

Ethical Approval:

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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Table (I): Effect of Compounds (ester and carboxamide) on Volume and count of EAC in treated groups

Group	Positive control		Group III		Group IV		Group V		Group VI	
	Mean	%Change	Mean	%Change	Mean	%Change	Mean	%Change	Mean	%Change
Volume of Ascites Fluid (ml)	4.56	-----	2.03	124.6%	1.94	135.05%	2.25	102.6%	1.91	138.74%
Count of EAC cells (×10 ⁶)	288	-----	181.44	58.76%	185.7	55.08%	189.3	52.13%	179.5	60.44%

***There are decrease in count and volume of EAC in treated and preventives groups**

Table (II): Effect of Compounds (ester and carboxamide) on life span prolongation

Life span prolongation	Positive control	Group III	Group IV	Group V	Group VI
Days	14	21	21	22	11
Life span (T/C %)	-----	150.0%	150.0%	157.14%	78.57%
Increase in life span	-----	50.0%	50.0%	57.14%	-21.42%

***There is increase in life span in both therapeutic and preventive groups.**

Table (III): Anti-oxidants Effect of Compounds (ester and carboxamide) in EAC cells

Variable	Positive control		Group III		Group IV		Group V		Group VI	
	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change
MDA (nmol/g.tissue)	14.22*** ± 0.32	-----	8.76*** ± 0.20	38.39 %	7.99*** ± 0.42	43.81%	5.22*** \pm 0.12	63.29 %	5.89*** ± 0.27	58.57%
NO (μmol/gT)	90.36*** ± 0.36	-----	46*** ± 3.10	49.09 %	61.58*** ± 3.24	31.85%	15.1*** \pm 0.51	64.89 %	15.98** * ± 12.35	55.20%
G.peroxidase (U/gT)	105*** ± 0.81	-----	191.6*** ± 2.60	- 82.47 %	580.2*** ± 26.60	- 166.85 %	341.2*** ± 28.46	- 224.9 5%	446.6** * ± 10.35	- 325.33 %

***P value <0.001 was considered very highly significant.

***There is a very highly significant decrease in both EAC MDA and NO levels in ester and carboxamide therapeutic and preventive groups.**

****There is a very highly significant increase in EAC G.peroxidase activity in both ester and carboxamide therapeutic and preventive groups.**

Table (IV): Anti-oxidants Effect of Compounds (ester and carboxamide) in liver of all studied groups

Variable	Positive control		Group III		Group IV		Group V		Group VI	
	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change
MDA	7.63*** ± 0.34	-----	6.73*** ± 0.44	11.7%	6.8*** ± 0.42	10.87%	4.4*** ± 0.46	42.33 %	3.78*** ± 0.17	50.35%
NO	43.08*** \pm 1.88	-----	33.06*** ± 2.44	23.25 %	35.15*** ± 0.89	18.40%	15.10*** ± 0.51	64.94 %	15.98** * ± 1.23	62.90%
G.peroxidase	120.5*** ± 28.53	-----	384.1*** ± 25.87	- 322.08 %	491.5*** ± 30.45	- 440.10 %	452.6*** ± 21.83	- 397.3 6%	565.9** * ± 23.85	- 521.86 %

***P value <0.001 was considered very highly significant.

***There is a very highly significant reduction in both liver MDA and NO levels in ester and carboxamide therapeutic and preventive groups.**

****There is a very highly significant increase in liver G.peroxidase activity in both ester and carboxamide therapeutic and preventive groups.**

Table (V): Effect of Compounds (ester and carboxamide) on caspase-3 activity and Bax levels in EAC cells

Variable	Positive control		Group III		Group IV		Group V		Group VI	
	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change
caspase-3	0.27*** ± 0.0047	-----	0.782*** ± 0.06	- 189.62 %	0.41*** ± 0.12	- 54.07%	0.52*** ± 0.13	-95.18	1.006** * ± 0.068	-272.59
Bax	2.14*** ± 0.12	-----	5.04*** \pm 0.31	- 135.83 %	2.46*** \pm 0.17	- 15.14%	4.17*** \pm 0.21	- 94.91%	3.58*** ± 0.55	-67.41%

***P value <0.001 was considered very highly significant.

****There is a very highly significant increase in EAC caspase-3 and Bax levels in both ester and carboxamide therapeutic and preventive groups.**

Table (VI): Effect of Compounds (ester and carboxamide) on caspase-3 activity and Bax levels in liver of all studied groups

Variable	Positive control		Group III		Group IV		Group V		Group VI	
	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change	Mean \pm SE.	% Change
caspase-3	1.705 ± 0.12	-----	2.81*** ± 0.30	8.48%	2.42*** ± 0.23	21.29%	2.92*** ± 0.10	4.87%	2.67*** ± 0.09	13.19%
Bax	9.92 ± 0.35	-----	14.63*** ± 0.92	- 47.47%	10.03*** ± 0.14	-1.10%	13.73*** ± 0.64	- 38.40%	11.38** * ± 0.45	-14.71%

***P value <0.001 was considered very highly significant.

****There is a very highly significant increase in liver caspase-3 and Bax levels in both ester and carboxamide therapeutic and preventive groups.**

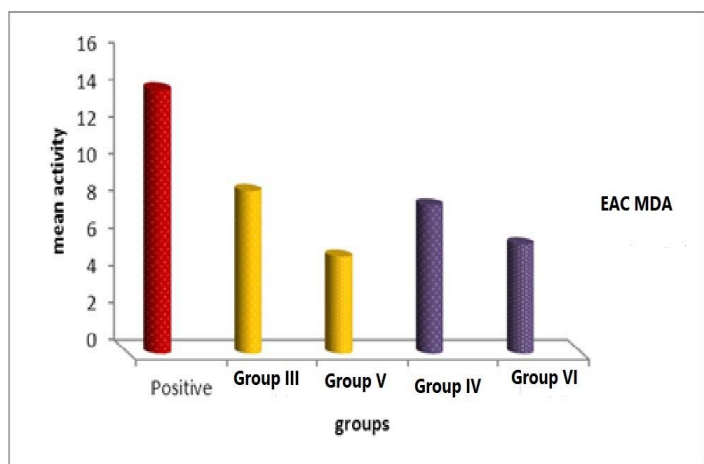


Figure (1): EAC MDA in all studied groups

***There is a very highly significant decrease in EAC MDA levels in ester and carboxamide therapeutic and preventive groups.**

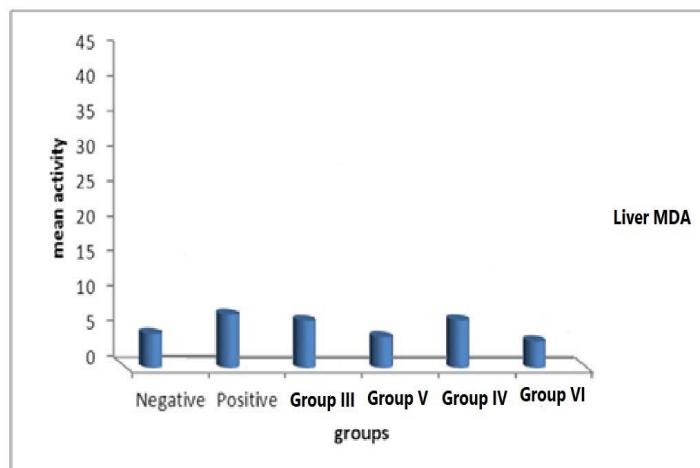


Figure (2): Liver MDA in all studied groups

***There is a very highly significant decrease in liver MDA levels in ester and carboxamide therapeutic and preventive groups.**

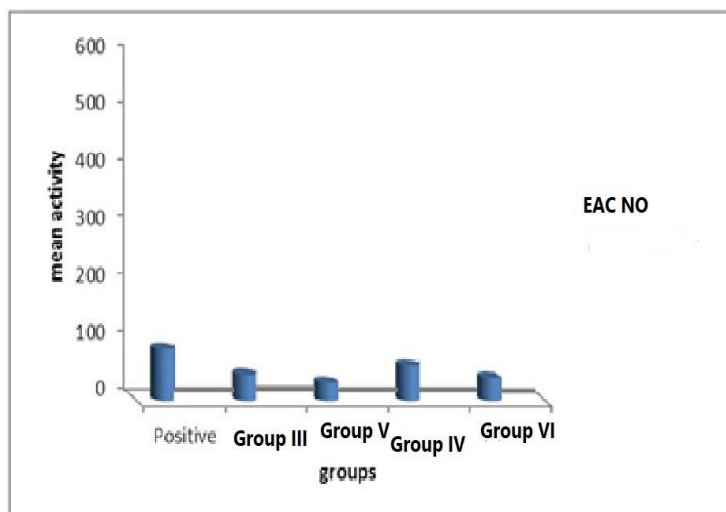


Figure (3): EAC NO in all studied groups

***There is a very highly significant decrease in EAC NO levels in ester and carboxamide therapeutic and preventive groups.**

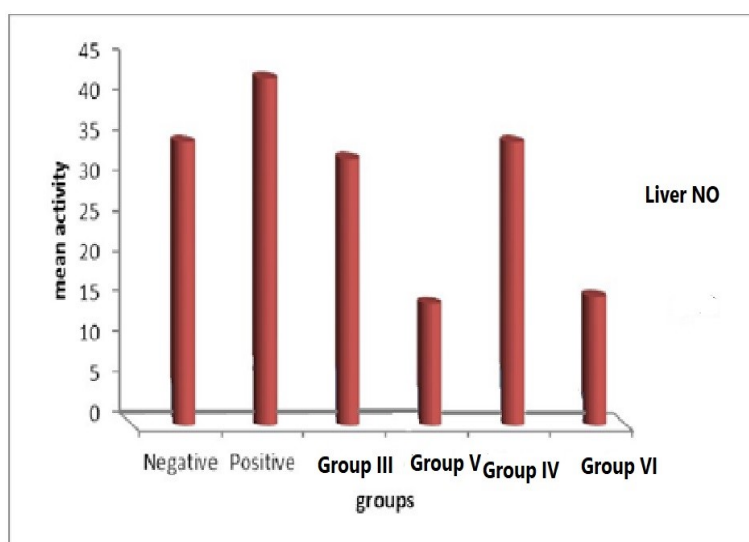


Figure (4): Liver NO in all studied groups

***There is a very highly significant decrease in liver NO levels in ester and carboxamide therapeutic and preventive groups.**

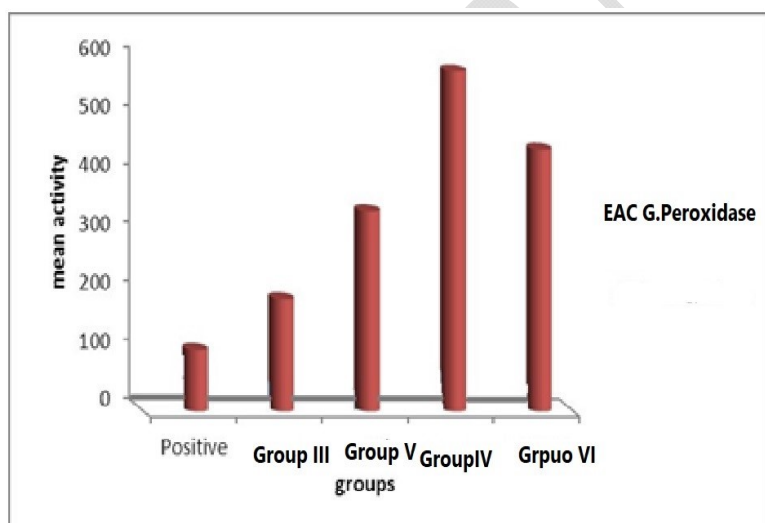


Figure (5): EAC G.peroxidase in all studied groups

***There is a very highly significant increase in EAC G.peroxidase levels in ester and carboxamide therapeutic and preventive groups.**

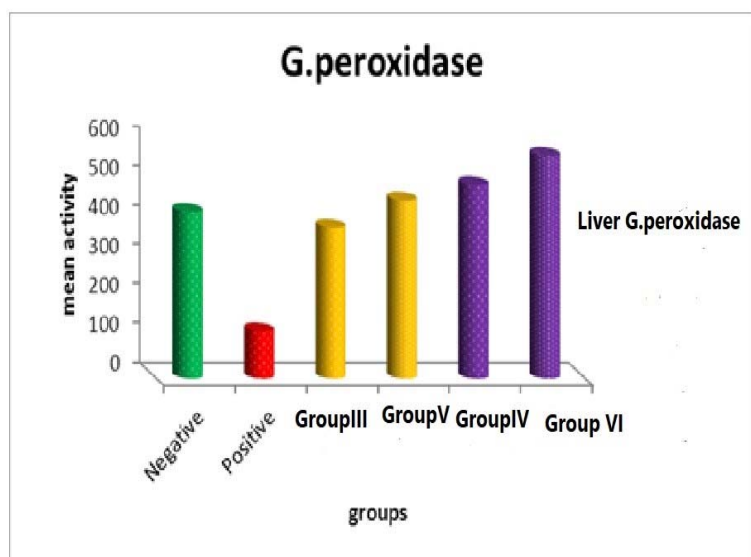


Figure (6): Liver G.peroxidase in all studied groups

***There is a very highly significant increase in liver G.peroxidase levels in ester and carboxamide therapeutic and preventive groups.**

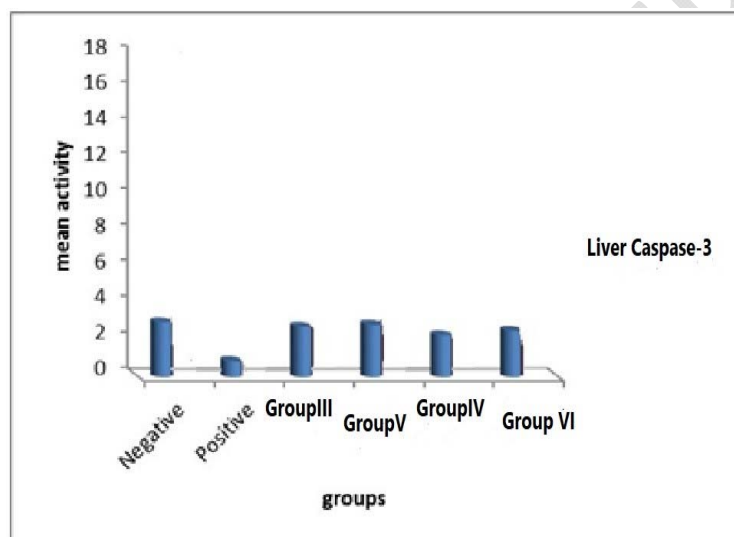


Figure (7): Liver Caspase-3 in all studied groups

***There is a very highly significant increase in liver caspase-3 levels in ester and carboxamide therapeutic and preventive groups.**

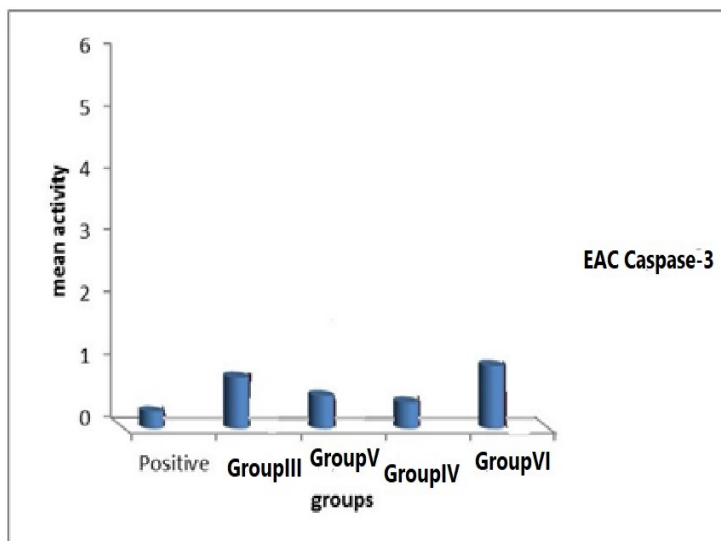


Figure (8): EAC Caspase-3 in all studied groups

***There is a very highly significant increase in EAC caspase-3 levels in ester and carboxamide therapeutic and preventive groups.**

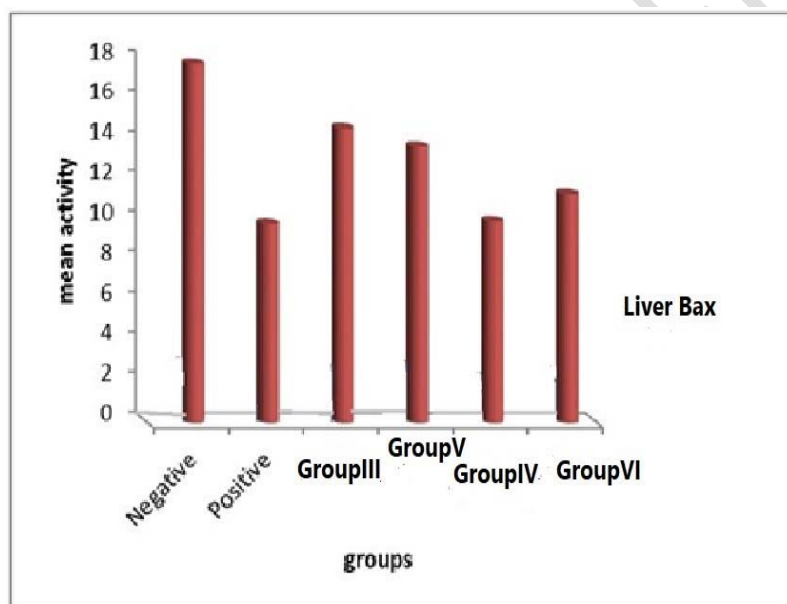


Figure (9): Liver Bax in all studied groups

***There is a very highly significant increase in liver Bax levels in ester and carboxamide therapeutic and preventive groups.**

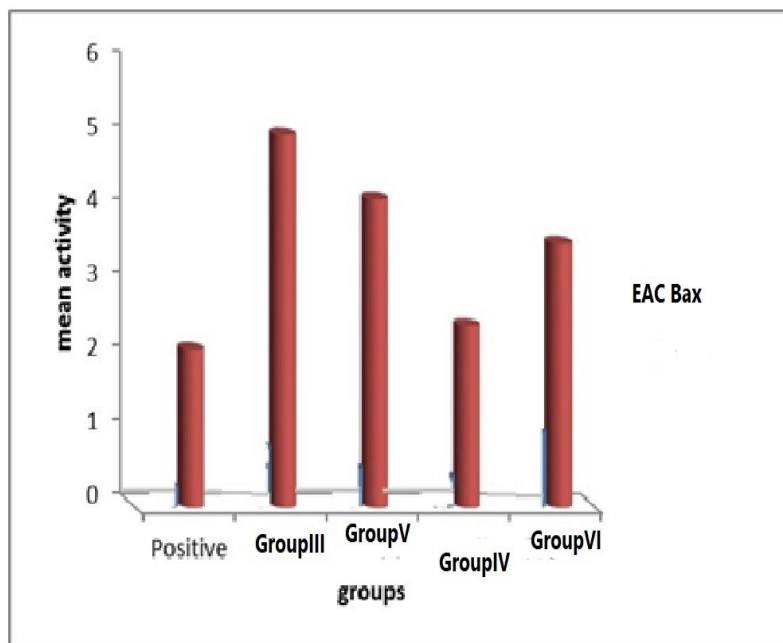


Figure (10): EAC Bax in all studied groups

***There is a very highly significant increase in EAC Bax levels in ester and carboxamide therapeutic and preventive groups.**