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# Potassium salt of 2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine and 9-bromo-2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine inhibits tumor growth *in vitro* and *in vivo*

KEYWORDS: Coumarins, Pyrimidine, Ehrlich ascites carcinoma cells.

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**ABSTRACT** *Coumarin and their derivatives are important and useful compounds with diverse pharmacological properties. In the present study, we evaluated the in vitro cytotoxic activity 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 MCF-7 (human breast cancer), HePG2 (Hepatocellular carcinoma), HCT116 (human colon cancer), PC3 (human prostate cancer). In order to find new drugs with anticancer activities prepared compounds were evaluated for their in vitro and in vivo anticancer activities. Coumarino[3, 4-b] pyrimidine -2-thioles (4a,b) were prepared via cyclocondensation of 6-substituted-3-ethoxycarbonylcoumarin (2a, b) with thiourea in the presence of anhydrous potassium carbonate to give the potassium salt of Coumarino[3, 4-b] pyrimidine -2-thioles (3a, b), followed by acidification of (3a, b), with 2 N hydrochloric acid . The compounds [3a & 3b] exhibited a significant anticancer activity towards MCF-7, HEPG2, HCT116 and PC3 cancer cell lines. Also, in vivo study of, [3a & 3b] compounds revealed a significant anticancer activity towards Ehrlich ascites carcinoma (EAC) cells by reduction of its volume to 55.5% and 73.3% ($p<0.001$), in the treated groups; respectively. And significantly decrease in the cell count by 65.9% and 78.9%, in treated groups ($p<0.001$); respectively, compared to the positive control group. It turned out that they reduced cell viability of cancer cells in a time and concentration dependent manner in vitro and in vivo studies.*

## I. Introduction

Cancer, a diverse group of diseases characterized by uncontrolled growth of abnormal cells, is a major worldwide problem. It is a fatal disease standing next to the cardiovascular disease. Although the cancer research has led to a number of new and effective solutions, the medicines used as treatments have clear limitations and unfortunately cancer is projected as the primary cause of death in the future currently there is a huge scientific and commercial interest in the discovery of new anticancer drugs. Therefore the search for potent, safe and selective anticancer compounds is a crucial aspect of modern cancer research [1].

Cancer chemoprevention is a rapidly growing area of oncology which can make a significant progress in the prevention and treatment of carcinogenesis by administration of various drugs with chemical or natural entities depending on their anti-mutagenic properties [2]. The search for new chemopreventive and anti-tumor agents that are more effective and less toxic has kindled great interest [3]. 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 [4]. As a result, the design and discovery of non-traditional, efficient and safe chemical classes of agents are prime targets in contemporary medicinal chemistry. Coumarins are compounds that display a special role in nature. Pharmacologically, Coumarins and their derivatives are included in the family of the flavonoids and exhibit a variety of interesting biological and pharmacological activity. Therefore, Coumarins and their derivatives have raised considerable interest because of their potential beneficial effects on human health [5]. They have been reported to possess the following activities: anti-HIV, anticoagulant, antibacterial, anticancer, anthelminthic, anti-inflammatory, and antioxidant activities [6].

As a result, Coumarins and derivatives have been the subject of extensive investigations. In the present study for new anticancer agents, we have evaluated the anti-tumor properties of recently developed synthetic coumarin derivatives among which two compounds revealed important activity: potassium salt of 2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine and 9-bromo-2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine against breast, liver, colon, prostate cancer cell lines (in vitro study) and against EAC cells (in vivo study).

## 2-Materials and methods

### 2. 1.Chemistry

In continuation of our previous paper [7-9] 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).

### 2.2- Experimental

Melting points were determined on a Boetius micro hostage apparatus and are uncorrected. An elemental analyzer, Hereaus CHN-OS-Rapid, was used for microanalyses. The IR spectra were recorded on a perkin-Elmer FTIR 1725 spectrometer. The mass spectra were taken on a VG12-250 instrument (70 ev EI ionization, source temperature 200 °C). The $^{1}$H-NMR and $^{13}$C-NMR spectra were recorded on a Varian unity 400 spectrometer at 399.925 and 200 MHZ, respectively, with TMS as internal standard.

**Potassium salt of 2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine (3a)**

A mixture of 2a (0.01 mol), Thiourea (0.01 mol), and anhy-

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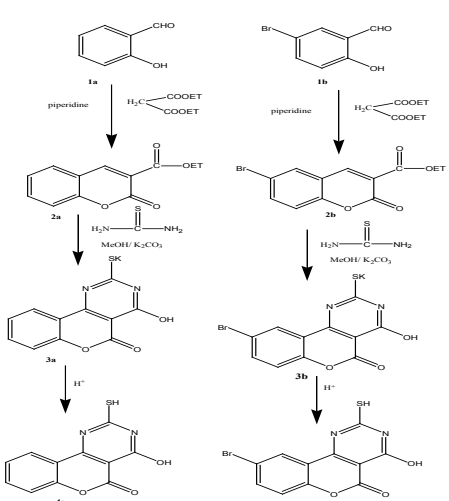
drous potassium carbonate (0.03 mol) was heated under reflux with stirring in methanol (70 ml) for 2 h. The solid formed was filtered of and dried to give 3a as yellow powder, yield 53%.

**2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine (4a)**
A potassium salt of 3a (0.01 mol) was dissolved in hot water (50 ml), then cooled and acidified with 2 N hydrochloric acid. The solid obtained was filtered off, washed with water, dried and purified by recrystallization (dimethyl formamide) to give 4a as orange crystals, yield 87%, m.p. 360 °C. $V_{max}$ (KBr): 3430-2253(br. OH), 3173(NH), 1747(C=O), 1632(C=N), 1109, 1056(C-O) $cm^{-1}$. $\delta_H$ (DMSO-$d_6$): 7.31-7.38(dt, 2H, Ar-H), 7.73-7.78(dt, 2H, Ar-H), 8.83(s, 1H, OH), 8.88(s, 1H, SH) ppm. $\delta_c$ (DMSO-d6): 156.40(C-2), 117.04(C-3), 154.86(C-4), 176.75(C-6), 151.72(C-8), 110.75(C-9), 125.30(C-10), 124.26(C-11), 135.40(C-12), 98.08(C-13), 153.83(C-14) ppm. MS: m/z= 248[($M^+$+2), 11.70], 247[($M^+$+1), 77.10], 246[($M^+$, 100]. Anal. $C_{11}H_6N_2O_3S$ for Calcd: C, 53.66; H, 2.44; N, 11.38; S, 13.00. Found: C, 53.52; H, 2.36; N, 11.35; S, 13.03.

**6-Bromo—3ethoxycarbonylcoumarin (2b)**
6-Bromo-3eyhoxcarbonycoumarin (2b) was prepared from 5-bromosalicylaldehde and diethyl malonate according to a literature method. M.p. 165 °C; IR (KBr): 1752(C-O of ester), 1705(C=O of puranone ), 1614, 1585(C=C), 1203, 1132, 1067, 1000(C-O) $cm^{-1}$. $^1$H-NMR (DMSO-$d_6$): δ 1.3(t, 3H, $CH_3$). 4.20 (q, 2H, $OCH_2$), 7.12-7.81(m, 3H, Ar-H), 8.42(s, 1H, H-4 of pyranone ring) ppm. Anal. $C_{12}H_9BrO_4$ for Calcd: C, 48.65; H, 3.04; Br, 26.69. Found: C, 48.46; H, 2.98; Br, 26.52.

**Potassium salt of 9-bromo—2-thioxo—4hyroxycoumarin [3, 4-b] pyrimidine (3b)**
A mixture of 2b (0.01 mol), Thiourea (0.01 mol), and anhydrous potassium carbonate (0.03 mol) was heated under reflux with stirring in methanol (70 ml) for 2 h. The solid formed was filtered of and dried to give 3b as yellow powder.

**9-bromo—2-thioxo—4hyroxycoumarin [3, 4-b] pyrimidine (4b)**
Compound 4b was obtained as orange crystals, yield 36%, m.p. = 323-325 °C. IR spectrum (KBr): 3220(NH), 3300-2890(br. OH), 1719(C-O), 1613, 1589, 1210, 1133, 1080 $cm^{-1}$. 1H.NMR spectrum (DMSO-$d_6$): δ 7.12-8.13(m, 3H, Ar-H). 8.61 (br. s, 1H, OH), 10.23(s, 1H, NH) ppm MS: m/z= 326 ($M^+$+2, 100),325(20.30), 310(9.80), 298(9.00), 269(10.80), 268(36.20), 267(11.70), 265(6.80), 246(17.80), 239(4.10), 225(4.70), 224(5.10), 210(3.20), 208(5.20), 199(21.20), 197(21.40), 196(5.10), 183(5.70), 171(9.10), 169(10.70), 119(12.50), 117(7.40), 103(7.20), 101(11.70), 88(12.40), 87(28.40), 75(22.80), 63(44.60), 61(22.20), 51(12.20), 50(22.40).. Anal. $C_{11}H_5BrN_2O_3S$ for Calcd: C, 40.74; H, 1.54; N, 8.64; Br, 24.38; S, 9.88. Found: C, 40.49; H, 1.39; N, 8.46; Br, 24.18; s, 9.63.



scheme 1

**In vitro study:**
**Cytotoxicity:** cytotoxic activity of compounds [3a & 3b] was performed on a panel of human tumor cell lines (MCF-7 (human breast cancer), HePG2 (Hepatocellular carcinoma), HCT116 (human colon cancer), PC3 (human prostate cancer) at different concentrations. The cytotoxicity was carried out using Sulphorhodamine-B (SRB) assay following the method reported by Philip et al., [10]. SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content.

**In vivo study:**
**Toxicity studies:** Approximate $LD_{50}$ of 3a and 3b in mice were determined according to the method Meier and Theakston [11].

**Dose response curve:** Dose response curve of 3a and 3b in mice was determined according to the method Crump et al., [12].

**Experimental design:** 30 female Swiss albino mice were divided into 3 groups each one contains of 10 mice: Group I "served as positive control; i.p. injected with $2.5x10^6$ of Ehrlich ascites carcinoma "EAC" cells. Group II "3a therapeutic group, injected i.p. with 5 mg/kg one day after EAC injection and repeated doses of 3a day after day; Group III "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, EAC cells were collected from mice, and viability study was assayed.

**Cell Viability and Counting of EAC cells:** the counting and viability of EAC cells was determined by the Trypan Blue Exclusion Method [13], where the total and viable cells (non-stained) were counted at magnification ×40; as the number of cells/ml was determined in the studied groups.

**Statistical analysis**
Statistical analysis was performed using SPSS software II version 14 [14]. 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:**
Cytotoxicity: The in vitro cytotoxic activities of compounds (3a and 3b) were showed in table (1) and figures (1-4). Minimum Inhibitory concentrations of synthesized compound 3a were found to be 25 µg/ml, 5 µg/ml, 25 µg/ml, and50 µg/ml against MCF-7, HePG2, HCT16, and PC3 cell lines; respectively. While, Minimum Inhibitory concentrations of synthesized compound 3b were found to be 50 µg/ml in all cell lines.

**Table 1: Minimum inhibitory concentration of compounds 3a and 3b against MCF-7, HEPG2, HCT116, and PC3 cell line.**

| | MCF-7 | HEPG2 | HCT116 | PC3 |
|---|---|---|---|---|
| Compound 3a | 25 µg/ml | 5 µg/ml | 25 µg/ml | 5 µg/ml |
| Compound 3b | 50 µg/ml | 50 µg/ml | 50 µg/ml | 50 µg/ml |

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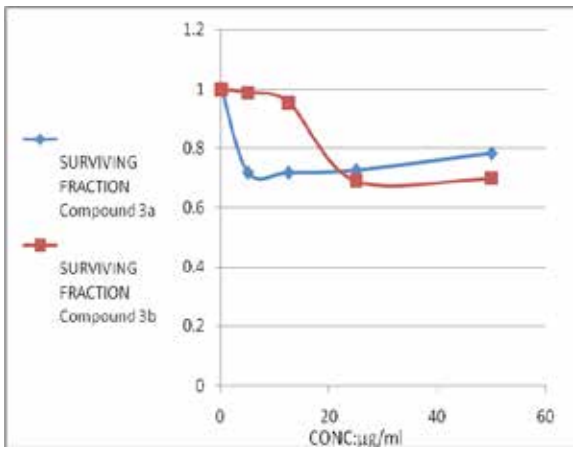


**Figure (1): Minimum inhibitory concentration of compounds 3a and 3b against MCF-7 cell line.**

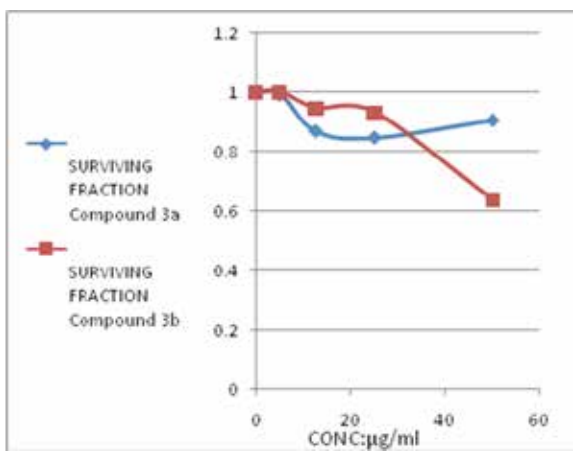


**Figure (2): Minimum inhibitory concentration of compounds 3a and 3b against HePG2 cell line.**

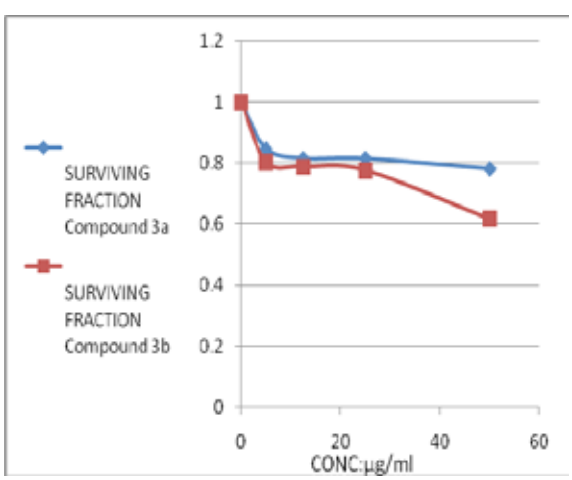


**Figure (3): Minimum Inhibitory Concentration of compounds 3a and 3b against HCT16 cell line.**

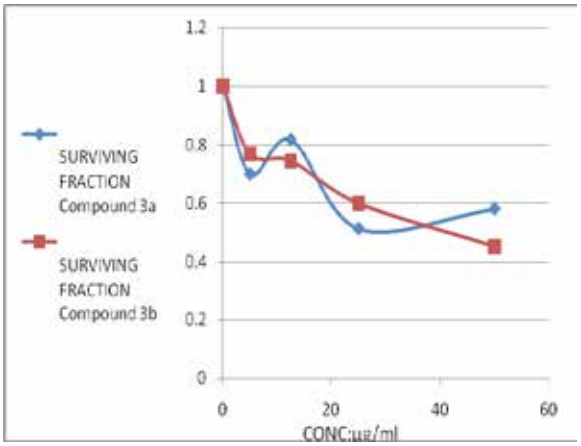


**Figure (4): Minimum Inhibitory Concentration of Compounds 3a and 3b against PC3 cell line.**

Determination of median lethal dose ($LD_{50}$) of two compounds: our results revealed that, doses up to 50 mg /kg and up to 100 mg /kg were considered safe for compounds 3a and 3b; respectively, where no mortality were observed.

**Dose-response curve:** the most effective doses were found to be 5 mg /kg and 7.5 mg /kg for compounds 3a and 3b; respectively, Fig. (5).

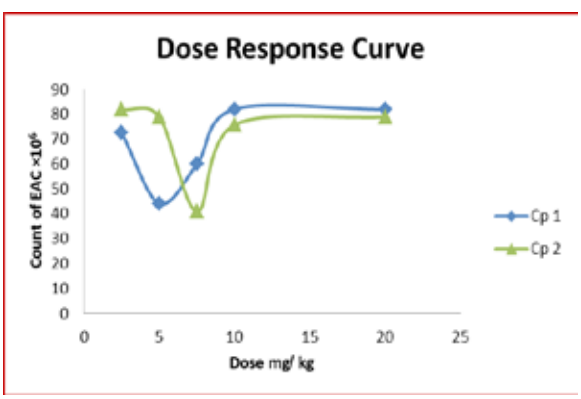


**Fig. (5) Dose response curve for compound 3a & 3b**

Volume and Counting of EAC cells: Table (2) summarizes the effect of compounds 3a and 3b on EAC cells volume and count. The mean volume of EAC in the positive control group was found to be 4.5 ±0.5 (ml). This value was significantly decreased by 55.5%, and by 73.3%, ($p<0.001$) for compounds 3a & 3b treated groups; respectively, Fig (6 a). Also, the mean count of EAC cells in the positive control group was found to be 182.6± 11.5 ($\times 10^6$), which significantly decreased by 65.9% and 78.9%, ($p<0.001$) for compounds 3a & 3b treated groups; respectively, compared to the positive control group, Fig (6 b).

**Table (2): Effect of compounds 3a & 3b on the volume and count of EAC in the studied groups:**

| Parameter | Positive control | Cpd 3a treated group | Cpd 3b treated group |
|---|---|---|---|
| Volume of Ascitis Fluid (ml) | 4.5 ± 0.5 | 2.04 ± 0.57*** | 1.2 ± 0.54*** |
| % change | ------ | 55.5% | 73.3% |
| Count of EAC cells ( $\times 10^6$) | 182.56± 12.13 | 62.22 ± 9.2*** | 38.39 ± 10.66*** |
| % change | -------- | 65.9% | 78.9% |

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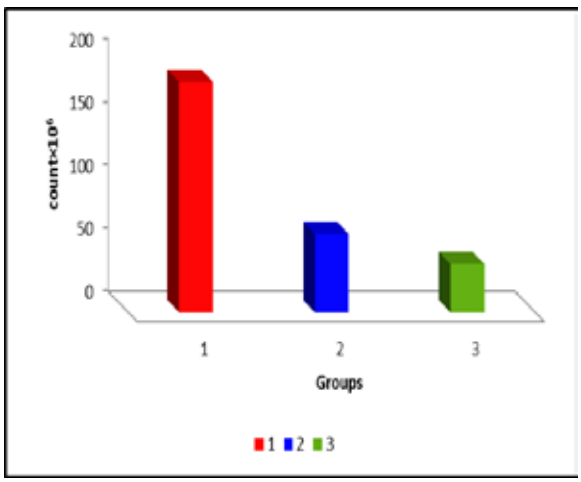
The significant difference: $P^{***} < 0.001 \rightarrow$high significant, $P^{**} < 0.01 \rightarrow$high significant $P^{*} < 0.05 \rightarrow$ significant



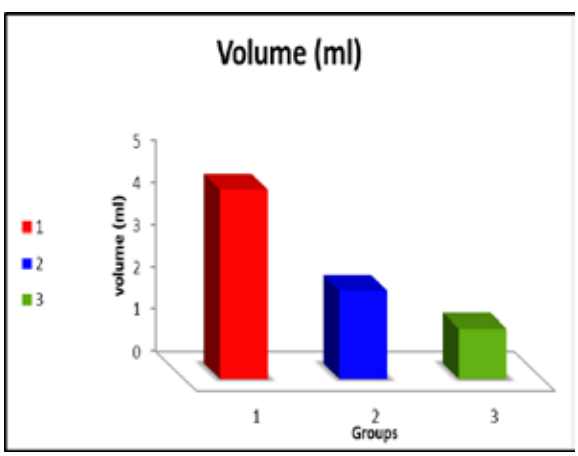




**Figure (6): (a) Effect of compound 3a and 3b on EAC volume and (b) Effect of compound 3a and 3b on EAC count.**

**4. Discussion:**

Cancer is a disease which may affect multicellular organisms and is characterized by the uncontrolled multiplication and is able to invade other tissues [15]. 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 [16].

Some new derivatives bearing coumarin ring including the furanocoumarins (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 [17]. 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 [18].

All these findings encouraged us to explore the synthesis of coumarino [3, 4-b] pyrimidine 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), 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 compared with doxorubicin as reference drug.

Our results agreed with MUSA et al., [19], who studied the cytotoxicity of new acetoxycoumarin derivatives against CRL 1548 liver cancer cell line, and A549 lung cancer.

In Vivo study; doses up to 50 mg /kg and up to 100 mg / kg were be safe in compounds 3a and 3b; respectively. We found that, 5 mg /kg and 7.5 mg /kg were considered to be the most effective dose of compounds 3a & 3b; respectively. In Vivo antitumor activity results against Ehrlich ascites carcinoma cells for compound 3a, 3b revealed that, the mean volume of EAC in the positive control group was found to be 4.5 ±0.5 (ml) as Amer [20] who reported that, the mean volume of EAC was 5.0±0.5 (ml). This value was significantly decreased by 55.6% and by 73.3% ($p<0.01$) in compounds 3a & 3b treated groups; respectively, as shown in Fig (6 a). Also, the mean count of EAC cells in the positive control group was found to be 182.6± 11.5 ($\times 10^6$), which significantly decreased by 65.9% and 78.9%, ($p<0.001$) in compounds 3a & 3b treated groups; respectively, compared to the positive control group, Fig (6 b). 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., [21] 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.

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 [22]. 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[23]. Coumarin (known as 1, 2-benzopyrone), consisting of fused benzene and α-pyrone ring, is an important group of low molecular weight [24].

Our results agreed with Stanway et al., [25] 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. Furo [2, 3 d] pyrimidines have been considered as templates for drug discovery for many years with the inhibition of dihydrofolate reductase (DHFR) as the primary target. More recently, furopyrimidines have been found to be active as kinase inhibitors [26]. 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) [27].

**5- Conclusion:**

The in vitro cytotoxic activity for the compounds 3 a, b

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against the human breast tumor cells (MCF-7), human hepatocellular cancer cells (HePG2), HCT16 (colon cancer), and PC3 (prostate cancer). Compound 3b exhibits minimum inhibitory concentration against all cell lines at higher doses than compound 3a. Also, in vivo effect of the compounds 3a, and 3b exhibited significant anticancer activity towards EAC cells by reduction of their volume and count. On the basis of these results, compound 3b may be considered as attractive leads in the future development of potential anticancer agent more than compound 3a.

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