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# Cytotoxicity and gene expression profiles of novel synthesized steroid derivatives as chemotherapeutic anti-breast cancer agents

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ABSTRACT

Anti-cancer agents which combine two biologically active compounds in one such as steroidal heterocyclic derivatives attain both hormone and cytotoxic effects on cancer cells. The aim of the present study is to synthesize and evaluate new potential chemotherapeutic anti-breast cancer agents. Several pyridazino-, pyrimido-, quinazolo-, oxirano- and thiazolo-steroid derivatives were synthesized. The structure of the novel steroid derivatives was confirmed using the analytical and spectral data. The most structurally promising of the novel synthesized steroid derivatives, compounds **8**, **12**, **17**, **20**, **22c**, **24c**, **30a** and **30b**, were investigated individually as anti-breast cancer agents against human breast cancer cells (MCF-7) using sulforhodamine B (SRB) assay. The tested compounds **17**, **20**, **22c** and **8** showed potent broad spectrum cytotoxic activity in vitro after 48 h incubation. Compound **17** ($IC_{50}$ = 2.5 μM) exhibited more inhibitory influence on MCF-7 growth than the reference drug doxorubicin (Dox) ($IC_{50}$ = 4.5 μM) after 48 h incubation. Also, the present study showed that all the tested steroid derivatives exhibited significant depletion with various intensities in gene expression of breast cancer related genes (VEGF, CYP19 and hAP-2γ). Noteworthy, compounds **17**, **20** and **22c** showed the most pronounced effect in this respect.



## 1. Introduction

Breast cancer is reported to be one of the commonest cancers, accounting for almost 20% of all malignancies world-wide, and over half a million women develop breast cancer every year. Breast cancer is the most common cause of cancer death among women worldwide, and especially young women in the developing world. If current rates of increase remain constant, a woman born today has a 1 in 10 chance of developing breast cancer.[1,2] It is well known that breast tumors are steroid hormone dependent neoplasms and represent important experimental models for investigational hormone-based therapies.

Cancer is a complex disease and a single mono-functional drug will not be effective for treating this most advanced disease. Combined drugs that impact multiple targets simultaneously are better at controlling complex disease systems, less prone to drug resistance and are the standard of care in cancer treatment. In order to improve the efficiency of using a two-drug cocktail, one approach involves the use of the so-called hybrid drugs, which comprises the incorporation of two drugs in a single molecule with the intention of exerting dual drug action.[3] Heterocyclic rings represent molecular framework that serve as a platform for developing pharmaceutical agents for various applications. The antitumor activity of many compounds containing heterocyclic ring have been reviewed.[4,5] The investigation of modified steroid derivatives condensed with various heterocyclic rings has drawn great attention.[6,7] Steroid heterocycles anticancer agents aspire to attain both hormone and cytotoxic effects on cancer cells. A variety of synthetic steroid derivatives have been contrived. Most these derivatives can interact with the steroid receptors because of a similarity of shape.[8,9]

Analysis of gene expression is increasingly important tool in many research fields including early detection of breast cancer. Reverse transcription-polymerase chain reaction (RT–PCR) is a highly

*Abbreviations:* aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; CBMN, cytokinesis-block micronucleus; DMF, dimethylformamide; DMSO, dimethylsulfoxide; Dox, doxorubicin; E2, estrogen; ER, estrogen receptor; ERα, estrogen receptor-alpha; ERβ, estrogen receptor-beta; CYP19, human aromatase; FBS, fetal bovine serum; hAP-2γ, human activation protein-2 gamma; HER-2/neu, human epidermal growth factor receptor 2; JCBN, joint commission on the biochemical nomenclature; RT–PCR, reverse transcription–polymerase chain reaction; RT, reverse transcription; SRB, sulforhodamine B; TLC, thin layer chromatography; TCA, trichloroacetic acid; TGF-α, tumor growth factor-α; TGF-β, tumor growth factor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

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sensitive and specific method useful for the detection of rare transcripts of limited amount of tumor cells.[10]

There is several genes play an important role in breast cancer development. Vascular endothelial growth factor (VEGF) is a key molecule in both tumor angiogenesis and the survival of tumor endothelial cells.[11] VEGF gene is expressed in the majority of tumor types, including breast cancer.[11] Aromatase activity in tumor or surrounding tissue plays a significant role in promoting this type of tumor growth due to the local production of estrogen, and thus aromatase is an important target in the treatment of estrogen receptor (ER) positive breast cancer.[12] Human aromatase gene (CYP19) expression in breast cancer cells is known to play an important role in growing cancer cells.[13] Breast cancer cell lines that have abundant human epidermal growth factor receptor (2HER-2/neu) protein concurrently over-express the human activation protein-2 gamma (hAP-2γ) gene discreetly suggesting that deregulation of these proteins is the preceding event in the cascade that culminate in mammary carcinoma.[14]

In view of the above facts, we set as a goal to prepare some new potential chemotherapeutic anti-breast cancer agents through the synthesis of new steroidal heterocyclic derivatives. The more structurally promising agents were examined for their cytotoxicity in vitro against breast cancer cell line (MCF-7) using SRB [sulforhodamine B] assay. Moreover, the expression of VEGF, CYP19 and hAP-2γ genes in the breast cancer cells was evaluated after treatment with the new agents.

## 2. Results and discussion

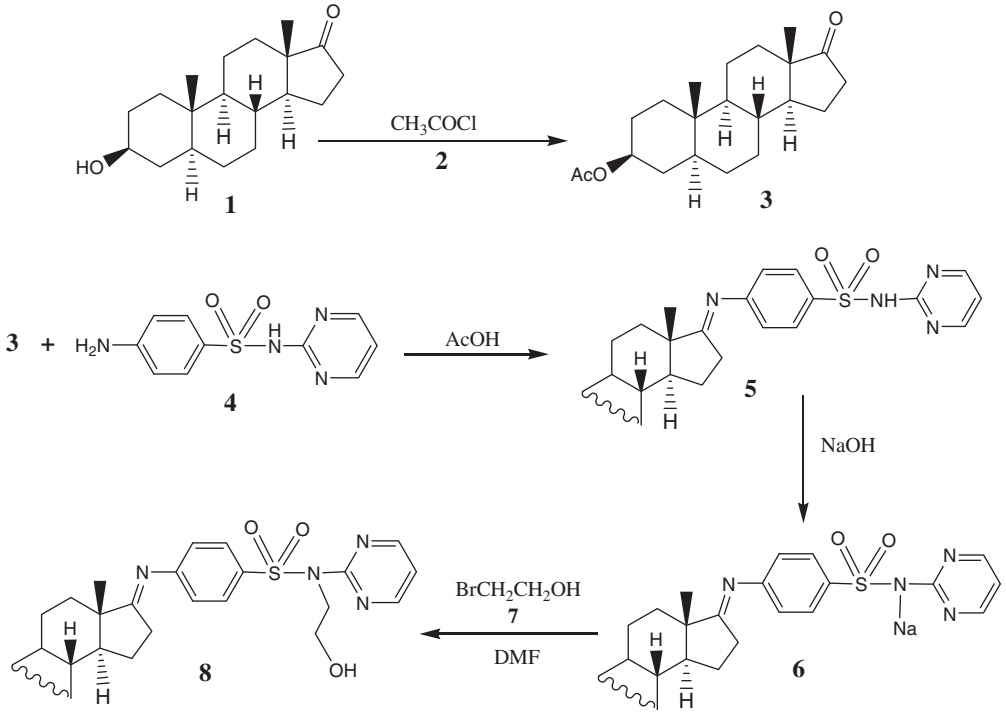
### 2.1. Chemistry

Sulfonamides are an important group of drugs. They exhibit a broad spectrum of antibacterial activities. Recently, some sulfonamide derivatives have been reported to have potential antitumor effects.[15,16] However, there are few literature references available for steroid sulfonamide derivatives. Also, pyrimidine and pyridazine rings represent molecular frameworks that serve as a platform for developing pharmaceutical agents for various applications.[17,18] Simple acetylation of 3β-hydroxy-5α-androstan-17-one (**1**) (*epi*-androsterone) afforded 3β-acetoxy-5α-androstan-17-one (**3**).[19] Compound (**3**) fused with equimolar amount of sulfadiazine **4** in the presence of glacial acetic acid to give the pyrimidinyl benzene sulfonamide derivative **5** in 94% yield (Scheme 1). Treatment of compound **5** with aqueous sodium hydroxide afforded the corresponding isolable sodium salt **6**. The reaction of compound **6** with 2-bromoethanol (**7**) in dimethylformamide (DMF) afforded the pyrimidinyl benzene sulfonamide ethanol derivative **8** in 57% yield (Scheme 1).

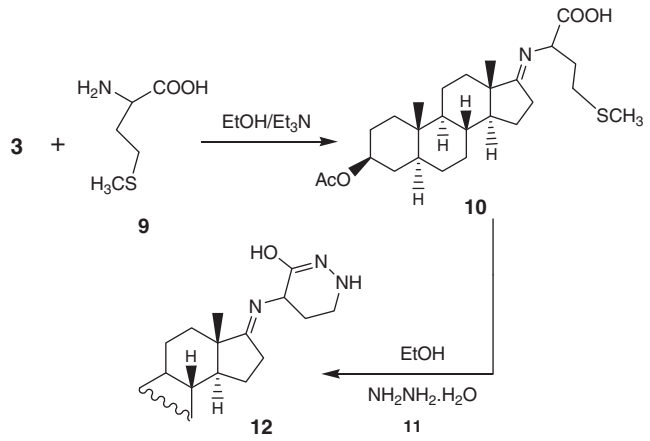
The reaction of compound (**3**) with equimolar amount of L-methionine (**9**) in ethanolic triethylamine solution afforded the corresponding 2-androstanylideneamino-4-methylthiobutanoic acid **10** in 71% yield (Scheme 2). Compound **10** reacted with hydrazine hydrate (**11**) in boiling ethanol solution to afford the corresponding pyridazinyl androstane derivative **12** in 83% yield (Scheme 2).

The azole moiety often shows some special biological activity when it is introduced to some biologically active compounds.[20,21] The basicity and hydrophilicity of an azole in theory might alter the biological function of a steroid.[22] Especially, 1,3-thiazoles are an important class of antitumor agents.[23,24] Bromination of compound (**3**) using cupric bromide (**13**) in dry methanol afforded the corresponding 3β-acetoxy-16-bromo-5α-androstan-17-one (**14**) in 73% yield. Compound (**14**) reacted with equimolar amount of cyanothioacetamide (**15**) in refluxing ethanol containing a catalytic amount of piperidine to give the thiazoloandrostane derivative **17** in 60% yield. Also, compound (**14**) reacted with equimolar amount of thiourea (**16**) in THF containing a catalytic amount of piperidine to give the aminothiazoloandrostane derivative **18** in 50% yield (Scheme 3).

Transition metal complexes containing heterocyclic ligand are commonly used in biological media as anticancer and play important roles in processes such as catalysis of drug interaction with




**Scheme 1.**

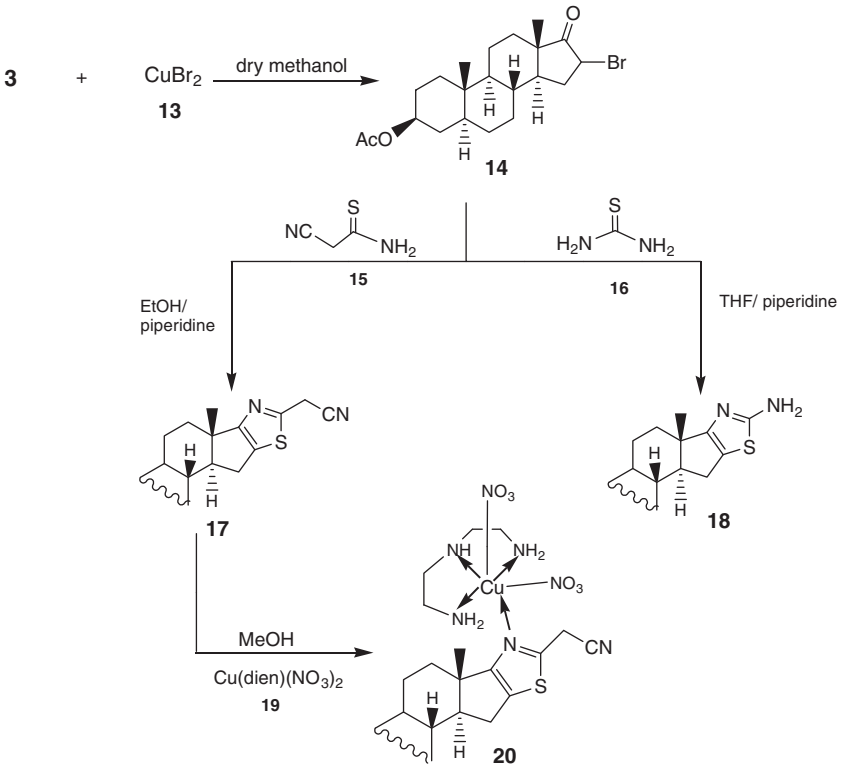



**Scheme 2.**

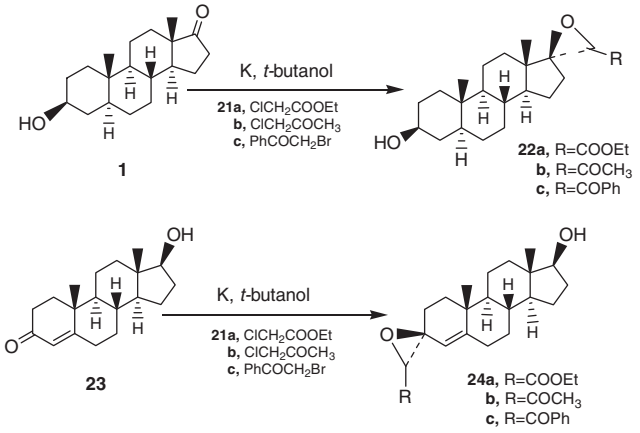
biomolecular activity.[25] Many metallodrugs are pro-drugs and they undergo ligand substitution and/or redox reactions before they reach the target site.[26] The pharmacological activity of Cu(II)-thiazole complexes was found to be much higher than that of the parent heterocycles.[27] The reaction of compound **17** with copper diethylenetriaminedinitrite [Cu(dien)$(NO_3)_2$] **19** in equimolar ratio (1:1) afforded copper complex of 3β-acetoxy thiazolo[16,17:4,5]androstane derivative **20** in 73% yield (Scheme 3). The resulted dark green compound is air stable crystalline powder and was characterized by elemental analysis (cf. materials and methods). The mass spectrum of compound **20** showed a molecular ion peak at $m/z$ = 703 (3%). The IR spectrum showed absorption bands at $\nu$ = 3385–3320 $cm^{-1}$ for the $NH_2$ and NH groups, and showed also absorption band at $\nu$ = 2220 $cm^{-1}$ for the cyano group. The absorption band at 1642 $cm^{-1}$ which is attributed to the (C=N) in the IR spectrum of compound **17** shifted to lower frequency at $\nu$ = 1630 $cm^{-1}$ upon coordination with copper. Also, the IR spectrum showed the characteristic broad band at $\nu$ = 1379 for the nitro complexes.[28] Moreover, the $^1$H NMR spectrum showed the presence of a singlet at $\delta$ = 2.0 (4H, $D_2O$-exchangeable) for the two $NH_2$ groups and showed also $D_2O$-exchangeable singlet (1H) at 8.20 for the NH group.

Introduction of the reactive oxirane ring in steroids results in dramatic changes in their biological activity.[29] Thus, it was of significant interest to trace the influence of replacement the ketone group by spirooxirane moiety. We have attempted a straightforward synthesis of spirooxiranoandrostane derivatives. *epi*-Androsterone (**1**) reacted with equimolar amount of either ethyl chloroacetate (**21a**), chloroacetone (**21b**) or phenacylbromide (**21c**) in potassium *tert*-butoxide to afford the corresponding spirooxiran-2,17β-androstane derivatives **22a**, **22b** and **22c** respectively (Scheme 4). Compounds **22a–c** cyclized at the less hindered C-17α face.[30] Thin layer chromatography examination of the reaction mixture revealed minor products probably due to the presence of other isomers. Isolation and identification of these minor products is beyond the scope of this study. To generalize such a methodology, the previous reactions with the α-haloketones **21a–c** were carried out by utility of testosterone (**23**) under the same experimental conditions, to afford the corresponding spirooxiran-2,3β-androstane derivatives **24a–c**, respectively.

The reaction of testosterone (**23**) with equimolar amount of thionyl chloride (**25**) in anhydrous diethylether gave the corresponding mixture of 17α- and 17β-chloroandrost-4-en-3-one **26**. Isolation and identification of this mixture is beyond the scope of this study. Compound **26** reacted with either anthranilic acid (**27a**) or 5-fluoroanthranilic acid (**27b**) in ethanol to give the corresponding androstenylaminobenzoic acid derivatives **28a** and **28b**, respectively (Scheme 5). Compounds **28a,b** reacted with equimolar amount of phenylisothiocyanate (**29**) in refluxing ethanol




**Scheme 3.**




**Scheme 4.**

containing a catalytic amount of triethylamine to afford the corresponding 17β-thioxoquinazolinandrost-4-en-3-one derivatives **30a,b**, respectively (Scheme 5).

## 2.2. Biological assays

### 2.2.1. In vitro evaluation of cytotoxic activity

In this study, the most structurally promising compounds of the novel synthesized steroid derivatives **8**, **12**, **17**, **20**, **22c**, **24c**, **30a** and **30b** were investigated individually as anti-breast cancer agents against the human breast cancer cells (MCF-7). The inhibition of proliferation of MCF-7 cells was determined using SRB assay in comparison to the chemotherapeutic anti-breast cancer drug, doxorubicin (Dox). The usage of DMSO as a solvent at a volume of 100 μL (the maximum volume used to dissolve the tested compounds) had insignificant effect on the viability of MCF-7 cells when treated for 24, 48 and 72 h.[7]

Dox significantly inhibited the growth of MCF-7 cells at all incubation times as compared to control values (untreated MCF-7 cells). After 24 h incubation, at 5, 12.5, 25 and 50 μg/mL concentrations (Fig. 1A and B), all tested compounds showed significant inhibition of MCF-7 cells growth as compared to the control values. As compared to Dox values, at concentration 12.5 μg/mL, compounds **8**, **17**, **22c**, **24c** and **30a** significantly inhibited the proliferation of

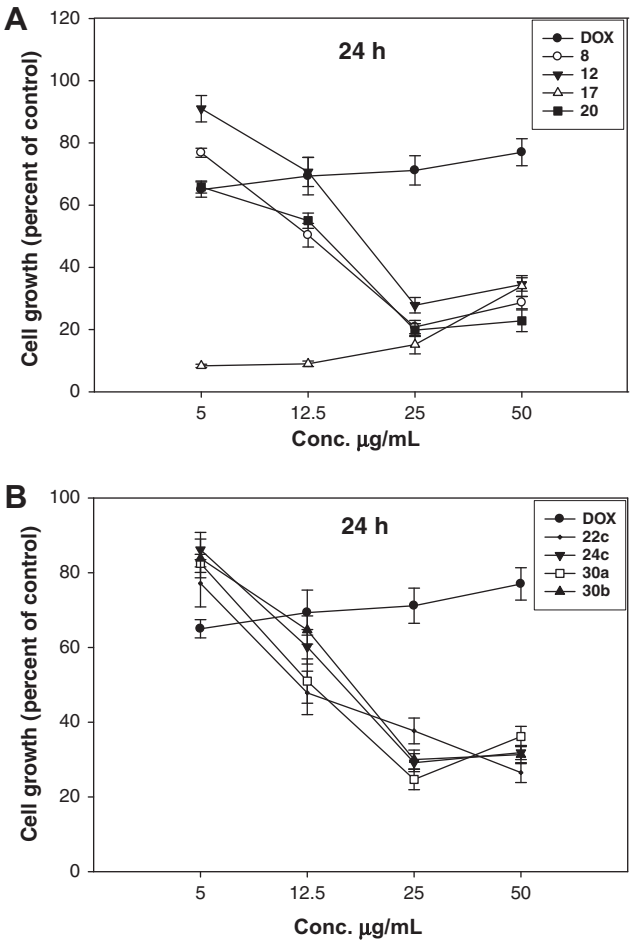
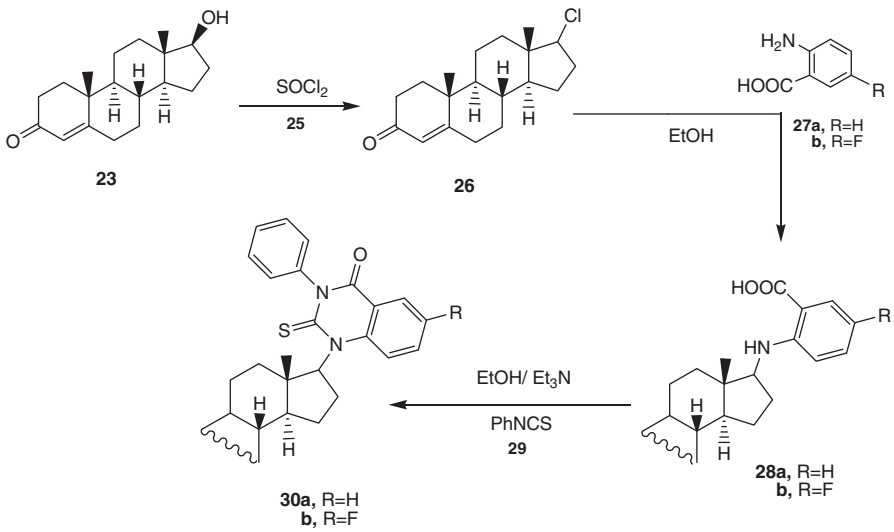



**Figure 1.** (A, B) Effects of tested compounds on MCF-7 cells at 24 h incubation time.

MCF-7 cells. Also, at concentrations 25 and 50 μg/mL, there was significant cell growth inhibition of MCF-7 cells by all tested compounds when compared with Dox values. Compound **17** is the most effective one in this respect with inhibition 91% at concentration 12.5 μg/mL and 85% at concentration 25 μg/mL.




**Scheme 5.**

At 48 h incubation, (Fig. 2A and B) there was significant cell growth inhibition of breast cancer cells by all tested compounds at 5, 12.5, 25 and 50 μg/mL concentrations as compared to the control values. As compared to Dox values, at concentration 5 μg/mL, compound **17** showed significant inhibition of proliferation of MCF-7 cells. At concentration 12.5 μg/mL, compounds **8**, **12**, **17**, **20** and **22c** significantly inhibited the growth of MCF-7 cells. Also, at 25 and 50 μg/mL concentrations, all tested compounds, except compound **24c** at concentration 25 μg/mL and compound **8** at concentration 50 μg/mL, showed significant inhibition of MCF-7 cells growth. The incubation time 48 h is the best incubation time for the cytotoxic effect of the tested compounds. At 72 h incubation, at 5, 12.5, 25 and 50 μg/mL concentrations (Fig. 3A and B), all tested compounds, except compound **20** at concentration of 5 μg/mL showed significant inhibition of MCF-7 cells growth as compared to the control values. As compared to Dox values, compound **17** significantly inhibited the cell proliferation of MCF-7 cells at concentration of 5 μg/mL (93%) and at concentration of 12.5 μg/mL (91%).

Table 1 revealed the $IC_{50}$ of the tested compounds. After 24 h incubation time, Dox inhibition values were less than 50% with the increase of its concentrations. After 48 h incubation time all tested compounds showed high cytotoxic activity against breast cancer cells (MCF-7). Compound **17** ($IC_{50}$ = 2.5 μM) is the more effective as it exhibited more inhibitory effect than Dox ($IC_{50}$ = 4.5 μM) after 48 h incubation time and showed similar effect as Dox after 72 h incubation time ($IC_{50}$ = 2.9 μM). Also, after incubation time 48 h, the effect of compound **20** ($IC_{50}$ = 4.7 μM) was approximately similar to Dox. Compounds **8** and **22c** have nearly similar effect ($IC_{50}$ = 7.5 μM and 7.3 μM respectively) but they exhibited less inhibition effect when compared to Dox. The results revealed that the tested compounds **17** and **20** showed the higher cytotoxic activity against breast cancer cells (MCF-7) and compound **17** is better than Dox after 24 and 48 h incubation times.

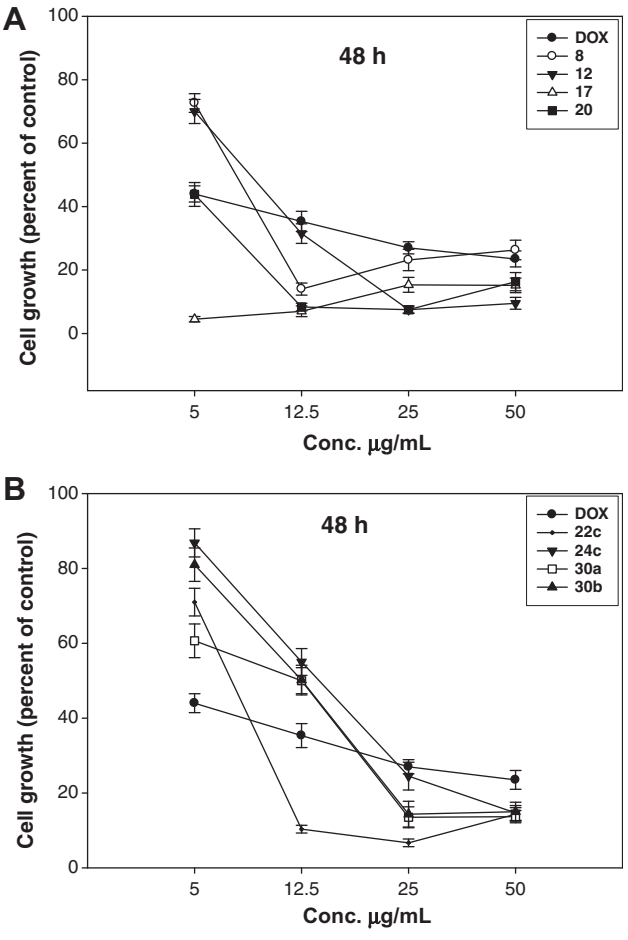



**Figure 2.** (A, B) Effects of tested compounds on MCF-7 cells at 48 h incubation time.

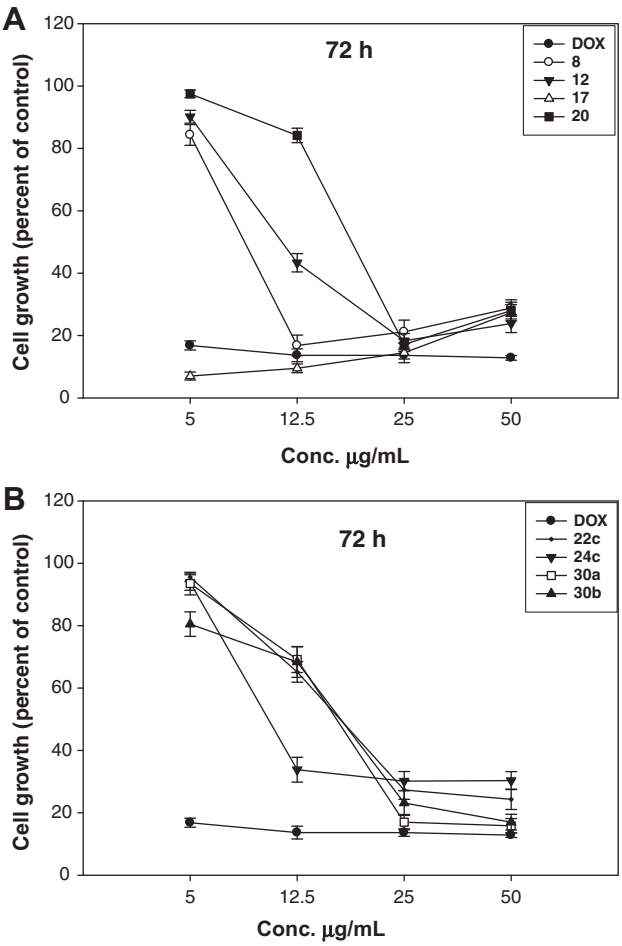



**Figure 3.** (A, B) Effects of tested compounds on MCF-7 cells at 72 h incubation time.

**Table 1**
The in vitro cytotoxic activity of compounds on MCF-7 cells ($IC_{50}$ in μM)

| Compounds | 24 h | 48 h | 72 h |
|---|---|---|---|
| Dox | — | 4.5 | 2.9 |
| **8** | 14.7 | 7.5 | 10 |
| **12** | 18.5 | 9.9 | 11.5 |
| **17** | 2.9 | 2.5 | 2.9 |
| **20** | 14 | 4.7 | 17.3 |
| **22c** | 17.1 | 7.3 | 17.3 |
| **24c** | 15.1 | 16 | 12 |
| **30a** | 13.1 | 12.5 | 17.5 |
| **30b** | 17.7 | 11.5 | 16 |

**2.2.1.1.Structure–activity relationship.** The analysis of the structure–activity relationships indicates firstly that the cytotoxicity of the tested compounds seems to be linked to the presence of heterocyclic moiety fused to the steroid moiety. This is identified clearly in comparison with the published cytotoxic activity of some steroids.[31] At 48 h incubation time, the acetonitrilothiazolyl androstane derivative **17** is the most cytotoxic compound followed by the copper complex of thiazolyl androstane derivative **20**, spirooxiranoandrostane derivative **22c** and pyrimidinyl androstane derivative **8**. The addition of copper complex to the acetonitrilothiazolyl

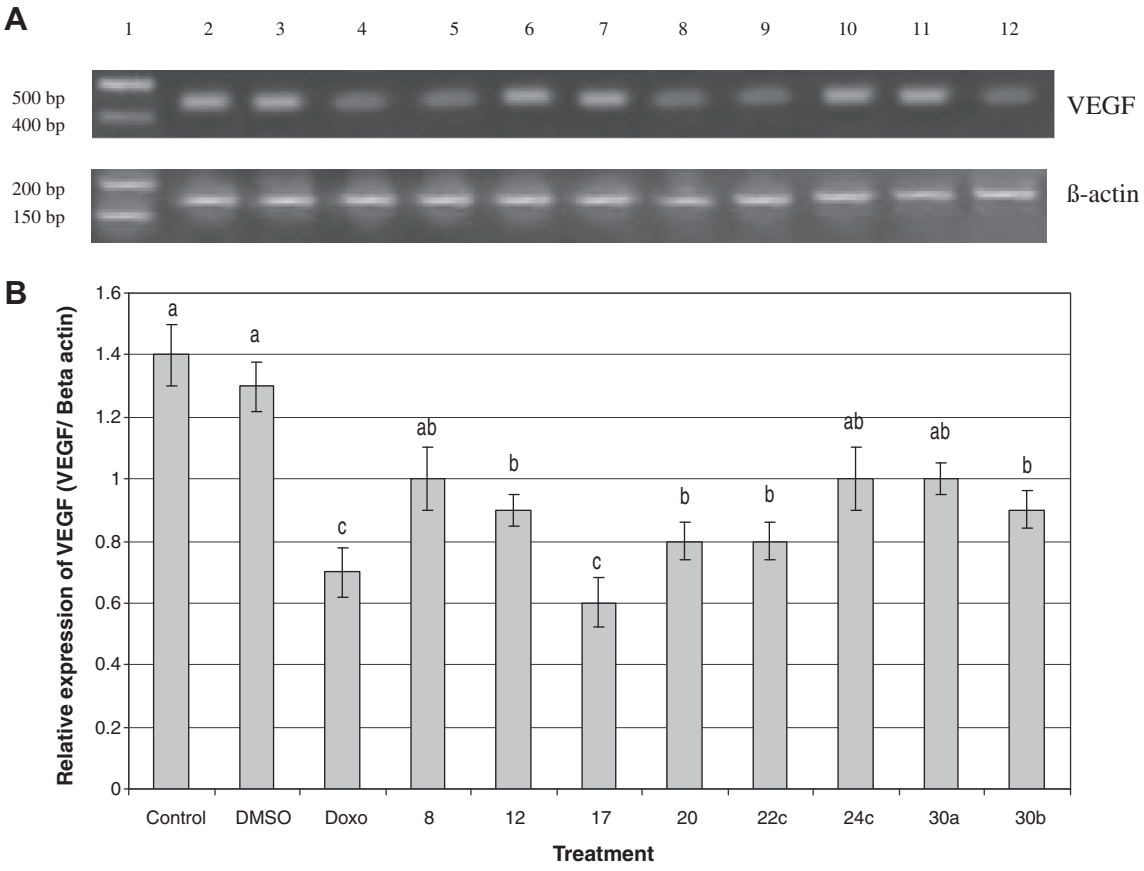



**Figure 4.** (A) RT–PCR confirmation of VEGF and β-actin genes expressed in breast cancer cell line (MCF-7). Lane 1 represents DNA marker, lane 2 represents control, lane 3 represents DMSO, lane 4 represents Dox, lane 5 represents compound **8**, lane 6 represents compound **12**, lane 7 represents compound **17**, lane 8 represents compound **20**, lane 9 represents compound **22c**, lane 10 represents compound **24c**, lane 11 represents compound **30a**, and lane 12 represents compound **30b**. (B) Expression of VEGF gene expressed in breast cancer cell line (MCF-7). The RNA recovery rate was estimated as the ratio between the intensity of VEGF gene and the β-actin gene. [a,b,c]Mean values within columns with unlike superscript letters were significantly different ($P$ <0.05). [a,b,ab,c]Mean values within columns with similar superscript letters were not significantly different ($P$ >0.05).

androstane moiety to form compound **20** decreased the cytotoxic activity of the acetonitrilothiazolyl androstane moiety of compound **17**. The acetonitrilothiazole moiety of compound **17** is more effective than the aminothiazole moiety of compound **18**. The presence of spirooxirane ring attached to C-17 of steroid moiety (compound **22c**) is more effective than its presence at C-3 (compound **24c**). The presence of fluorine atom attached to the quinazolinyl androstene derivative (compound **30b**) made no effective change in the cytotoxicity of compound **30a**. The addition of pyrimidine ring to the steroid moiety (compound **8**) is more effective than the addition of pyridazine ring (compound **12**). The results verified the importance of the presence of thiazole, pyrimidine and oxirane moieties as pharmacophores for the anti-cancer activity.

### 2.2.2. Molecular biology assay

**2.2.2.1. Gene expression patterns.** The cell line samples of 48 h incubation time, the more effective incubation time in our in vitro study, were selected for the gene expression analysis. The effect of steroids derivatives **8**, **12**, **17**, **20**, **22c**, **24c**, **30a** and **30b** on VEGF, CYP19 and hAP-2γ genes were evaluated by calculation of the ratio of its expression level to that of β-actin by semiquantitive analysis and in comparison to Dox. The usage of DMSO as a solvent at a volume of 100 μL had insignificant effect on the expression levels of the selected genes compared with control values (Figs. 4–6).

The present results revealed that Dox decreased significantly the expression levels of the selected genes which related to breast cancer. The expression levels of VEGF, CYP19 and hAP-2γ genes were down-regulated in breast cancer cell lines (MCF-7), treated with Dox compared with control values (Figs. 4–6). It is known that Dox in addition to its cytostatic effect has important adverse effects such as genotoxicity.[32] Duffaud et al.[33] reported that Dox was a genotoxic drug and gave positive results with the cytokinesis-block micronucleus (CBMN) test and chromosome aberrations test.

On the other hand, the results of the present study showed that all the tested synthetic steroid derivatives showed significant decrease in gene expression of VEGF, CYP19 and hAP-2γ genes compared to control values. Compounds **17**, **20** and **22c** were more effective as anticancer agents than the other steroid derivatives. These compounds were significantly ($P$ <0.001) decreased the level expression of all selected genes compared with control values, the level of expression was relatively similar to that of Dox. Moreover, the other steroid derivatives **8**, **12**, **24c**, **30a** and **30b** were also able to decrease the expression level of all selected genes in comparison to control values. However, the ability of these steroid derivatives in decreasing the level of expression of tested genes was less than that of compounds **17**, **20** and **22c** as well as Dox (Figs. 4–6). The process of tumors growth is affected by several growth factors such as aFGF (acidic fibroblast growth factor), bFGF (basic fibroblast growth factor), TGF-α (tumor growth factor-α), TGF-β (tumor growth factor-β), TNF-α (tumor necrosis factor-α), VEGF (vascular endothelial growth factor), and their receptors act as stimulators for various steps involved in this complex process. Among the various factors mentioned above, VEGF is considered to be the prime

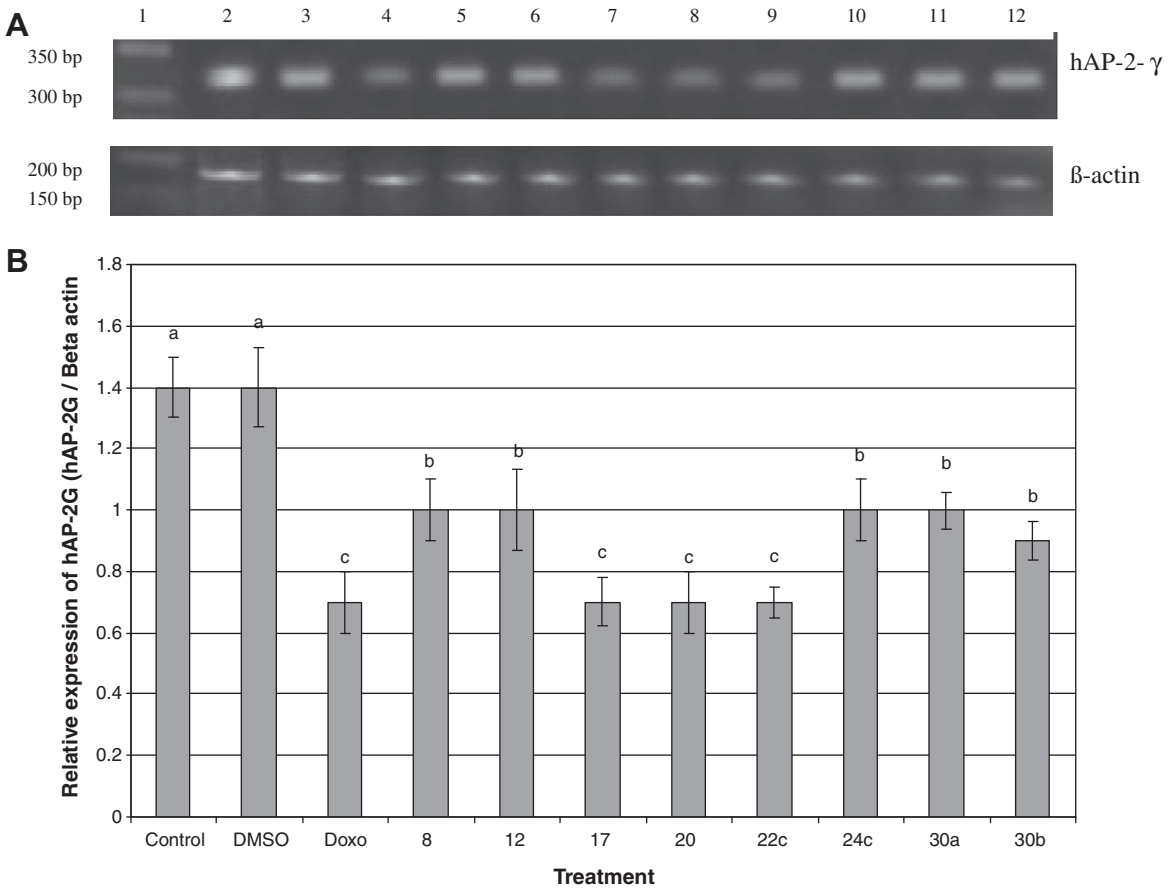



**Figure 5.** (A) RT–PCR confirmation of hAP-2-γ and β-actin genes expressed in breast cancer cell line (MCF-7). Lane 1 represents DNA marker, lane 2 represents control, lane 3 represents DMSO, lane 4 represents Dox, lane 5 represents compound **8**, lane 6 represents compound **12**, lane 7 represents compound **17**, lane 8 represents compound **20**, lane 9 represents compound **22c**, lane 10 represents compound **24c**, lane 11 represents compound **30a**, and lane 12 represents compound **30b**. (B) Expression of hAP-2-γ gene expressed in breast cancer cell line (MCF-7). The RNA recovery rate was estimated as the ratio between the intensity of hAP-2-γ gene and the β-actin gene. [a,b,c]Mean values within columns with unlike superscript letters were significantly different ($P$ <0.05). [a,b,c]Mean values within columns with similar superscript letters were not significantly different ($P$ >0.05).

regulator of angiogenesis, vasculogenesis and vascular permeability in vivo.[34]

VEGF is an endothelial cell-specific mitogen (MAP kinase), which induces angiogenesis and vascular permeability in vivo. Angiogenesis is a complex, multi-step process that results in the formation of new blood vessels from preexisting vasculature.[34] During tumor angiogenesis endothelial cells degrade the basement membrane at the postcapillary venule by releasing enzymes, migrate through the membrane to form a sprout, and finally proliferate to extend the blood vessel into the tumor. Therefore, we expect that, the role of the current synthetic steroid derivatives on prevention of tumor progression as a result of down regulation of VEGF gene may be attributed to inhibition of the releasing enzymes which are essential to extend the blood vessel into the tumor. In the present study the expression of CYP19 gene was significantly decreased due to treatment with synthetic steroid derivatives **17**, **20** and **22c**, and Dox compared to control. Kirma et al.[35] found that the over expression of CYP19 gene in the mammary gland is promoted with secretion of estrogens (E2). E2 up-regulates aromatase expression by a nongenomic action of estrogen receptors (ERα) via cross-talk with MAP kinase growth factor-mediated pathways. Where, secretion of estrogen causes increase in aromatase expression through the phosphorylation of MAP protein.[36] This would suggest that a complex mechanism is involved in regulating aromatase expression in breast cancer tissue. As mentioned above, VEGF is an endothelial cell-specific mitogen (MAP kinase), which induces angiogenesis and vascular permeability in vivo. Therefore, VEGF could play a role with the estrogen to enhance the tumor progression. Consequently, inhibition of the VEGF gene due to synthetic steroid derivatives treatment may suppress the CYP19 gene activity.

Regarding hAP-2γ gene, the present results found that expression of this gene was significantly decreased when treated with the synthetic steroid derivatives especially **17**, **20** and **22c**, and Dox compared to control. Breast cancer cell line (MCF-7) has abundant HER-2/*neu* protein concurrently over-expressed hAP-2γ gene.[37] The regulation of hAP-2γ expression was linked to HER-2/neu over-expression-mediated mammary carcinoma cell line (MCF-7).[14] Over-expression of estrogen receptor (ERβ) gene leads to the elevation of HER2 expression in MCF-7 breast cancer cells. Based on this information, VEGF gene may also interact with CYP19 and hAP-2γ genes under modulation of estrogen and/or estrogen receptors resulting in regulation of tumor cells.

### 2.3. Conclusion

The present study described a facile synthesis of novel promising anticancer steroid derivatives and investigated also the importance of incorporating heterocyclic moiety to the steroid nucleus to form new effective anticancer agents. The tested compounds namely **17**, **20**, **22c** and **8** dissolved in DMSO showed promising cytotoxic activity in vitro after 48 h incubation. Compound **17**

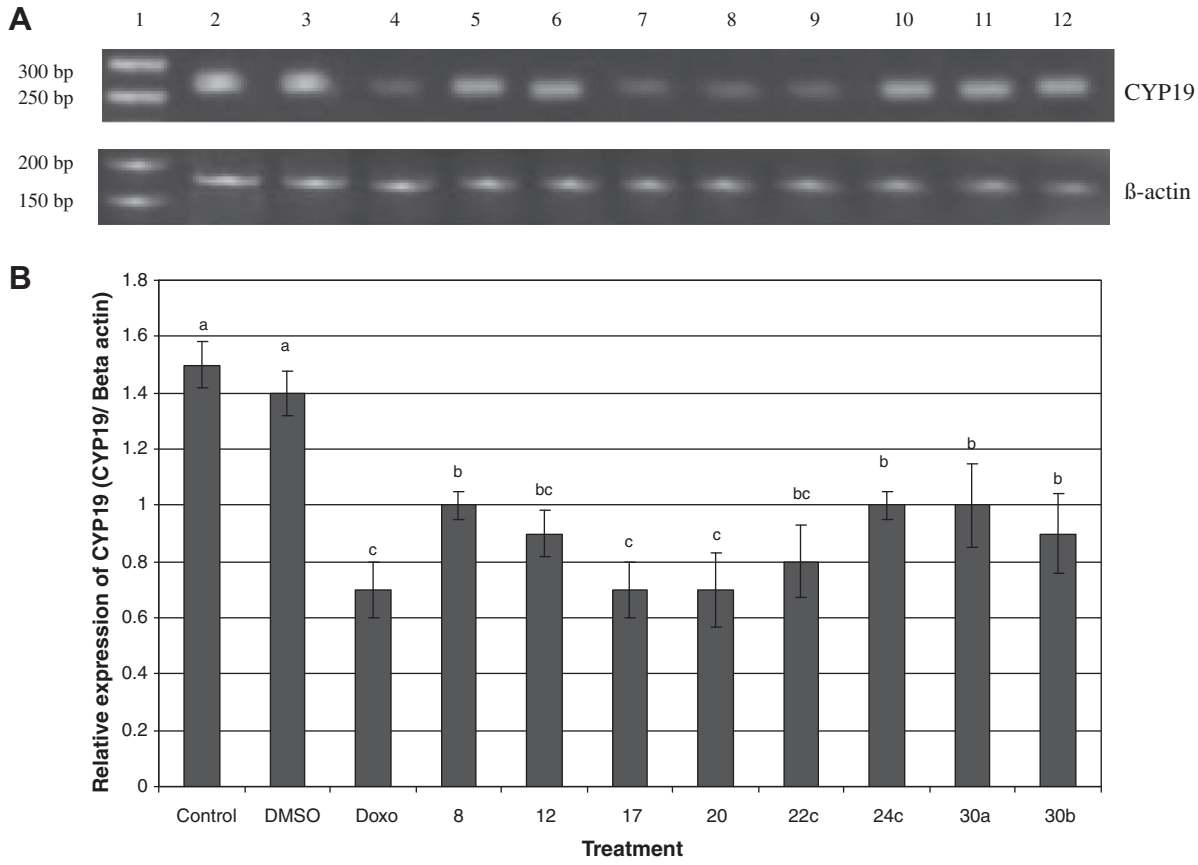



**Figure 6.** (A) RT–PCR confirmation of CYP19 and β-actin genes expressed in breast cancer cell line (MCF-7). Lane 1 represents DNA marker, lane 2 represents control, lane 3 represents DMSO, lane 4 represents Dox, lane 5 represents compound **8**, lane 6 represents compound **12**, lane 7 represents compound **17**, lane 8 represents compound **20**, lane 9 represents compound **22c**, lane 10 represents compound **24c**, lane 11 represents compound **30a**, and lane 12 represents compound **30b**. (B) Expression of CYP19 gene expressed in breast cancer cell line (MCF-7). The RNA recovery rate was estimated as the ratio between the intensity of CYP19 gene and the β-actin gene. [a,b,c]Mean values within columns with unlike superscript letters were significantly different ($P$ <0.05). [a,b,bc,c]Mean values within columns with similar superscript letters were not significantly different ($P$ >0.05).

($IC_{50}$ = 2.5 μM) exhibited more inhibition effect of MCF-7 growth than Dox ($IC_{50}$ = 4.5 μM) after 48 h incubation time. Also, our results confirmed that, 48 h incubation time is the best incubation time for the cytotoxic effect of the tested compounds. The present study showed that all the tested synthetic steroid derivatives showed significant decrease with various intensities in gene expression of breast cancer related genes (VEGF, CYP19 and hAP-2γ). Compounds **17**, **20** and **22c** were more effective in this respect. Finally, the antitumor activity displayed by these compounds may be of interest for further studies of the mechanism of action and toxicity profile of the promising tested compounds before application in phase 1 of clinical study in the hope of finding more active and selective anticancer agents.

## 3. Materials and methods

### 3.1. Chemistry

Starting steroids, *epi*-androsterone and testosterone, were purchased from Sigma Company, USA. All solvents were anhydrated by distillation prior to using. All melting points were measured using an Electrothermal apparatus and are uncorrected. The IR spectra were recorded in (KBr discs) on a shimadzu FT-IR 8201 PC spectrometer and expressed in $cm^{-1}$. The $^{1}H$ NMR and $^{13}C$ NMR spectra were recorded with Jeol instrument (Japan), at 270 and 125 MHz respectively, in DMSO-$d_6$ as solvent and chemical shifts were recorded in ppm relative to TMS. The spin multiplicities were abbreviated by the letters: s-singlet, d-doublet, t-triplet, q-quartet and m (multiplet, more than quartet). Mass spectra were recorded on a GCMS-QP 1000 ex spectra mass spectrometer operating at 70 ev. Elemental analyses were carried by the Microanalytical Data Unit at the National Research Centre, Giza, Egypt and the Microanalytical Data Unit at Cairo University, Giza, Egypt. The reactions were monitored by thin layer chromatography (TLC) which was carried out using Merck 60 F254 aluminum sheets and visualized by UV light (254 nm). The mixtures were separated by preparative TLC and gravity chromatography. All described compounds showed the characteristic spectral data of cyclopentanoperhydrophenanthrene nuclei of androstane series were similar to those reported in literature.[38,39] For the nomenclature of steroid derivatives, we used the definitive rules for the nomenclature of steroids published by the Joint Commission on the Biochemical Nomenclature (JCBN) of IUPAC.[40,41] Compound **3**, 3β-acetoxy-5α-androstan-17-one was prepared by simple acetylation of *epi*-androsterone **1**.[19]

#### 3.1.1. Synthesis of 3β-acetoxy-17-ylideneamino(*N*-2′-pyrimidylbenzenesulfonamide)-5α-androstane (5)

To a suspension of 3β-acetoxy-5α-androstan-17-one (**3**) (0.33 g, 1 mmol) in glacial acetic acid (1.5 mL), equimolar amount of sulfadiazine **4** (0.25 g, 1 mmol) was added. The reaction mixture was heated in an oil bath at 120 °C for 10 min and then left to cool at room temperature. The fused result triturated with absolute ethanol (5 mL) and the reaction mixture was heated again under reflux for

20 min. The solid product obtained upon cooling was collected by filtration and crystallized from absolute ethanol to afford dark yellow crystals of compound **5**, yield 0.53 g (94%), mp 220–222 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3354 (NH), 3037 (CH-aromatic), 2930, 2859 (CH-aliphatic), 1734 (C=O, acetate), 1651 (C=N), 1583 (C=C), 1241 ($SO_2N$). $^1H$ NMR (DMSO-$d_6$, ppm): $\delta$ = 0.71 (s, 3H, $CH_3$-19), 1.12 (s, 3H, $CH_3$-18), 1.53 (m, 1H, $C_5$-αH), 2.06 (s, 3H, $COCH_3$), 3.95 (m, 1H, $C_3$-αH), 6.50 (t, 1H, $J$ = 4.8 Hz, pyrimidine), 6.91 (d, 2H, $J$ = 7.2 Hz, aromatic-H), 7.53 (d, 2H, $J$ = 7.2 Hz, aromatic-H), 8.40 (d, 2H, $J$ = 4.8 Hz, pyrimidine), 11.22 (s, 1H, $SO_2NH$). $^{13}C$ NMR (DMSO-$d_6$, ppm): $\delta$ = 31.29 (C-1), 30.32, (C-2), 72.85 (C-3), 34.43 (C-4), 38.50 (C-5), 27.11 (C-6), 27.86 (C-7), 35.31(C-8), 50.56 (C-9), 44.00 (C-10), 21.34 (C-11), 34.3 (C-12), 38.80 (C-13), 63.61 (C-14), 35.18 (C-15), 33.64 (C-16), 184.3 (C-17), 19.3 (C-18), 20.02 (C-19), 170.37 (C=O), 21.10 (*C*$H_3$-acetate), 112.67, 157.81, 158.74, 169.31 (C-pyrimidine), 125.19, 129.76, 130.31, 153.51 (C-phenyl). M.S (EI): $m/z$ (%): 564 ($M^{+\cdot}$, 33), 546 ($C_{31}H_{38}N_4O_3S$, 41), 504 ($M^+$–$CH_3COOH$, 40), 470 ($C_{27}H_{36}NO_4S$, 35), 234 ($C_{10}H_8N_3O_2S$, 30), 109 ($C_8H_3$, 50), 67 ($C_3H_3N_2$, 75), 55 ($C_2H_3N_2$, 100). Calcd for $C_{31}H_{40}N_4O_4S$ (564.739): C, 65.90; H, 7.14; N, 9.92; S, 5.68. Found: C, 66.10; H, 7.34; N, 10.11; S, 5.93.

### 3.1.2. Synthesis of 3β-acetoxy-17-ylideneamino(*N*-2′-pyrimidyl benzenesulfonamidesodium salt)-5α-androstane (6) and 3β-acetoxy-17-ylideneamino[2′-(*N*-2″-pyrimidylbenzenesulfonamide) ethanol]-5α-androstane (8)

**3.1.2.1. General procedure.** To an aqueous solution of sodium hydroxide (0.00354 g in 3.54 mL $H_2O$), compound **5** (0.5 g, 0.88 mmol) was added, the solution was stirred and gently warmed until all of compound **5** was dissolved and the pH of solution dropped to about 8. The resulted solid product was collected by filtration, dried and crystallized from ethanol (95%) to afford compound **6** as yellow crystals, yield 0.45 g (86%). To a solution of compound **6** (0.4 g, 0.6 mmol) in DMF (20 mL), 2-bromo ethanol **7** (0.085 g, 0.6 mmol) was added. The reaction mixture was heated to 80 °C with stirring and maintained at this temperature for 8 h with continued stirring. The reaction was monitored by TLC, concentrated under vacuum and poured over 50 mL $H_2O$. The formed solid product was filtered off, dried and crystallized from absolute ethanol. *Compound* ***8****:* Yellow crystals, yield 0.23 g (57%), mp 205–206 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3425 (OH), 3033 (CH-aromatic), 2936, 2861 (CH-aliphatic), 1733 (C=O, acetate), 1651 (C=N), 1585 (C=C), 1244 ($SO_2N$). $^1H$ NMR (DMSO-$d_6$, ppm): $\delta$ = 0.76 (s, 3H, $CH_3$-19), 1.17 (s, 3H, $CH_3$-18), 1.47 (m, 1H, $C_5$-αH), 2.01 (s, 3H, $COCH_3$), 3.86 (m, 1H, $C_3$-αH), 3.35, 3.98 (2t, 4H, $J$ = 4.5 Hz, $2CH_2$-ethanol), 4.55 (t, 1H, $J$ = 4.5 Hz, OH, $D_2O$-exchangeable), 6.56 (dd, 1H, $J$ = 2.4 Hz, $J$ = 4.2 Hz, pyrimidine), 7.58 (d, 2H, $J$ = 9.0 Hz, aromatic-H), 7.97 (d, 2H, $J$ = 9 Hz, aromatic-H), 8.15 (dd, 1H, $J$ = 2.4 Hz, $J$ = 4.2 Hz, pyrimidine), 8.45 (q, 1H, $J$ = 2.4 Hz, pyrimidine). $^{13}C$ NMR (DMSO-$d_6$, ppm): $\delta$ = 31.83 (C-1), 29.01, (C-2), 73.43 (C-3), 34.43 (C-4), 38.57 (C-5), 27.11 (C-6), 27.86 (C-7), 35.83 (C-8), 51.1 (C-9), 44.53 (C-10), 21.87 (C-11), 34.3 (C-12), 38.8 (C-13), 63.61 (C-14), 35.18 (C-15), 33.64 (C-16), 184.3 (C-17), 19.3 (C-18), 20.02 (C-19), 170.37 (C=O), 21.60 (*C*$H_3$), 51.12, 57.62 (*C*$H_2CH_2OH$), 112.67, 157.81, 158.74, 169.31 (C-pyrimidine), 125.19, 129.76, 130.31, 153.51 (C-phenyl). M.S (EI): $m/z$ (%): 608 ($M^{+\cdot}$, 35), 563 ($M^{+\cdot}$–$CH_2CH_2OH$, 16), 548 ($M^{+\cdot}$–$CH_3COOH$, 14), 109 ($C_8H_3$, 30), 55 ($C_2H_3N_2$, 100). Calcd for $C_{33}H_{44}N_4O_5S$ (608.791): C, 65.10; H, 7.28; N, 9.20; S, 5.27. Found: C, 64.87; H, 7.12; N, 9.01; S, 5.03.

### 3.1.3. Synthesis of 2-(3β-acetoxy-5α-androstan-17-ylidenea mino)-4-(methylthio)butanoic acid (10)

Equimolar amounts of 3β-acetoxy-5α-androstan-17-one (**3**) (0.20 g, 0.6 mmol) and L-methionine (**9**) (0.09 g, 0.6 mmol) in absolute ethanol (50 mL) containing a catalytic amount of triethylamine (2 mL) were heated under reflux for 10 h until all the starting materials had disappeared as indicated by TLC. The reaction mixture was allowed to stand overnight at room temperature. The solid product that formed was collected by filtration and crystallized from absolute ethanol to afford yellow crystals of compound **10**, yield 0.20 g (71%), mp 105–107 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3424 (br, OH, carboxylate), 2965, 2830 (CH-aliphatic), 1738, 1728 (2C=O), 1640 (C=N). $^1H$ NMR (DMSO-$d_6$, ppm): $\delta$ = 0.76 (s, 3H, $CH_3$-19), 1.18 (s, 3H, $CH_3$-18), 1.58 (m, 1H, $C_5$-αH), 1.90 (q, 2H, $J$ = 4.3 Hz, $CH_2CH_2S$), 2.01 (s, 3H, $COCH_3$), 2.07 (s, 3H, $SCH_3$), 2.37 (t, 1H, $J$ = 4.9 Hz, *CH*COOH), 2.49 (t, 2H, $J$ = 4.8 Hz, $CH_2CH_2S$), 3.93 (m, 1H, $C_3$-αH), 8.21 (s, 1H, COOH). $^{13}C$ NMR (DMSO-$d_6$, ppm): $\delta$ = 34.52 (C-1), 30.99, (C-2), 72.85 (C-3), 35.21 (C-4), 38.57 (C-5), 27.12 (C-6), 28.00 (C-7), 38.57 (C-8), 53.44 (C-9), 44.42 (C-10), 22.99 (C-11), 33.70 (C-12), 39.80 (C-13), 69.28 (C-14), 35.28 (C-15), 34.50 (C-16), 184.80 (C-17), 17.23 (C-18), 20.43 (C-19), 177.40 (C=O), 21.10 (*C*$H_3$), 35.52 (C-1′), 32.99, (C-2′), 28.85 (C-3′), 17.14 ($SCH_3$). M.S (EI): $m/z$ (%): 463 ($M^{+\cdot}$, 28), 418 ($M^{+\cdot}$–COOH, 50), 416 ($M^{+\cdot}$-SMe, 40), 301 ($C_{20}H_{29}O_2$, 25), 61 ($C_2H_5S$, 100). Calcd for $C_{26}H_{41}NO_4S$ (463.673): C, 67.35; H, 8.91; N, 3.02; S, 6.92. Found: C, 67.55; H, 9.15; N, 3.25; S, 7.11.

### 3.1.4. Synthesis of 3β-acetoxy-17-ylideneamino(1′,4′,5′,6′-tetrahydro-3′-hydroxypyridazin-4′-yl)-5α-androstane (12)

To a solution of compound **10** (0.2 g, 0.4 mmol) in absolute ethanol (35 mL), hydrazine hydrate (**11**) (0.1 g, 2.1 mmol) was added. The reaction mixture was heated under reflux for 15 h until all the reactants had disappeared as indicated by TLC. The reaction mixture poured with stirring over crushed ice. The solid product that formed was collected by filtration and crystallized from absolute ethanol to afford compound **12** as pale yellow crystals, yield 0.15 g (83%), mp 266–267 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3390 (OH), 3365 (NH), 2937, 2856 (CH-aliphatic), 1732 (C=O, acetate), 1648 (C=N). $^1H$ NMR (DMSO-$d_6$, ppm): $\delta$ = 0.75 (s, 3H, $CH_3$-19), 1.07 (s, 3H, $CH_3$-18), 1.42 (m, 1H, $C_5$-αH), 1.54 (t, 1H, $J$ = 6.4 Hz, C-4′ pyridazine), 2.03 (s, 3H, $COCH_3$), 2.16 (t, 2H, $J$ = 6.4 Hz, C-6′ pyridazine), 2.20 (q, 2H, $J$ = 6.4 Hz, C-5′ pyridazine), 3.96 (m, 1H, $C_3$-αH), 4.55 (s, 1H, OH, $D_2O$-exchangeable), 8.0 (s, 1H, 1NH, $D_2O$-exchangeable). $^{13}C$ NMR (DMSO-$d_6$, ppm): $\delta$ = 34.52 (C-1), 30.99, (C-2), 72.85 (C-3), 35.21 (C-4), 38.57 (C-5), 27.12 (C-6), 28.00 (C-7), 38.57 (C-8), 53.44 (C-9), 44.42 (C-10), 22.99 (C-11), 33.70 (C-12), 39.80 (C-13), 69.28 (C-14), 35.28 (C-15), 34.50 (C-16), 184.80 (C-17), 17.23 (C-18), 20.43 (C-19), 161.40 (C=O), 21.10 (*C*$H_3$), 174.0, 60.30, 24.23, 48.50 (C-pyridazine). M.S (EI): $m/z$ (%): 429 ($M^{+\cdot}$, 12), 369 ($M^{+\cdot}$–$CH_3COOH$, 14), 234 ($C_{13}H_{20}N_3O$, 25), 111 ($C_4H_5N_3O$, 12), 99 ($C_4H_7N_2O$, 20), 55 ($C_3H_3O$, 100). Calcd for $C_{25}H_{39}N_3O_3$ (429.595): C, 69.90; H, 9.15; N, 9.78. Found: C, 70.14; H, 9.42; N, 9.98.

### 3.1.5. Synthesis of 3β-acetoxy-16-bromo-5α-androstan-17-one (14)

A mixture of compound (**3**) (0.33 g, 1 mmol) and cupric bromide (**13**) (0.71 g, 3 mmol) in dry methanol (40 mL) was refluxed for 24 h. The formed light blue solution was left to cool at room temperature and then poured over ice/water mixture. The formed solid product was filtered off, dried and crystallized from methanol to form compound **14** as pale green crystals. Yield 0.30 g (73%), mp 110–112 °C, IR (KBr, $cm^{-1}$): $\nu$ = 2936, 2860 (CH-aliphatic), 1734,1728 (2C=O). $^1H$ NMR (DMSO-$d_6$, ppm): $\delta$ = 0.77 (s, 3H, $CH_3$-19), 1.10 (s, 3H, $CH_3$-18), 1.46 (m, 1H, $C_5$-αH), 2.45 (s, 3H, $COCH_3$), 3.95 (m, 1H, $C_3$-αH), 4.46 (t, 1H, $J$ = 7.2 Hz, CH-16). M.S (EI): $m/z$ (%): 331 ($M^{+\cdot}$-HBr, 30), 290 ($C_{19}H_{30}O_2$, 26), 59 ($C_2H_3O_2$, 100). Calcd for $C_{21}H_{31}BrO_3$ (411.373): C, 61.31; H, 7.60. Found: C, 61.52; H, 7.78.

### 3.1.6. Synthesis of 3β-acetoxy-1′,3′-thiazolo[4′,5′:17,16]-5α-androstan-2′-acetonitrile (17)

To a solution of compound **14** (0.15 g, 0.3 mmol), in absolute ethanol (20 mL) containing a catalytic amount of piperidine (0.5 mL), cyanothioacetamide (**15**) (0.036 g, 0.3 mmol) was added. The reaction mixture was heated under reflux for 5 h until all the reactants had disappeared as indicated by TLC. The reaction mixture was cooled, poured over ice/ water mixture, neutralized with dilute hydrochloric acid and then left in a refrigerator at 4 °C for 24 h. The solid product that formed, was filtered off, dried and crystallized of absolute ethanol to afford brown crystals of compound **17**, yield 0.09 g (60%), mp 170–171 °C, IR (KBr, $cm^{-1}$): $\nu$ = 2926, 2854 (CH-aliphatic), 2211 (CN), 1733 (C=O, acetate), 1642 (C=N), 1593 (C=C). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.87 (s, 3H, $CH_3$-19), 1.16 (s, 3H, $CH_3$-18), 1.57 (m, 1H, $C_5$-αH), 2.43 (s, 3H, $COCH_3$), 3.49 (s, 2H, $CH_2CN$), 3.87 (m, 1H, $C_3$-αH). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 32.70 (C-1), 29.01, (C-2), 73.60 (C-3), 34.60 (C-4), 37.70 (C-5), 27.00 (C-6), 27.60 (C-7), 38.57 (C-8), 55.0 (C-9), 44.42 (C-10), 22.99 (C-11), 33.70 (C-12), 39.80 (C-13), 69.28 (C-14), 35.28 (C-15), 130.60 (C-16), 150.50 (C-17), 17.23 (C-18), 20.43 (C-19), 170.37 (C=O), 21.60 ($CH_3$), 165.20 (C-2′), 20.70 (*C*$H_2$CN), 117.80 (CN). M.S (EI): *m*/*z* (%): 411 ($M^{+\cdot}$-1, 8), 382 ($M^{+\cdot}$-2$CH_3$, 3), 347 ($C_{21}H_{30}O_2S$, 5), 273 ($C_{18}H_{25}O_2$, 85), 272 ($C_{19}H_{28}O$, 100), 55 ($C_3H_3O$, 68). Calcd for $C_{24}H_{32}N_2O_2S$ (412.588): C, 69.87; H, 7.82; N, 6.79; S, 7.77. Found: C, 69.60; H, 7.61; N, 6.59; S, 7.58.

### 3.1.7. Synthesis of 3β-acetoxy-2′-amino-1′,3′-thiazolo [4′,5′:17,16]-5α-androstane (18)

To a solution of compound **14** (0.15 g, 0.3 mmol) in anhydrous tetrahydrofuran (20 mL) containing a catalytic amount of piperidine (1.5 mL), equimolar amount of thiourea (**16**) (0.027 g, 0.3 mmol) was added with stirring. After complete addition, the reaction mixture was stirred for 72 h at room temperature and monitored by TLC. The reaction mixture poured over crushed ice and then left in a refrigerator at 4 °C overnight. The solid product was collected by filtration and crystallized from absolute ethanol. *Compound **18***: Pale yellow crystals, yield 0.07 g (50%), mp 120–122 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3420 ($NH_2$), 2925, 2853 (CH-aliphatic), 1735 (C=O, acetate), 1643 (C=N), 1590 (C=C). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.70 (s, 3H, $CH_3$-19), 1.16 (s, 3H, $CH_3$-18), 1.52 (m, 1H, $C_5$-αH), 2.43 (s, 3H, $COCH_3$), 3.93 (m, 1H, $C_3$-αH), 4.38 (s, 2H, $NH_2$, $D_2O$-exchangeable). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 32.70 (C-1), 29.01, (C-2), 73.60 (C-3), 34.60 (C-4), 37.70 (C-5), 27.00 (C-6), 27.60 (C-7), 38.57 (C-8), 55.0 (C-9), 44.42 (C-10), 22.99 (C-11), 33.70 (C-12), 39.80 (C-13), 69.28 (C-14), 35.28 (C-15), 130.60 (C-16), 150.50 (C-17), 17.23 (C-18), 20.43 (C-19), 170.37 (C=O), 21.60 ($CH_3$), 165.20 (C-2′), 20.70 (*C*$H_2$CN), 117.80 (CN). M.S (EI): *m*/*z* (%): 388 ($M^{+\cdot}$, 23), 234 ($C_{13}H_{18}N_2S$, 22), 178 ($C_9H_{11}N_2S$, 2), 55 ($C_3H_3O$, 100). Calcd for $C_{22}H_{32}N_2O_2S$ (388.567): C, 68.00; H, 8.30; N, 7.21; S, 8.25. Found: C, 67.77; H, 8.08; N, 7.02; S, 8.04.

### 3.1.8. Synthesis of 3β-acetoxy-*N*-(diethylenetriamine)copper(Π) dinitrite-1′,3′-thiazolo[4′,5′: 17,16]-5α-androstane (20)

To a suspension of Cu(dien)$(NO_3)_2$ **19** (0.14 g, 0.48 mmol) in methanol (5 mL), a solution of compound **17** (0.2 g, 0.48 mmol) in methanol (3 mL) was added dropwise with stirring. The reaction mixture was stirred for 12 h at room temperature, until both of the starting materials dissolved. The resulted blue solution was filtered to remove any insoluble material. Anhydrous diethyl ether was added to the filtrate dropwise with stirring until the clear solution became cloudy. The reaction mixture was left for 24 h in a refrigerator at 4 °C, the solid product that formed, was isolated by filtration, washed with anhydrous diethyl ether several times, dried in air and crystallized from methanol to afford compound **20** as dark blue crystals yield, 0.25 g (73%). IR (KBr, $cm^{-1}$): $\nu$ = 3385–3320 ($NH_2$, NH), 2928, 2857 (CH-aliphatic), 2220 (CN), 1735 (C=O, acetate), 1630 (C=N), 1590 (C=C), 1379 ($NO_3$). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.76 (s, 3H, $CH_3$-19), 1.09 (s, 3H, $CH_3$-18), 1.49 (m, 1H, $C_5$-αH), 2.0 (s, 4H, $2NH_2$, $D_2O$-exchangeable), 2.43 (s, 3H, $COCH_3$), 2.77 (m, 4H, $2CH_2$), 2.80 (m, 4H, $2CH_2$), 3.49 (s, 2H, $CH_2CN$), 3.90 (m, 1H, $C_3$-αH), 8.20 (s, 1H, NH, $D_2O$-exchangeable). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 32.70 (C-1), 29.01, (C-2), 73.60 (C-3), 34.60 (C-4), 37.70 (C-5), 27.00 (C-6), 27.60 (C-7), 38.57 (C-8), 55.0 (C-9), 44.42 (C-10), 22.99 (C-11), 33.70 (C-12), 39.80 (C-13), 69.28 (C-14), 35.28 (C-15), 130.60 (C-16), 150.50 (C-17), 17.23 (C-18), 20.43 (C-19), 170.37 (C=O), 21.60 ($CH_3$), 165.20 (C-2′), 20.70 (*C*$H_2$CN), 117.80 (CN), 41.70 (C-1′), 51.01, (C-2′). M.S (EI): *m*/*z* (%): 703 ($M^{+\cdot}$, 3), 507 (5), 332 (30), 273 ($C_{19}H_{29}O$, 100), 231 ($C_{13}H_{15}N_2S$, 27). Calcd for $C_{28}H_{45}CuN_7O_8S$ (703.317): C, 47.82; H, 6.45; N, 13.94; S, 4.56. Found: C, 47.55; H, 6.20; N, 13.70; S, 4.36.

### 3.1.9. Synthesis of spiro[oxiran-2′,17β-5α-androstane] derivatives (22a–c) and spiro[oxiran-2′,3β-5α-androstene] derivatives (24a–c)

**3.1.9.1. General procedure.** To a solution of either *epi*-androsterone (**1**) (0.29 g, 1 mmol) or testosterone (**23**) (0.28 g, 1 mmol) in potassium *tert*-butoxide [prepeared by the reaction of dry *tert*-butanol (30 mL) with potassium metal (0.5 g)],[42] equimolar amount of either ethyl chloroacetate (**21a**) (0.12 g, 1 mmol), chloroacetone (**21b**) (0.09 g, 1 mmol), or phenacylbromide (**21c**) (0.19 g, 1 mmol) was added. The reaction mixture, in each case, was stirred at room temperature for 24 h and then heated for 6 h in water bath at 70 °C. After cooling at room temperature, the reaction mixture poured over ice/water mixture and the resulted semi-solid was subjected to extraction with chloroform (2 × 30 mL). The organic layer was dried over anhydrous magnesium sulfate and then filtered. The oil product that formed in each case on removal of the solvent in vacuum, was solidified by boiling in petroleum ether (60–80 °C), collected by filtration and crystallized from the appropriate solvent.

**3.1.9.2. Ethyl 3β-hydroxy-spiro[oxiran-2′,17β-5α-androstan]-3′-carboxylate (22a).** Yellow crystals from absolute ethanol, yield 0.25 g (67%), mp 135–136 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3469 (OH), 2931, 2861 (CH-aliphatic), 1730 (C=O). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.81 (s, 3H, $CH_3$-19), 1.08 (s, 3H, $CH_3$-18), 1.55 (m, 1H, $C_5$-αH), 1.27 (t, 3H, *J* = 7.4 Hz, ester-$CH_3$), 2.02 (s, 1H, OH, $D_2O$-exchangeable), 3.47 (s, 1H, oxirane-CH), 3.85 (m, 1H, $C_3$-αH), 4.45 (q, 2H, *J* = 7.4 Hz, ester-$CH_2$). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 31.32 (C-1), 30.9 (C-2), 69.23 (C-3), 38.99 (C-4), 39.99 (C-5), 29.0 (C-6), 32.1 (C-7), 35.27 (C-8), 50.63 (C-9), 36.8 (C-10), 20.51 (C-11), 35.6 (C-12), 44.36 (C-13), 53.86 (C-14), 27.1 (C-15), 22.5 (C-16), 71.4 (C-17), 12.05 (C-18), 16.89 (C-19), 31.25 (C-1′), 8.27 (C-2′), 205.96 (C=O), 67.0 (C-oxirane). M.S (EI): *m*/*z* (%): 376 ($M^{+\cdot}$, 24), 303 ($M^{+\cdot}$-$CO_2$Et, 26), 290 ($C_{19}H_{30}O_2$, 100), 275 ($C_{17}H_{23}O_3$, 14), 246 ($C_{16}H_{22}O_2$, 34), 107 (56). Calcd for $C_{23}H_{36}O_4$ (376.530): C, 73.37; H, 9.64. Found: C, 73.15; H, 9.39.

**3.1.9.3. 3′-Acetyl-spiro[oxiran-2′,17β-5α-androstan]-3β-ol (22b).** Pale yellow crystals from dioxane, yield 0.25 g (73%), mp 125–126 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3458 (OH), 2930, 2859 (CH-aliphatic), 1723 (C=O). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.71 (s, 3H, $CH_3$-19), 1.06 (s, 3H, $CH_3$-18), 1.46 (m, 1H, $C_5$-αH), 1.99 (s, 1H, OH, $D_2O$-exchangeable), 2.14 (s, 3H, $CH_3$), 3.44 (s, 1H, oxirane-CH), 3.90 (m, 1H, $C_3$-αH). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 32.22 (C-1), 30.5 (C-2), 70.03 (C-3), 38.45 (C-4), 39.29 (C-5), 29.2 (C-6), 32.46 (C-7), 35.20 (C-8), 51.33 (C-9), 36.8 (C-10), 20.53 (C-11), 35.6 (C-12), 44.33 (C-13), 53.86 (C-14), 27.1 (C-15), 22.5 (C-16), 72.7 (C-17), 12.05 (C-18), 17.59 (C-19), 218.23 (C=O), 34.26 ($COCH_3$), 67.34 (C-oxirane), M.S (EI): *m*/*z* (%): 346 ($M^{+\cdot}$, 23), 327 ($M^{+\cdot}$-$COCH_3$, 16), 166 ($C_{10}H_{14}O_2$, 2), 57 ($C_3H_5O$, 100). Calcd for $C_{22}H_{34}O_3$ (346.504): C, 76.26; H, 9.89. Found: C, 76, 03; 10.17.

**3.1.9.4. 3′-Benzoyl-spiro[oxiran-2′,17β-5α-androstan]-3β-ol (22c).** Orange crystals from dioxane, yield 0.30 g (75%), mp 155–156 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3465 (OH), 3058 (CH-aromatic), 2945, 2861 (CH-aliphatic), 1729 (C=O), 1595 (C=C). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.70 (s, 3H, $CH_3$-19), 1.06 (s, 3H, $CH_3$-18), 1.47 (m, 1H, $C_5$-αH), 1.98 (s, 1H, OH, $D_2O$-exchangeable), 3.86 (m, 1H, $C_3$-αH), 4.38 (s, 1H, oxirane-CH), 7.49-7.92 (m, 5H, aromatic-H). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 31.32 (C-1), 30.9 (C-2), 69.23 (C-3), 38.99 (C-4), 39.99 (C-5), 29.0 (C-6), 32.1 (C-7), 35.27 (C-8), 50.63 (C-9), 36.8 (C-10), 20.51 (C-11), 35.6 (C-12), 44.36 (C-13), 53.86 (C-14), 27.1 (C-15), 22.5 (C-16), 71.4 (C-17), 12.05 (C-18), 16.89 (C-19), 197.05 (C = O), 67.0 (C-oxirane), 136.21, 128.7, 127.50, 133.2 (C-phenyl). M.S (EI): $m/z$ (%): 408 ($M^{+\cdot}$, 29), 303 ($M^{+\cdot}$-COPh, 4), 247 ($C_{17}H_{27}O$, 4), 160 ($C_{10}H_8O_2$, 5), 105 (PhCO, 67), 55 ($C_3H_3O$, 100). Calcd for $C_{27}H_{36}O_3$ (408.573): C, 79.37; H, 8.88. Found: C, 79.59; H, 9.17.

**3.1.9.5. Ethyl 17β-hydroxy-spiro[oxiran-2′,3β-androst-4-en]-3′-carboxylate (24a).** Dark yellow crystals from absolute ethanol, yield 0.23 g (62%), mp 135–137 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3528 (OH), 3024 (CH-aromatic), 2939, 2874 (CH-aliphatic), 1736 (C=O), 1612 (C=C). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.72 (s, 3H, $CH_3$-19), 1.18 (s, 3H, $CH_3$-18), 1.36 (t, 3H, $J$ = 7.5, ester-$CH_3$), 1.97 (s, 1H, OH, $D_2O$-exchangeable), 3.34 (t, 1H, $C_{17}$-αH, $J$ = 8.8 Hz), 4.20 (s, 1H, oxirane-CH), 4.48 (q, 2H, $J$ = 7.5, ester-$CH_2$), 5.56 (s, 1H, $C_4$-H). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 31.95 (C-1), 31.27 (C-2), 57.70 (C-3), 123.90 (C-4), 140.03 (C-5), 33.0 (C-6), 32.1 (C-7), 35.12 (C-8), 49.98 (C-9), 36.80 (C-10), 22.51 (C-11), 35.05 (C-12), 44.36 (C-13), 53.42 (C-14), 27.1 (C-15), 22.50 (C-16), 79.80 (C-17), 11.18 (C-18), 16.89 (C-19), 31.25 (C-1′), 8.27 (C-2′), 205.96 (C=O), 63.40 (C-oxirane). M.S (EI): $m/z$ (%): 374 ($M^{+\cdot}$, 24), 356 ($M^{+\cdot}$-$H_2O$, 5), 301 ($M^{+\cdot}$-$CO_2Et$, 7), 289 ($C_{19}H_{29}O_2$, 100), 271 ($C_{19}H_{27}O$, 2), 177 ($C_{13}H_{21}$, 0.6). Calcd for $C_{23}H_{34}O_4$ (374.514): C, 73.76; H, 9.15. Found: C, 73.56; H, 8.89.

**3.1.9.6. 3′-Acetyl-spiro[oxiran-2′,3β-androst-4-en]-17β-ol (24b).** Pale yellow crystals from dioxane, yield 0.25 g (73%), mp 80–81 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3422 (OH), 3024 (CH-aromatic), 2944, 2870 (CH-aliphatic), 1720 (C=O), 1602 (C=C). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.73 (s, 3H, $CH_3$-19), 1.13 (s, 3H, $CH_3$-18), 1.97 (s, 1H, OH, $D_2O$-exchangeable), 2.08 (s, 3H, $CH_3$), 3.39 (t, 1H, $C_{17}$-αH, $J$ = 8.8 Hz), 4.32 (s, 1H, oxirane-CH), 5.57 (s, 1H, $C_4$-H). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 31.95 (C-1), 31.27 (C-2), 57.70 (C-3), 123.90 (C-4), 140.03 (C-5), 33.0 (C-6), 32.1 (C-7), 35.12 (C-8), 49.98 (C-9), 36.80 (C-10), 22.51 (C-11), 35.05 (C-12), 44.36 (C-13), 53.42 (C-14), 27.1 (C-15), 22.50 (C-16), 79.80 (C-17), 11.18 (C-18), 16.89 (C-19), 197.96 (C = O), 34.26 ($COCH_3$), 63.40 (C-oxirane). M.S (EI): $m/z$ (%): 344 ($M^{+\cdot}$, 23), 301 ($M^{+\cdot}$-$COCH_3$, 55), 178 ($C_{11}H_{14}O_2$, 25), 79 ($C_6H_7$, 100). Calcd for $C_{22}H_{32}O_3$ (344.488): C, 76.70; H, 9.36. Found: C, 76, 93; 9.61.

**3.1.9.7. 3′-Benzoyl-spiro[oxiran-2′,3β-androst-4-en]-17β-ol (24c).** Orange crystals from dioxane, yield 0.32 g (80%), mp 120–122 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3526 (OH), 3058 (CH-aromatic), 2947, 2873 (CH-aliphatic), 1690 (C=O), 1613 (C=C). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.60 (s, 3H, $CH_3$-19), 1.07 (s, 3H, $CH_3$-18), 2.04 (s, 1H, OH, $D_2O$-exchangeable), 3.45 (t, 1H, $C_{17}$-αH, $J$ = 8.6 Hz), 4.41 (s, 1H, oxirane-CH), 5.56 (s, 1H, $C_4$-H), 7.49–7.92 (m, 5H, aromatic-H). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 31.95 (C-1), 31.27 (C-2), 57.70 (C-3), 123.90 (C-4), 140.03 (C-5), 33.0 (C-6), 32.1 (C-7), 35.12 (C-8), 49.98 (C-9), 36.80 (C-10), 22.51 (C-11), 35.05 (C-12), 44.36 (C-13), 53.42 (C-14), 27.1 (C-15), 22.50 (C-16), 79.80 (C-17), 11.18 (C-18), 16.89 (C-19), 197.96 (C=O), 63.40 (C-oxirane), 136.21, 127.70, 128.80, 133.20 (C-phenyl). M.S (EI): $m/z$ (%): 406 ($M^{+\cdot}$, 29), 301 ($M^{+\cdot}$-COPh, 5), 246 ($C_{17}H_{27}O$, 12), 124 ($C_8H_{12}O$, 41), 105 (PhCO, 23), 57 ($C_3H_5O$, 100). Calcd for $C_{27}H_{34}O_3$ (406.557): C, 79.76; H, 8.43. Found: C, 79.99; H, 8.72.

**3.1.10. Synthesis of 17-chloroandrost-4-en-3-one (26), 2′-(3-oxoandrost-4-en-17-yl-amino)benzoic acid (28a) and 2′-(3-oxoandrost-4-en-17yl-amino)-5′-fluorobenzoic acid (28b)**

To a solution of testosterone (**23**) (0.28 g, 1 mmol) in anhydrous diethyl ether (20 mL) in ice bath, equimolar amount of thionyl chloride (**25**) (0.12 g, 1 mmol) was added dropwise with stirring. After complete addition, the reaction mixture was stirred in ice bath for 5 h until all starting materials had disappeared as indicated by TLC. Then, the reaction mixture was left at room temperature over night. The solid product that formed was filtered off, dried and crystallized from absolute ethanol to afford compound **26** as yellow crystals, yield 0.26 g (86%). Equimolar amounts of compound **26** (0.31 g, 1 mmol) and anthranilic acid (**27a**) (0.137 g, 1 mmol) or 5-fluoroanthranilic acid (**27b**) (0.15 g, 1 mmol) in absolute ethanol (50 mL) were refluxed for 6-8 h until all the starting materials had disappeared as indicated by TLC. The reaction mixture, then left to cool at room temperature, poured over crushed ice and left in a refrigerator at 4 °C overnight. The formed solid product, in each case, was collected by filtration and crystallized from the appropriate solvent.

*Compound **28a***: Dark yellow crystals from absolute ethanol, yield 0.35 g (87%), mp 110–112 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3453 (NH), 3359 (OH), 3048 (CH-aromatic), 2938, 2865 (CH-aliphatic), 1686, 1675 (2C=O), 1613 (C=C). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.68 (s, 3H, $CH_3$-19), 1.15 (s, 3H, $CH_3$-18), 2.52 (t, 1H, $C_{17}$-H, $J$ = 8.5 Hz), 4.51 (s, 1H, NH, $D_2O$-exchangeable), 5.63 (s, 1H, $C_4$-H), 6.53-7.40 (m, 4H, aromatic-H), 10.45 (s, 1H, OH, $D_2O$-exchangeable). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 35.12 (C-1), 34.20 (C-2), 198.22 (C-3), 123.0 (C-4), 171.06 (C-5), 32.60 (C-6), 31.90 (C-7), 36.20 (C-8), 49.9 (C-9), 34.9 (C-10), 23.01 (C-11), 35.40 (C-12), 40.92 (C-13), 52.90 (C-14), 24.70 (C-15), 28.40 (C-16), 65.10 (C-17), 16.20 (C-18), 22.50 (C-19), 170.50 (C=O), 113.0, 116.10, 131.10, 134.82, 151.20, 169.40 (C-phenyl). M.S (EI): $m/z$ (%): 407 ($M^{+\cdot}$, 28), 136 ($C_7H_6NO_2$, 20), 91 ($C_6H_5N$, 25), 57 ($C_3H_5O$, 100). Calcd for $C_{26}H_{33}NO_3$ (407.545): C, 76.62; H, 8.16; N, 3.44. Found: C, 76.82; H, 8.40; N, 3.65.

*Compound **28b**:* Dark yellow crystals from absolute ethanol, yield 0.4 g (90%), mp 100–101 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3453 (NH), 3360 (OH), 3020 (CH-aromatic), 2924, 2844 (CH-aliphatic), 1690, 1672 (2C=O), 1595 (C=C). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.68 (s, 3H, $CH_3$-19), 1.09 (s, 3H, $CH_3$-18), 2.51 (t, 1H, $C_{17}$-H, $J$ = 8.8 Hz), 4.51 (s, 1H, NH, $D_2O$-exchangeable), 5.63 (s, 1H, $C_4$-H), 6.53-7.06 (m, 3H, aromatic-H), 10.45 (s, 1H, OH, $D_2O$-exchangeable). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 35.12 (C-1), 34.20 (C-2), 198.01 (C-3), 124.0 (C-4), 171.06 (C-5), 32.60 (C-6), 31.90 (C-7), 36.20 (C-8), 49.9 (C-9), 34.9 (C-10), 23.01 (C-11), 35.40 (C-12), 40.50 (C-13), 53.60 (C-14), 24.70 (C-15), 28.40 (C-16), 65.10 (C-17), 16.20 (C-18), 22.50 (C-19), 172.20 (C=O), 115.0, 116.30, 121.10, 145.0, 151.30, 169.20 (C-phenyl). M.S (EI): $m/z$ (%): 425 ($M^{+\cdot}$, 36), 406 ($M^{+\cdot}$-F, 25), 370 ($C_{23}H_{29}FNO_2$), 139 ($C_7H_4FO$), 91 ($C_6H_5N$, 25), 55 ($C_3H_3O$, 100). Calcd for $C_{26}H_{33}FNO_3$ (425.536): C, 73.38; H, 7.58; N, 3.29. Found: C, 73.80; H, 7.75; N, 3.54.

**3.1.11. Synthesis of 17-[4′(1*H*)-oxo-3′-phenyl-2′-thioxoquinazolin-1-yl] androst-4-en-3-one (30a), 17-[6-fluoro-4′(1*H*)-oxo-3′-phenyl-2′-thioxoquinazolin-1-yl]androst-4-en-3-one (30b)**

**3.1.11.1. General procedure.** To a solution of either compound **28a** (0.21 g, 0.51 mmol) or compound **28b** (0.22 g, 0.51 mmol), in absolute ethanol (30 mL) containing a catalytic amount of triethylamine (1 mL), phenyl isothiocyanate (**29**) (0.068 g, 0.51 mmol) was added. The reaction mixture, in each case, was heated under reflux for 8–10 h until all the reactants had disappeared as indicated by TLC. The reaction mixture poured over crushed ice and then kept in a refrigerator at 4 °C overnight. The solid product that formed in each case, was filtered off, dried and crystallized from the proper solvent.

*Compound* ***30a****:* Pale yellow crystals from absolute ethanol, yield 0.20 g (74%), mp 308–310 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3048 (CH-aromatic), 2976, 2846 (CH-aliphatic), 1683 (C=O), 1652 (C=O, amide), 1598 (C=C), 1199 (C=S). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.68 (s, 3H, $CH_3$-19), 1.15 (s, 3H, $CH_3$-18), 2.55 (t, 1H, $C_{17}$-H, $J$ = 8.2 Hz), 5.63 (s, 1H, $C_4$-H), 6.95–7.84 (m, 9H, aromatic-H). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 35.12 (C-1), 33.60 (C-2), 198.01 (C-3), 123.23 (C-4), 171.06 (C-5), 32.60 (C-6), 31.90 (C-7), 36.20 (C-8), 49.90 (C-9), 34.90 (C-10), 23.01 (C-11), 35.05 (C-12), 41.40 (C-13), 53.40 (C-14), 25.90 (C-15), 22.50 (C-16), 79.80 (C-17), 16.20 (C-18), 22.50 (C-19), 160.90 (C=O), 177.0 (C=S), 121.75, 123.94, 124.05, 124.79, 126.67, 128.97, 129.04, 132.17, 137.60, 159.0 (C-phenyl). M.S (EI): $m/z$ (%): 524 ($M^{+\cdot}$, 35), 270 ($C_{19}H_{26}O_2$, 24), 92 ($C_6H_6N$, 100), 77 ($C_6H_5$, 14). Calcd for $C_{33}H_{36}N_2O_2S$ (524.716): C, 75.54; H, 6.92; N, 5.34; S, 6.11. Found: C, 75.32; H, 6.75; N, 5.62; S, 6.37.

*Compound* ***30b***: Pale yellow crystals from absolute ethanol, yield 0.23 g (85%), mp 85–86 °C, IR (KBr, $cm^{-1}$): $\nu$ = 3029 (CH-aromatic), 2963, 2856 (CH-aliphatic), 1687 (C=O), 1661 (C=O, amide), 1529 (C=C), 1195 (C=S). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.68 (s, 3H, $CH_3$-19), 1.08 (s, 3H, $CH_3$-18), 2.51 (t, 1H, $C_{17}$-H, $J$ = 8.7 Hz), 5.48 (s, 1H, $C_4$-H), 6.79-7.64 (m, 8H, aromatic-H). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 35.12 (C-1), 33.60 (C-2), 198.01 (C-3), 123.23 (C-4), 171.06 (C-5), 32.60 (C-6), 31.90 (C-7), 36.20 (C-8), 49.90 (C-9), 34.90 (C-10), 23.01 (C-11), 35.05 (C-12), 41.40 (C-13), 53.40 (C-14), 25.90 (C-15), 22.50 (C-16), 79.80 (C-17), 16.20 (C-18), 22.50 (C-19), 160.90 (C=O), 177.0 (C=S), 121.75, 121.94, 123.05, 124.79, 128.67, 128.97, 129.04, 132.17, 137.60, 159.0 (C-phenyl). M.S (EI): $m/z$ (%): 542 ($M^{+\cdot}$, 22), 523 ($M^{+\cdot}$-F, 25), 312 ($C_{17}H_3FN_2OS$, 26), 270 ($C_{19}H_{26}O_2$, 24), 92 ($C_6H_6N$, 100), 77 ($C_6H_5$, 14). 55 ($C_3H_3O$, 100). Calcd for $C_{33}H_{35}FN_2O_2S$ (542.707): C, 73.03; H, 6.50; N, 5.16; S, 5.91. Found: C, 73.29; H, 6.75; N, 5.43; S, 6.17.

### 3.2. In vitro cytotoxic assay

#### 3.2.1. Chemicals

Dimethylsulfoxide (DMSO), Sodium bicarbonate, Fetal Bovine Serum (FBS), Penicillin/Streptomycin, Trypan blue, Trypsin, Sulphorhodamine-B (SRB), Trichloroacetic acid (TCA), Acetic acid, isopropanol, and Ethanol (70%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A stock solution of TCA 50% was prepared and stored 50 μL of the stock was added to 200 μL RPMI-1640 medium/well to yield a final concentration of 10% used for protein precipitation.

RPMI-1640 medium (Sigma Chemical Co., St. Louis, Mo, and USA): The medium was used for culturing and maintenance of the human tumor cell lines. It was prepared as follows: 10.4 g medium was weighed, mixed with 2 g sodium bicarbonate, completed to 1 L with distilled water and shaked carefully till complete dissolution. The medium was then sterilized by filtration in a Millipore bacterial filter (0.22 μM). The prepared medium was kept in a refrigerator (4 °C) and checked at regular intervals for contamination. Buffers: Tris base 10 mM (pH 10.5): It was used for SRB dye solubilization. 121.1 g of tris base was dissolved in 1000 mL of distilled water and pH was adjusted by HCl acid (2 M).

#### 3.2.2. Cell propagation and maintenance

Breast cancer cell lines (MCF-7) were obtained in liquid nitrogen (−180 °C) from the American Type Culture Collection. The tumor cell lines were maintained in the National Cancer Institute, Cairo, Egypt. A cryotube containing frozen cells was taken out of the liquid nitrogen container and then thawed in a water bath at 37 °C. The cryotube was opened under strict aseptic conditions and its contents were supplied by 5 mL supplemented medium. The tube was incubated for 2 h then its contents were centrifuged at 1200 rpm for 10 min. The supernatant was discarded and the cell pellet was suspended and seeded in 5 mL supplemented medium in T25 Nunclon sterile tissue culture flasks. The cell suspension was incubated and followed up daily with replacing the supplemented medium every 2–3 days. Incubation was continued until a confluent growth was achieved and the cells were freshly sub-cultured before each experiment.

The medium was discarded and the cell monolayer was washed twice with 5 mL phosphate buffer saline. All the adherent cells were dispersed from their monolayer by the addition of 1 mL trypsin solution (0.025% trypsin w/v) for 2 min. The flask was left in the incubator till complete detachment of all the cells and checked with the inverted microscope (Olympus 1x70, Tokto, Japan). Trypsin was inactivated by the addition of 5 mL of the supplemented medium. The trypsin content was discarded by centrifugation at 1200 rpm for 10 min. The supernatant was discarded and the cells were separated into single cell suspension by gentle dispersion several times, then suspended and seeded in 5 mL supplemented medium in T25 Nunclon sterile tissue culture flasks.

#### 3.2.3. Determination and counting of viable cells

Trypan blue solution (50 μL of 0.05%) was added to 50 μL of the single cell suspension. The cells were examined under the inverted microscope using the haemocytometer. Non stained (viable) cells were counted using the following equation:

$$\text{Viable cells/mL} = \text{number of cells in 4 quarters} \times 2\ (\text{dilution factor}) \times 10^4/4$$

The cells were then diluted to give the concentration of single cell suspension required for each experiment.

#### 3.2.4. Growth inhibition assay

The cytotoxic effect of Dox and the tested compounds was investigated using SRB assay. The human breast cancer (MCF-7) cells were used when 90% confluence was reached in T25 flasks. The cells were seeded in 96-well microplates, after the cell concentrations were adjusted to ($5 \times 10^4$–$10^5$ cells/well) in 100 μL RPMI-1640 culture medium and warmed at 37 °C and supplemented with penicillin/streptomycin and FBS. The cells were treated with Dox or either one of the tested compounds which were dissolved individually in DMSO in four concentrations (5, 12.5, 25, 50 μg/mL) and re-incubated for 24, 48 and 72 h. Control cells were treated with vehicle alone and for each drug concentration, 6 wells were used. Then the cells were fixed with 50 μL cold 50% trichloroacetic acid for 1 h at 4 °C. Wells were washed 5 times with distilled water and stained for 30 min at room temperature with 50 μL 0.4% SRB dissolved in 1% acetic acid. The wells were then washed 4 times with 1% acetic acid. The plates were air-dried and the dye was solubilized with 100 μL/well of 10 mM tris base (pH 10.5) for 5 min on a shaker (Orbital shaker OS 20, Boeco, Germany) at 1600 rpm. The optical density (O.D.) of each well was measured spectrophotometrically at 564 nm with an ELIZA microplate reader (Meter tech. Σ 960, USA). The results were expressed as percent of cell growth inhibition compared with the control and calculated with the following equation:[43]

$$1 - \left\{\frac{\text{Absorbance of treated}}{\text{Absorbance of control}}\right\} \times 100$$

### 3.3. Gene expression analysis

According to Brun et al.[44] and Kronmiller et al.[45] serial analysis of gene expression via semi-quantitative reverse transcription polymerase chain reaction (RT–PCR) were used for mRNA analysis to determine the expression level of the selected genes.

### 3.3.1. First-strand cDNA synthesize using extracted RNA

After cytotoxicity study, 48 h incubation time cell samples which stored at −80 °C prior to extraction were used to extract total RNA. The total RNA was isolated using the BioFlux RNA Extraction Kit (China). Total RNA pellets were dissolved in DNase, RNase-free water and quantitated using a spectrophotometer. The quality of RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide; the 18S and 28S rRNA bands were visualized under ultraviolet light. To synthesize the first-strand cDNA, 5 μg of the complete Poly(A)$^+$ RNA isolated from samples was reverse transcribed into cDNA in a total volume of 20 μL using 1 μL oligo (poly(deoxythymidine)) primer.[44] The composition of the reaction mixture consisted of 50 mM $MgCl_2$, 10× reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris–HCl; pH 8.3), 200 U/μL reverse transcriptase (RNase H free), 10 mM of each dNTP, and 50 μM of oligo (dT) primer. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and finished with denaturation step at 99 °C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through RT-PCR.[44,45]

### 3.3.2. RT–PCR assay

The first strand cDNA from different cell line samples was used as templates for the semi-quantitative RT–PCR with a pair of specific primers in a 25 μL reaction volume. The quantitative values of RT–PCR of VEGF (VEGF-F: 5′-TCG GGC CTC CGA AAC CAT GA-3′, VEGF-R: 5′-CCT GGT GAG AGA TCT GGT TC-3′), Cyp19 (Cyp19-F: 5′-TGT CTC TTT GTT CTT CAT GCT ATT TCT C-3′, Cyp19-R: 5′-TCA CCA ATA ACA GTC TGG ATT TCC-3′) and hAP-2γ (hAP-2γ-F: 5′-CCA GAC CTC ATC TTG GAG GAC-3′, hAP-2γ-R: 5′-CAG CTG GAC TCT GGT CTC CAG-3′) genes were normalized on the bases of β-actin (β-actin-F: 5′-TTG CCG ACA GGA TGC AGA A-3′, β-actin-R: 5′-GCC GAT CCA CAC GGA GTA CT-3′) expression. The reaction mixture for RT–PCR was consisted of 10 mM dNTP's, 50 mM $MgCl_2$, 10× PCR buffer (50 mM KCl; 20 mM Tris–HCl; pH 8.3), 1 U/μL taq polymerase, and autoclaved water. The PCR cycling parameters of the studied genes were performed as follows: VEGF: 40 cycles: 95 °C, 10 min; 94 °C, 30 s; 59 °C, 1 min; 68 °C, 2 min; 68 °C, 7 min; CYP19: 50 cycles: 95 °C, 10 min; 95 °C, 15 s; 60 °C, 1 min; 72 °C, 1 min; 72 °C, 7 min, hAP-2-γ: 20 cycles: 94 °C, 5 min; 94 °C, 1 min; 65 °C, 2 min; 72 °C, 30 s; 72 °C, 7 min, and β-actin: 40 cycles: 95 °C, 15 min; 94 °C, 15 s; 60 °C, 30 s; 72 °C, 1 min; 72 °C, 7 min. The PCR products (VEGF: 400 bp; CYP19: 278 bp; hAP-2-γ: 306 bp and β-actin; 165 bp) were then loaded onto 2.0% agarose gel, with PCR products derived from β-actin of the different samples.

### 3.4. Statistical analysis

The data were analyzed using version 11.0 of SPSS, 2001. All the data are expressed as mean ± standard deviation. Analysis of the data was done using Anova One Way to detect the significant difference among the studied compounds. A level of $P$ >0.05 was defined as statistically significant.

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