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Mode of Action of Potassium Salt of 2-Thioxo-4-Hydroxycoumarin [3, 4-b] Pyrimidine and 9-Bromo-2-Thioxo-Hydroxycoumarin [3, 4-b] Pyrimidine Against Ehrlich Ascites Carcinoma Cells

Faten Zahran[1*], Akaber T. Keshta[1], I. M. EL-Deen[2], M. M. El-behary[2]

1. Biochemistry Department, Faculty of Science, Zagazig University, Egypt

2. Chemistry Department, Faculty of Science, Port-Saied University.

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Abstract: Coumarin and pyrimidine derivatives have attracted intense interest in recent years because they have anti-tumor, antioxidant activities, and induce apoptosis. Our study aims to evaluate the antitumor and anti-oxidant activities of new Coumarin derivatives: Potassium salt of 2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine and 9-bromo-2-thioxo-hydroxycoumarin [3, 4-b] pyrimidine against in vivo tumor model. Coumarino [3, 4-b] pyrimidine -2-thioles (4 a,b) were prepared and their cytotoxicity were determined. The compounds [3a & 3b] exhibited a significant anti-oxidant activity towards Ehrlich ascites carcinoma (EAC) cells by reduction the MDA by 30.3% & 54.9% and NO concentration by 19.4% & 39.6% ($p<0.001$), respectively; compared to the positive control group. Whereas significantly increase in the CAT activity by 150%, 700%, respectively; and SOD activity by 102.9% and 379.9%, ($p<0.001$) respectively; in [3a & 3b] treated groups compared to the positive control group. Anticancer agent kills tumors at least partially through induction of apoptosis. Furthermore, the treatments with 3a and 3b showed a significantly increase in Caspase-3 activity by 85.9% and 269.23%, ($p<0.001$), respectively; and cytochrome c by 85.9% and 269.23%, ($p<0.001$), respectively; compared positive control group. The synthesized compounds have potent antioxidant activity and good inducer for apoptosis by induction of caspase-3 and releasing of cytochrome c.

Keywords: Coumarins, Pyrimidine, 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 [1]. The progression from normal cells to cells that can form a discernible mass to outright cancer involves multiple steps [2]. It 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 [3]. Many therapeutic anticancer have been developed which has relied on surgery, chemotherapy, radiotherapy, hormone therapy and more recently immunotherapy [4]. Therefore the search for potent, safe and selective anticancer compounds is a crucial aspect of

Corresponding author:

Faten Zahran, Biochemistry Department, Faculty of Science, Zagazig University, Egypt. E-mail: dr_fzahran@yahoo.com.

modern cancer research [5]. 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 [6]. Coumarins attract great attention due to their wide range of biological properties, including anticancer, antileukemic, antibacterial and anti-inflammatory activities [7]. Also, some of novel pyrimidine derivatives showed moderate to potent antioxidant, anti-inflammatory, antibacterial, antifungal and anthelmintic activity [8]. In the broad sense, most chemotherapeutic drugs work by impairing cell division, effectively targeting fast- dividing cells. They prevent mitosis by various mechanisms including damaging DNA and inhibition of the cellular machinery involved in cell division [9]. One theory as to why these drugs kill cancer cells is that they induce a programmed form of cell death known as apoptosis [10].

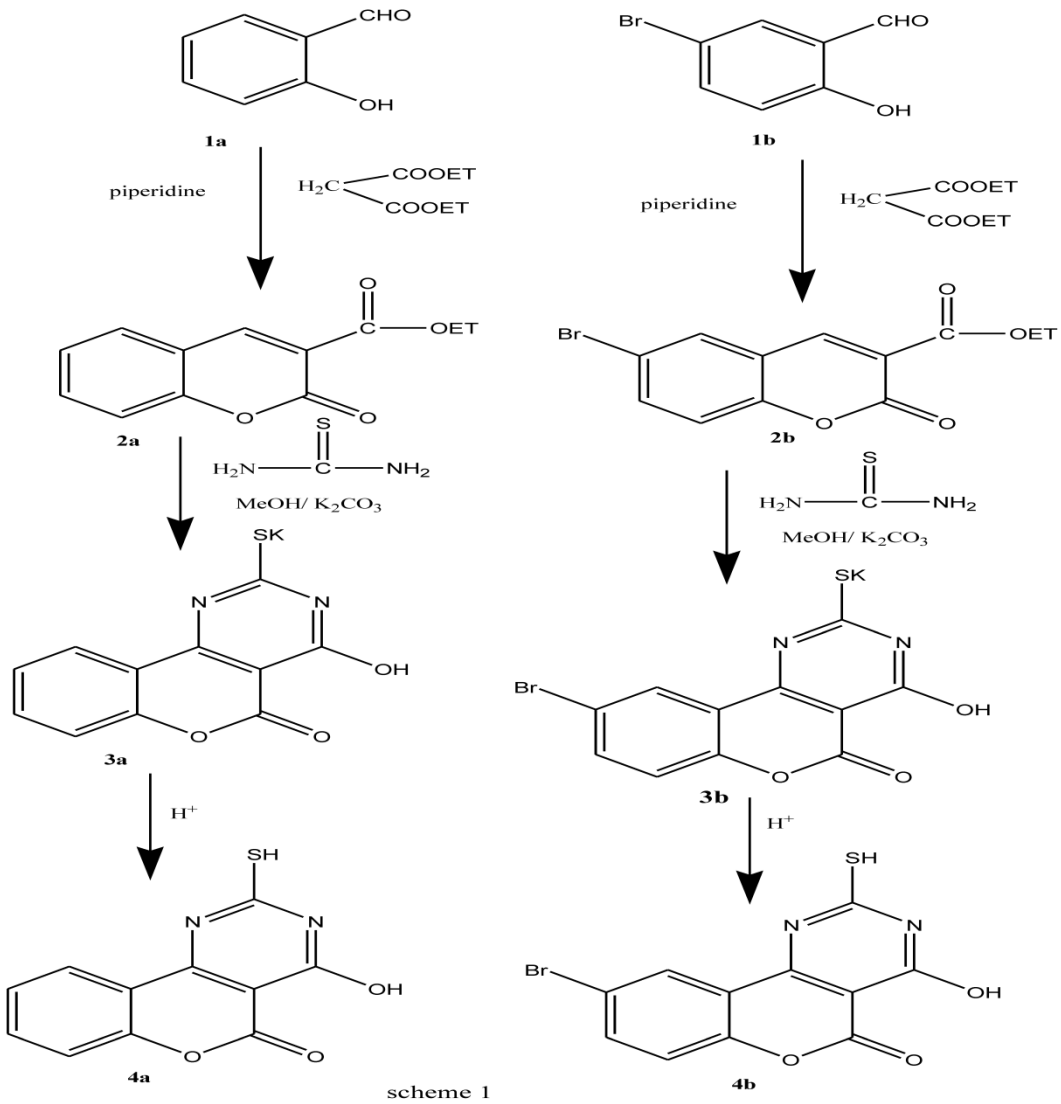
The Coumarin (benzopyran-2-one, or chromen-2-one) ring display interesting pharmacological properties has intrigued chemists and medicinal chemists for decades to explore the natural Coumarins or synthetic analogs for their applicability as drugs [11]. Pyrimidine derivatives and heterocyclic annelated pyrimidines have attracted a great deal of interest owing to their medicinal activities. These medicinal activities include anticancer, antiviral, antitumor, and anti-inflammatory [12].

Our study aims to evaluate the anti-tumor, and the anti-oxidant properties of recently developed synthetic coumarin derivatives: potassium salt of 2-thioxo-4-hydroxycoumarin [3,4-b] pyrimidine and 9-bromo-2-thioxo-4-hydroxyc-oumarin [3, 4-b] pyrimidine against Ehrlich ascites carcinoma "EAC" cells, and study the mechanism of killing cancer cells.

2. Materials and Methods

2.1 Chemistry

In continuation of our previous paper [13-15] towards the synthesis of fused O, N heterocyclic compounds, we report an efficient synthesis of potassium salt of 2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine and 9-bromo-2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine from salicylaldehyde and 5-bromo salicylaldehyde. Condensation of salicylaldehyde (**1a, b**) with diethyl malonate in presence of piperidine afforded the corresponding 3 -ethoxycarbonylcoumarine (**2 a, b**). The reaction of **2** with thiourea in presence of anhydrous potassium carbonate in methanol under reflux produced the potassium salt of 2-thio-oxo-hydroxycoumarin [3, 4-b] pyrimidine (**3 a, b**). Dissolving 3 in water and acidifying with 2 N hydrochloric acid led to the formation of 2-thio-oxo-hydroxycoumarin [3, 4-b] pyrimidine (**4a, b**) (***Scheme 1***).



scheme 1

2.2 In vivo study

***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, Zagazig 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*.

***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.

***Experimental design*:** 30 female Swiss albino mice were divided into 3 groups each one contains of 10 mice: Group I "served as negative control group" injected I.P. with sterile saline for 10 days (day after day); group II "positive control"; i.p. injected with $2.5x10^6$ of Ehrlich ascites carcinoma "EAC" cells. Group III "**3a** therapeutic group, injected i.p. with 5 mg/kg one day after EAC injection and repeated doses of **3a** day after day; Group IV "**3b** therapeutic group", injected i.p. with 7.5mg/kg one day after EAC injection and repeated doses of **3b** injected day after day. After the end of

the experiment, blood and EAC cells were collected from mice, and antioxidants assays, Caspase-3 activity, and cytochrome c were assayed.

***Life span prolongation*:** Life span calculation was carried out according to the method described by Mazumdar *et al.* [16].

***Estimation of Malondialdehyde*:** The lipid peroxidation products were estimated by the formation of thiobarbituric acid (TBA) and quantified in term of MDA where, thiobarbituric acid (TBA) reacts with MDA in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product, the absorbance of the resultant pink product can be measured at 534 nm in a spectrophotometer [17].

***Estimation of Nitric Oxide*:** The Bio-diagnostic Nitrite Assay Kit provides an accurate and convenient method [18] for measurement of endogenous nitrite concentration as indicator of nitric oxide production in biological fluids. In acid medium and in the presence of nitrite the formed nitrous acid diazotise sulphanilamide and the product are coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish-purple color which can be measured at 540 nm in a spectrophotometer.

***Assessment of catalase enzyme activity (CAT)*:** In the presence of hydrogen peroxides remaining H_2O_2 reacts with 3.5-dichloro-2 hydroxy benzene sulfonic acid (DHBS) and 4-amino phenazone (AAP) to from achromophore with color intensity inversely proportional to amount of catalase in the original sample [19].

***Assessment of Suproxid Dismutase (SOD)*:** This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye [20].

***Caspase-3 Colorimetric Assay*:** The activity of caspase-3 was determined by the colorimetric caspase-3 kit according to the manufacturer's instructions (R& D system, Inc.) [21]. This assay is based on spectrophotometrically detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate7-amino-4- trifluoromethyl coumarin conjugated *p*NA (DEVD-*p*NA) in equal amount of cells protein lysates. Briefly, 1×10^6 cells were collected and lysed with 50 µl of chilled lysis buffer and incubated on ice for 10 min. Cell lysates were centrifuged at maximum speed for 5 min at 4°C, after which 50 µl of 2× reaction buffer/dithiothreitol (DTT) mix and 5 µl of 1 mM caspase-3 substrate (DEVD-pNA) were added to each reaction and incubated at 37 °C for 1 hr. The *p*NA light emission was quantified using a microplate reader at 400- or 405- nm.

***Quantitative determination of mouse cytochrome c "Cyt.c" in cell lysates*:** The Quantikine Rat/Mouse Cytochrome c Immunoassay is a 2.5 hour solid phase ELISA designed to measure rat and mouse Cytochrome c according to the method [22]. A monoclonal antibody specific for rat/mouse Cytochrome c has been pre-coated onto a micro-plate. Conjugate, Standards, Control, and samples are pipetted into the wells and any rat/mouse Cytochrome c present is bound by the immobilized antibody and the enzyme-linked monoclonal antibody specific for rat/mouse Cytochrome c.

***Statistical analysis*:** Statistical analysis was performed using SPSS software II version 14 [23]. 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 $p < 0.05$ was considered statistically significant.

3. Results

***Effect of compounds (3a, 3b) on life span prolongation in studied groups*:**

Life span prolongation in all the studied groups is shown in Table (6). The mean life span prolongation in the positive control group was found to be 16 days. **3a** and **3b** treated groups showed a significant increase in the life span prolongation to 19 days by 18.7% (T/ C ratio = 118.8%), and 21 days by 31.3% (T/ C ratio = 131.3%); respectively; compared to the positive control group.

***Effects of compounds (3a, 3b) on anti-oxidants in blood and EAC samples*:**

Table (2), summarize the antioxidant effect of compounds (**3a**, **3b**), in EAC cells. The mean values of MDA levels in positive control group were found to be 22.77 ±1.3 (nmol/mL). 3a and 3b treated groups showed a significant decrease by 30.3% & 54.9% ($p<0.001$) respectively; compared to the positive control group; *Fig.* (1). Also, the mean values of NO levels in positive control group were found to be 93.39 ±4.58 (µmol/mL). 3a and 3b treated groups showed a significant decrease by 19.4% & 39.6% ($p<0.001$) respectively; compared to the positive control group; *Fig.* (2). But, CAT activity in positive control group was found to be 0.06 ±0.01 (U/g tissue). CAT activity showed a significantly increase in **3a** treated group by 150%, and 700% in **3b** treated group, compared to positive control, ($p<0.001$), *Fig.* (3). Moreover, the mean value of SOD activity in positive control group was found to be 67.32±5.17 (U/g tissue). **3a** and **3b** treated groups showed a significant increase by 102.9% and 379.9%, ($p<0.001$) respectively; compared to the positive control group, *Fig.* (4).

Table (3) summarize the effect of compound **3a** and **3b** on MDA, NO, CAT, SOD in blood samples. The mean values of MDA in plasma samples were a significantly increased in the positive control group by 219.8%, ($p<0.01$) compared to the negative control group 10.85 ± 1.43 (nmol/mL). While, the mean MDA levels were significantly decreased by 48.58% and 71.23% in **3a** and **3b** treated group; ($p<0.01$) respectively, compared to the positive control group, *Fig* (5). Also, plasma NO concentration were a significantly increased in the positive control group by 83.58%, ($p<0.01$) compared to the negative control group 20.05 ± 1.24 (µmol/mL). But; the mean NO levels were significantly decreased by 41.64% in **3a** treated group; 55.15% in **3b** treated groups, ($p<0.01$), compared to the positive control group, *Fig*. (6). On the other hand, CAT activities were decreased by 50.61%, ($p<0.001$) in the positive control group compared to the negative control group 310.25 ± 50.61 (U/ml). While, their activities showed a significantly increase by 193.98%, and 300.57%, ($p<0.001$); respectively; compared to the positive control group, *Fig.* (7). SOD activities were decreased to 97.55 ± 9.69 (U/mL) in the positive control group compared to the negative control group 214.27 ± 10.62 by 54.47%, ($p<0.001$). In **3a** and **3b** treated groups, SOD activities were a significantly increased by 253.6%, and 531.96%, ($p<0.001$), respectively; compared to positive control group, *Fig.* (8).

***Effects of compounds (3a, 3b) on Caspase3 activity and cytochrome levels in EAC cells*:**

Table (4) demonstrated the apoptotic effect of compounds **3a** and **3b** by measurement Caspase-3 activity and cytochrome c levels in the EAC cells in all studied groups. Caspase-3 activity in positive control was found to be 0.270 (ng/mL). Furthermore, the treatments with **3a** and **3b** (5 mg/kg, and 7.5 mg/kg, I.P.) showed a significantly increase in its activity by 85.9% and 269.23%, ($p<0.001$), respectively; compared to the positive control group, *Fig.* (9). Also, cytochrome *c* levels in positive control were found to be 0.88±0.37 (ng/mL). Furthermore, the treatments with **3a** and **3b** showed a significantly increase in cytochrome *c*, by 81.8% and 328.4%, ($p<0.001$), respectively; compared to the positive control group, *Fig.* (10).

***Correlations between different Studied Parameters among different Groups*:**

To confirm our results, correlations between different parameters were carried out. We found, there were significant positive and negative correlations between parameters.

4. Discussion

Cancer is considered one of the major causes of mortality in the world. 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 [24]. This paper describes the evaluation of the anti-invasive, anti-oxidant properties of recently developed synthetic coumarino [3,

4-b] pyrimidine derivatives. Heterocycles such as chromone, 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 [25]. The most effective doses of two synthetic compounds **3a, and 3b** were to be safe compounds and exhibited *in vitro* anti-cancer against some different cell lines such as [MCF-7(human breast cancer), HePG2 (Hepatocellular carcinoma), HCT116 (human colon cancer), PC3 (human prostate cancer)] to assess their cytotoxicity effects. The results indicated that compound 3a, b have cytotoxicity potency. Compound 3a showed a very potent activity against MCF-7, HePG2, HCT116, and PC3 with minimum inhibitory concentration (MIC) [25, 5, 25, and 5 µg/ml, respectively] but compound 3b showed low activity than compound 3a with minimum inhibitory concentration 50 µg/ml for all cell lines according to our pervious paper [26]. Our results found that, these doses were significant prolonged the life span to 19 days by 18.7% (T/ C ratio = 118.8%) in 3a treated group, and 21 days by 31.3% (T/ C ratio = 131.3%) in 3b treated group; compared to the positive control group. 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 [27]. Our results found that, MDA and NO levels in EAC cells in both **3a and 3b** treated groups showed a significant decrease by 30.3% & 54.9%.; by 19.4% & 39.6% ($p<0.001$) respectively; compared to the positive control group. While, CAT & SOD activities showed a significantly increase in **3a** treated group by 150%, 102.9% and 700% 379.9%, in **3b** treated group, compared to positive control, ($p<0.001$), table (2), Fig. (1-4). Confirming the results above, anti-oxidant activity of **3a and 3b** compounds were evaluated in the plasma; table (3) and Fig. (5-8). The therapeutic strategies should aim to reducing free-radical formation and scavenging free radicals [28]. Coumarins possess, antioxidant activities, probably due to their structural analogy with flavonoids and benzophenones [29]. 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, the α-pyrone rings of coumarins significantly enhanced the capacity of inhibiting oxidative reactions of coumarins [30]. Moreover, in a pyrimidine ring, as the number of nitrogen atoms increases the ring π electrons become less energetic; electrophilic ar omatic substitution gets more difficult, while nucleophilic aromatic substitution resonance stabilization properties of pyrimidine may lead to the addition and ring cleavage reactions rather than substitutions [31]. Our results are in a line with many authors, ***Abu- Hashem et al.,*** [32] who synthesized a series of pyrimidine derivatives that showed a potent anti-oxidative activity by lipid peroxidation assay. Also, **Bhalgat *et al.,*** [33] synthesized a series of novel dihydropyrimidine Carbonitrile compounds and evaluated for antioxidant activities. These compounds revealed potent antioxidant activity due to the presence of NH and –SH groups. As reported by *Kumar et al.,* [34] who, synthesized a novel series of 4, 6-bisaryl-pyrimidin- 2-amine derivative. All of these compounds were evaluated for their antioxidant activity by nitric oxide and hydrogen peroxide free radical scavenging method using ascorbic acid as a standard drug. The compounds show potent antioxidant activity as compare with the standard drug due to the presence of Cl and Br as the electron withdrawing group. The substitution on six-member heterocyclic nucleus was made by different substitutions of Cl, and Br, which increased the penetration of molecules into the lipid membrane so that they increase the antioxidant activity by combining with the reactive oxygen species, which is generated by the different disease conditions. By making these changes on the nucleus, we are able to find out the most potent substituted antioxidant compounds [35]. The provide information on the mechanisms by which coumarin induces cell cycle arrest and apoptosis in cancer cells [36].

Also, the preliminary results of pyrimidine derivatives indicated they exhibit more potent antitumor inhibitory activity [37]. The apoptotic effect of compounds **3a and 3b** were evaluated by measurement Caspase-3 activity and

cytochrome c in the EAC cells in all studied groups. Furthermore, the treatments with **3a and 3b** (5 mg/kg, and 7.5 mg/kg, I.P.) showed a significantly increase in Caspase-3 activity, by 85.9% and 269.23%, ($p<0.001$), respectively; compared to the positive control group. And significantly increase in cytochrome c, by 85.9% and 269.23% ($p<0.001$), respectively; 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 [38]. Coumarins and coumarin derivatives as well as diallyl polysulfides 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 [39]. 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. Cumarines decreased the mitochondrial membrane potential and promoted the release of cytochrome c and the activation of caspase-3 before leading to apoptosis [36]. 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. The most promising compounds were tested against two non-tumoral cell lines (CRL-8799 and CRL-11233) and, among them, bis (4- methoxybenzyl)-pyrido[2,3d]pyrimidine-2,4-diamine showed the best profile in these assays [40]. More recently, furo-pyrimidines have been found to be active as kinase inhibitors [41]. Also, The pyrazolo[3,4-d]pyrimidine nucleus is considered as an isostere to the purine nucleus and hence exhibits promising antitumor activity by acting as ATP competitive inhibitor for many kinase enzymes. Indeed, many pyrazolo[3,4-d]pyrimidines were reported to exhibit potent anti-tumor activity . Their cytotoxic activities might be attributed to inhibition of several enzymes such as Sarcoma (Src) kinase, tyrosine kinase, mammalian target of rapamycin (mTOR), cyclin dependent kinase (CDK) and glycogen synthase kinase (GSK) [42]. Our data were in a line with many authors, **Chen *et al.,*** [43] who reported that, Gemcitabine (4-amino-1-(2-deoxy-2, 2-difluoro-β-Derythropentofuranosyl) pyrimidin-2(1H)-on) is currently the best treatment available for pancreatic cancer, as such agent that has been shown to induce apoptosis in other tumor cells via down-regulation of Bcl-2/Bax and promoting the release of Cytochrome c, but with very low toxicity. Emodin potentiates the apoptosis induced by gemcitabine, which was demonstrated by activation of caspase-3 in the combination group. Also, **Lukasik *et al.,*** [44] studied the cellular mechanism of action of 4-(1H-Indazol-1-yl)-N-phenylpyrimidin-2-amine. Induction of caspase 3/7 activity was measured in HCT116 cancer cells after treatment with this compound for 24 h. Significantly induced caspase 3/7 activity at its GI50 mM compared with the untreated cells, with enhanced activity at higher concentrations. In the light of our in vivo results, the bromo-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 [45]. This indicates that compound 3b has in vivo antitumor activity against EAC more than compound 3a; this may be attributed due to the presence of bromine atom in compound 3b. As **Kempen *et al.,*** [45] 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.

5. Conclusion

The *in vivo* effect of the compounds 3a, and 3b exhibited significant anticancer and anti-oxidant activities towards EAC cells by induction of apoptosis. On the basis of these results, compound 3b may be considered as attractive leads in the future development of potential anticancer and anti-oxidant agent more than compound 3a.

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Table (1): Effect of Compounds (3a, 3b) on life span prolongation

Parameter	Positive control	treated group (Cpd 3a)	treated group (Cpd 3b)
Life span prolongation	Life span prolongation	Life span prolongation	Life span prolongation
Days	16	19	21
% change	---------	18.7%	31.3%
T/C ratio (%)	--------	131.3	118.8

Table (2): Anti-oxidants Effect of Compounds (3a, 3b) in EAC cells

Variables	Positive Control Group		Treated Group (3 a)		Treated Group (3 b)		P
	Mean ± SD.	% Change	Mean ± SD.	% Change	Mean ± SD.	% Change	
MDA (nmol/mL)	22.77 ±1.3	------	15.87 ±0.87	30.3%	10.25 ±1.78	54.9%	0.001**
NO (µmol/mL)	93.39 ±4.58	------	75.27 ±5.26	19.4%	57.26 ±6.58	39.6%	0.001**
Catalase (U/g tissue)	0.06 ±0.01	------	0.15 ±0.02	150%	0.48±0.05	700%	0.001**
SOD (U/g tissue)	67.32±5.17	------	136.62±21.27	102.9%	323.08±45.04	379.9%	0.001**

The significant difference: $P^{**} < 0.01$→high significant $P^{*} < 0.05$→ significant

Table (3): Anti-oxidants Effect of Compounds (3a, 3b) in blood samples

Variables	Negative control	Positive control (EAC)		Treated Group (3 a)		Treated Group (3 b)		P
	Mean ± SD.	Mean ± SD.	% Change	Mean ± SD.	% Change	Mean ± SD	% Change	
MDA (nmol/mL)	10.85 ± 1.43	34.70 ± 3.94	219.8%	17.84 ± 1.73	48.58%	9.98 ± 1.58	71.23%	0.001**
NO (µmol/mL)	20.05 ± 1.24	36.81 ± 4.51	83.58%	21.48 ± 0.98	41.64%	16.51 ± 2.51	55.15%	0.001**
Catalase (U/ml)	310.25 ± 50.61	153.24± 30.0	50.61%	450.5 ± 2.47	193.98%,	613.84 ± 66.24	300.57%	0.001**
SOD (U/ml)	214.27 ± 10.62	97.55± 9.69	54.47%	344.96 ± 42.82	253.6%	616.48 ± 43.18	531.96%	0.001**

The significant difference: $P^{**} < 0.01$→high significant $P^{*} < 0.05$→ significant

Table (4): Effect of Compounds (3a, 3b) on caspase-3 activity and cytochrome c levels in EAC cells

Variables	Positive Control Group		Treated Group (3 a)		Treated Group (3 b)		P
	Mean ± SD.	% Change	Mean ± SD.	% Change	Mean ± SD.	% Change	
Caspase-3 activity (U/ml)	0.270	------	0.502	85.9%	0.997	269.23%	0.001**
Cyto. C (ng/ml)	0.88±0.37	------	1.60±.29	81.8%	3.77±0.66	328.4%	0.001**

The significant difference: $P^{**} < 0.01 \rightarrow$ *high significant* $P^{*} < 0.05 \rightarrow$ *significant*

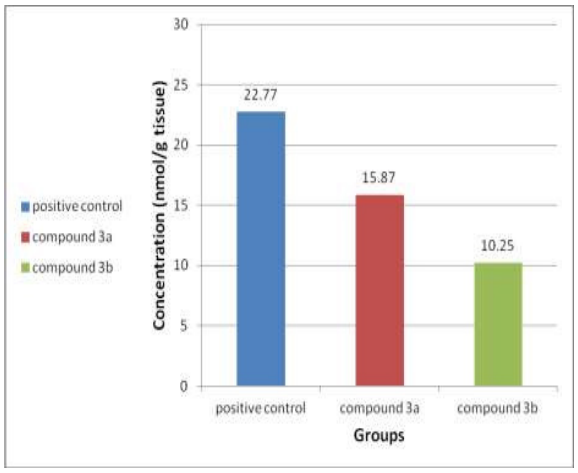

Fig. (1): Effect of compound 3a and 3b on MDA in EAC in Mice Groups.

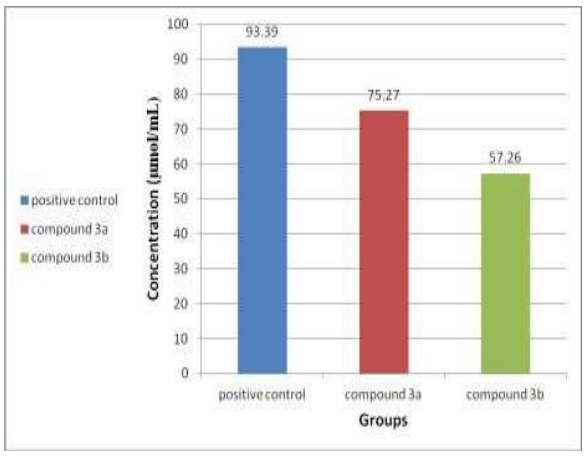

Fig. (2): Effect of compound 3a and 3b on NO in EAC in Mice Groups.

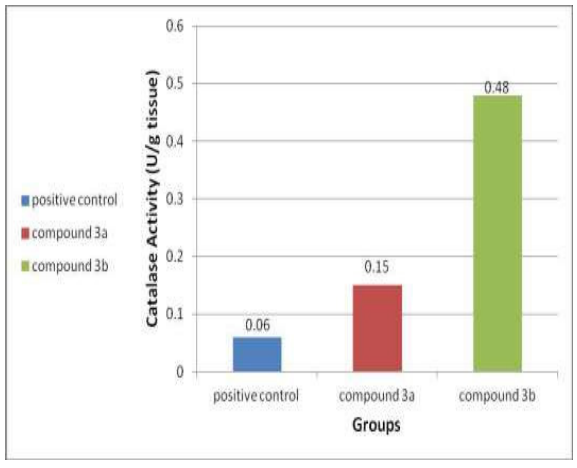

Fig. (3): Effect of compound 3a and 3b on CAT activity in EAC in Mice Groups.

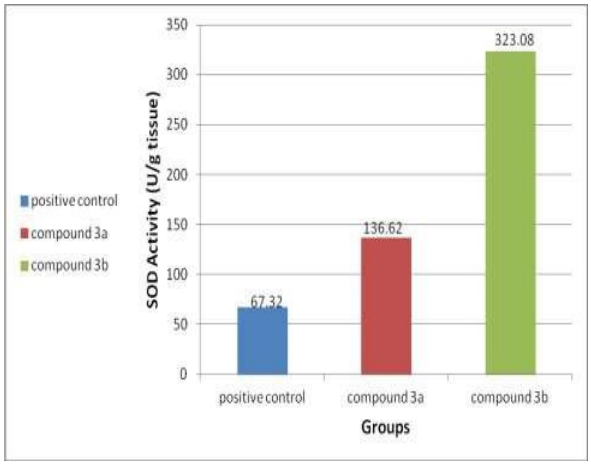

Fig. (4): Effect of compound 3a and 3b on SOD activity in EAC in Mice Groups.

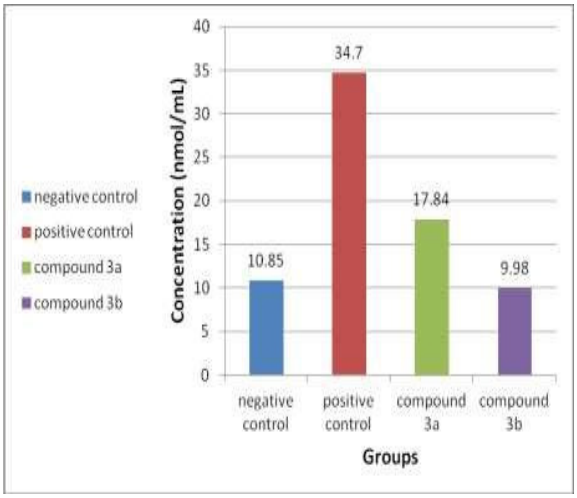

Fig. (5): Effect of compound 3a and 3b on MDA in blood in Mice Groups.

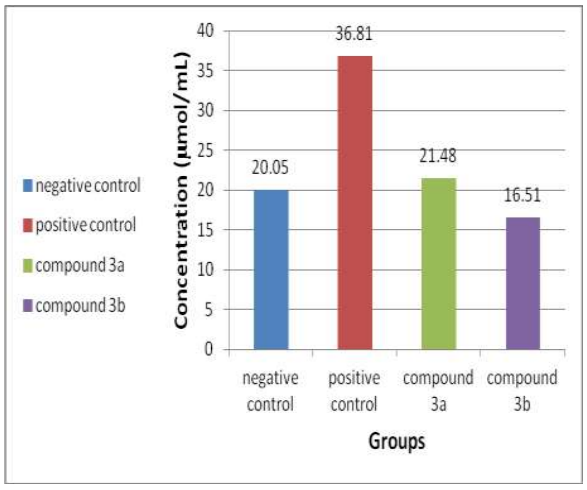

Fig. (6): Effect of compound 3a and 3b on NO in blood in Mice Groups.

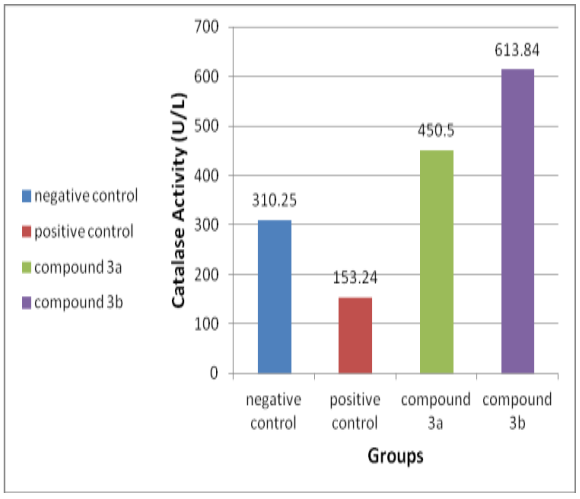

Fig. (7): Effect of compound 3a and 3b on CAT activity in blood in Mice Groups.

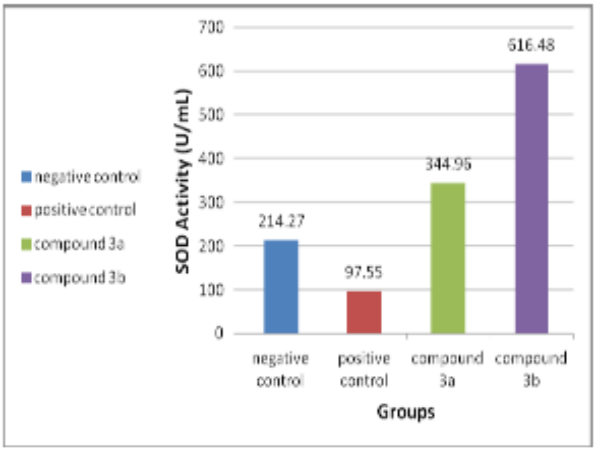

Fig. (8): Effect of compound 3a and 3b on SOD activity in blood in Mice Groups.

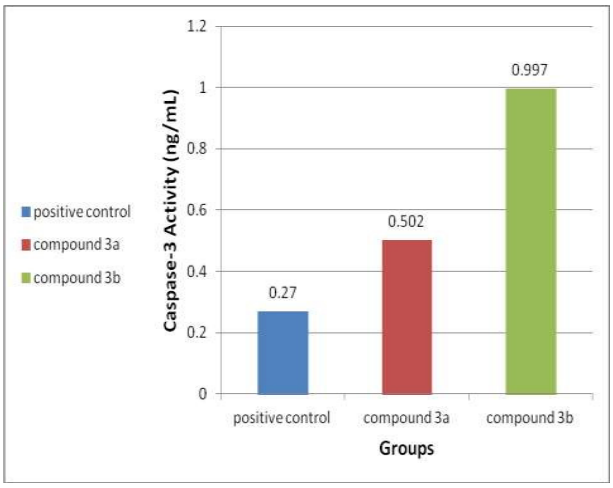

Fig. (9): Effect of compound 3a and 3b on caspase-3 activity in EAC in Mice Groups.

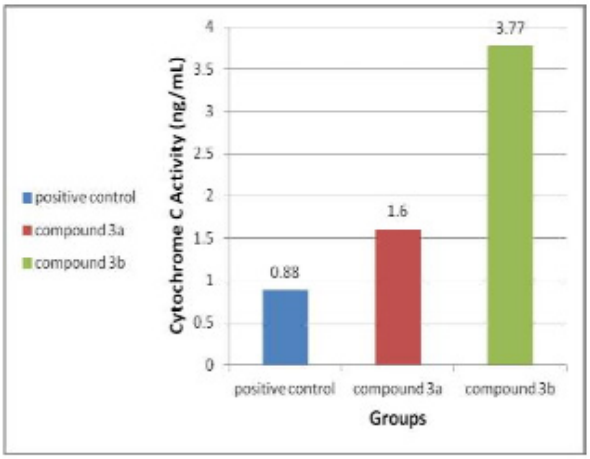

Fig. (10): Effect of compound 3a and 3b on cytochrome c levels in EAC in Mice Groups.