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ARTICLE

Exploring the Anti-Breast Cancer Potential of Flavonoid Analogs

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Abstract

In the course of our search for new antitumor agents in breast cancer, novel flavone derivatives were synthesized, characterized and examined for the antitumor activities against breast cancer cell lines. In initial screening, analogs **7a** [3-(5-amino-1,3,4-thiadiazol-2-yl)methoxy)-2-phenyl-4*H*-chromen-4-one] and **7b** [3-(5-amino-1,3,4-thiadiazol-2-yl)methoxy)-2-(4-methoxyphenyl)-4*H*-chromen-4-one] was found to be effective against estrogen receptor negative cell line (MDA-MB 453) followed by their evaluation in five dose assay. In addition, mechanistic study of **7a** and **7b** was done with cytometric analysis and electrophoretic study and it was observed that apoptosis is a mechanism of cell death, confirmed morphologically by acridine orange/ethidium bromide double staining and TUNEL analysis. Further *In-vivo* evaluation of anti-tumor activity of compound **7a** and **7b** by Ehrlich Ascites Carcinoma (EAC) model and related studies confirm the anti-breast cancer potential of flavonoid analogs.

Keywords: Synthesis Flavones, Breast Cancer, MDA-MB 453, Anticancer agent, Flavonoid

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1. Introduction

Breast cancer is the most commonly diagnosed malignancy among women with more than one million new cases diagnosed per year throughout the world. [1,2] Despite advances in the early detection of breast cancer and the advent of novel targeted therapies, breast cancer still remains a significant public health problem due to the involvement of multiple aberrant and redundant signaling pathways in the tumorigenesis and the development of resistance to the existing therapeutic agents. The currently available breast cancer therapies achieve meaningful clinical results in only 30–40% of the patients [3]. The efficacy of current chemotherapeutics is low and undesirable side effects are still unacceptably high [4]. Hence, the development of novel, efficient, and less toxic anti-breast cancer agents remains an important and challenging goal of medicinal chemists worldwide.

The female hormone estrogen stimulates breast cell division leading to the increase in risk of permanent damage to DNA [5]. Compounds that can regulate the apoptosis of cancer cells are of a high medical significance [6]. Natural products (NPs) have played a valuable role in the drug discovery and development [7-9]. Newman and Cragg [10] reported that in the case of cancer around 79% of FDA-approved drugs during a period of 1981-2010 are either natural products or their based/mimicked-compounds. NPs are chosen through evolutionary process via lead optimization to interact with various enzymes/proteins and thus represent biologically relevant regions of the vast chemical space [11-13]. Flavopiridol, a semisynthetic flavones analog, acts as CDK9 inhibitor, is FDA-approved orphan drug for acute myeloid leukaemia. It has been reported that Myricetin, (flavonoid compound) could decrease pancreatic cancer growth via induction of cell apoptosis [14]. LY294002 (Flavonoid analogue) entered clinical trials as a potential antineoplastic agent [15]. Effects of phytoestrogens in cancer prevention have been reported for decades [16-18]. Since then many molecular mechanisms underlying these effects have been identified. Targets of phytoestrogens comprise steroid receptors, steroid metabolising enzymes, elements of signal transduction and apoptosis pathways, and even the DNA

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processing machinery [18]. Phytoestrogens include chalcones (A), flavones (B) and isoflavones (C) which are non-steroidal compounds possessing anti-estrogenic activity (**Fig.1**) [19].

In light of these findings and in continuation of our research for novel anti-cancer agents [20-23] in the present study, new series of flavone derivatives has been synthesized and screened *in vitro* for cytotoxicity by sulphorhodamine B assay. Five dose assay in Estrogen Receptor negative cell line (MDA-MB 453) and determination of IC_{50} by SRB assay was also performed. In addition, mechanistic study was done with cytometric analysis and electrophoretic determination of apoptosis. Further *in-vivo* activity evaluation of anti-tumor activity of selected synthetic compounds by Ehrlich Ascites Carcinoma (EAC) model and related studies was performed.

2. Rationale and Design

Estrogens have long been recognized to play a key role in the development, growth, and function of female sex organs, and mammary gland [24-25]. Estrogens have also an important role in the skeletal, cardiovascular, and central nervous systems [26-28]. Since estrogens are known to play a predominant role in breast cancer development and growth [29-30], a logical approach for the treatment of estrogen-sensitive breast cancer is the use of anti-estrogens, which block the interaction of estrogens with their specific receptors.

Tamoxifen, the compound in general use for treatment of breast cancer, possesses mixed agonist-antagonist activities, thus limiting its efficacy as a blocker of estrogen action since it exerts estrogenic activity at various organs in different species [31-34]. The most serious adverse events attributable to tamoxifen are the increased risk of uterine cancer and thromboembolic phenomena. Furthermore, up to one half of ER-positive breast cancers and the majority of ER-negative cancers are not prevented with tamoxifen [35]. The use of a pure selective estrogen receptor modulator (SERM) as preventive and therapeutic agent should also have positive effects on the skeletal and cardiovascular systems while decreasing the risk as well as treating breast and uterine cancer [36].

Acolbifene (EM-652.HCl) is a fourth generation SERM of the benzopyran class which has been found to have no estrogen agonist effects in either the breast or endometrium [37-40]. Acolbifene and its prodrug

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(EM-800) have been associated with reduction of growth of tumor xenografts [41] as well as the incidence of DMBA-induced rat mammary cancer [42]. The lack of estrogen agonist activity in the uterus of EM-800 as well as reported activity in tamoxifen-resistant metastatic disease [40] made it an attractive agent for assessment for treatment and prevention. Preclinical and clinical data indicate that Acolbifene (EM-652) possesses characteristics superior to tamoxifen and raloxifene for breast and uterine cancer prevention and treatment as well as for hormone replacement therapy at menopause [43-45]. Encouraged with the above findings we have concentrated our efforts to synthesis the further derivatives of Acolbifene (EM-652), where we tried to modified 4-hydroxyphenyl (C-3) of acolbifene with different 1,3,4-thiadiazoles and imidazo[2,1-b][1,3,4]thiadiazoles with hope to get better analogue of this category (**Fig.2**).

3. Chemistry

General scheme to synthesize proposed flavone derivatives is described in **Scheme 1**. The most common synthetic routes to the chromone structure occur *via* a chalcone intermediate or *via* the Baker-Venkataraman rearrangement. The chalcone pathway implicates the base-catalyzed aldol condensation of 2- hydroxy acetophenones with aromatic or conjugated aldehydes. The condensation of substituted o-hydroxy acetophenone **1** and **2** with substituted benzaldehyde **3** was carried out by vigorous stirring in sodium hydroxide solution (1gm/5 ml) and 95% ethanol to give chalcone **4(a-g)**. The resulting chalcone **4(a-g)** is cyclized to the corresponding 3-hydroxyflavone **5(a-g)**, using alkaline hydrogen peroxide solution, *via* the Algar-Flynn- Oyamada reaction. Further treatment of **5(a-g)** with chloroacetic acid in sodium hydroxide solution gives chromone carboxylic acid **6(a-g)**. Later chromone **6(a-g)** is converted in to the corresponding thiadiazoles **7(a-g)** using $POCl_3$ and thiosemicarbazide. Substituted phenacyl bromides eventually react with thiadiazoles **8(a-g)** to yield imidazo-thiadiazoles **7(a-n)**.

The structures of final derivatives **7(a-g)** and **8(a-n)** were confirmed spectral by IR, ^{1}H NMR, ^{13}C NMR and Mass study. IR absorption peak at ~3000 cm^{-1} for aromatic C-H, disappearance of acid peak at 1700 cm^{-1} and primary amine peak above 3200 cm^{-1} confirm the synthesis of **7(a-g).** C=N of thiadiazole and imidazole are identified individually at around 1600 cm^{-1} and 1500 cm^{-1}. ^{1}HNMR spectra revealed all the

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corresponding peaks at δ = 6-8 ppm for aromatic protons while methoxy protons shows peak at δ= 3 - 4.5 ppm. ^{13}C NMR and Mass gave valuable information about the cyclization of substituted chalcone to flavone and thiadiazole to imidazo thiadiazole ring system.

4. Results and Discussion

4.1 *In vitro* Cytotoxicity study

4.1.1 *In vitro* Cytotoxicity study of compounds 7(a-g) and 8(a-n) by Sulphorhodamine B assay using MDA-MB 453 cells (Breast Cancer Cell Line)

Preliminary screening of compounds **7 (a-g)** and **8 (a-n)** was determined in MDA-MB 453 cells (**Breast Cancer Cell Line**) by sulphorhodamine B assay. The percentage cytotoxicity for all compounds was assessed at different time point 24, 48 and 72h as shown in **Table 1**. Compound **7a** and **7b** showed maximum % Cytotoxicity compared to other compounds.

4.1.2 Five dose assay of selected compounds in MDA-MB453 cells (Breast Cancer Cell Line) by SRB assay

Amongst all the tested compounds **7 (a-g)** and **8 (a-n)**,7a and 7b was the most effective, the cytotoxic potency of the compounds **7a** and **7b** at different time points on MDA-MB453 cells exhibited marked growth inhibition at 72 h when compared to 24 and 48 h, in a dose-dependent fashion. In case of standard Di-Allyl Di Sulphide (DADS) a dose dependent and time dependent increase in the cytotoxicity activity was observed as evident by the IC_{50} values. Therefore, it was selected for detailed mechanistic study (**Table 2**).

4.1.3 Electrophoretic determination of apoptosis

For confirmation of nuclear fragmentation, DNA laddering assay was performed. As illustrated in **Fig. 3**, agarose gel electrophoresis of DNA extracted from **MDA-MB453 cells** treated with different concentration of **7a** and **7b** (50, 150, 300μm) for 72h, revealed a progressive increase in the non-random fragmentation of DNA. **7b** at 150 and 300μM showed a characteristic fragmentation in DNA, whereas **7a** showed an effective degradation in DNA at 300μM when compared to 50 and 150 μM treated concentration. The extracted DNA was electrophoresed on 1% agarose gel at 60 V, 400 mA for 90

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min.the gel were captured under UV illumination in Gel Doc system (Syngene gel documentation system).

4.1.4 Influence of 7a and 7b on the cell cycle progression through cell cycle after 48 h treatment on MDA-MB453 cells

The assay was performed to assess the cell cycle specificity of compound (7a and 7b) on **MDA-MB453 cells**. The data in **Figure 4** demonstrates that the compound **7a** arrested cells in G2/M stage of the cell cycle as evidenced by a significant increase in G2/M population when cells were exposed to 50 μM. But, a significant increase in G0/G1 and S-phase population was also observed when the **7a** concentration is elevated to 150 μM, which could be due to increased cell death (~45% cell death, please compare the IC_{50} **Table. 2**) at this concentration. Similar to compound **7a**, compound **7b** also arrested the cells in G2/M phase of the cell cycle at 50 μM concentration.

DADS, which is used as a positive control, arrested cells in G2/M phase of the cell cycle. Though there is a slight increase in S-phase population, the difference between control and DADS treated cells is not significant. Furthermore, no significant difference was seen in G0/G1 phase of the cell cycle.

4.1.5 Maximum tolerable dose of selected compounds (7a and 7b) by acute toxicity study

Maximum tolerable dose was determined by acute toxicity study according to OECD guideline-425. After 14 days administration of both compounds there were no sign of mortality but slight decline in body weight (<9.0%) was found in 2000mg/kg treated mice whereas 500 mg/kg dosed animals were found to be safe without any sign of mortality hence the MTD was calculated.

4.2 *In Vivo* evaluation of anti-cancer activity by Ehrlich's ascites (EAC) Model

4.2.1 Effect of 7a and 7b on Viability of tumor cells

On day 15th ascitic fluid was collected and checked for percentage viability of cells, there were increased number of viable cells in negative control compared to day 0. Standard decreased the increase in number of % viable cells compared to negative control. The treatment with 7a and 7b at 25 mg/kg and 10 mg/kg significantly reduced the % increase in viable cells compared to negative control. The efficacy of 7b at 10mg/kg was comparable with standard (**Fig.5**).

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4.2.2 Effect of 7a and 7b on change in body weight of (EAC) inoculated Mice

Substantial gain in body weight of EAC inoculated negative control mice was found to be maximum gain of (35.00±1.06) on day 15 when compared to day 0. Standard Di-Allyl Di Sulphide (DADS) at 50mg/kg reduced the body weight (24.80±0.16) compared to negative control. The treatment with 7a and 7b at 10mg/kg and 25mg/kg significantly reduced the % increase in the body weight when compared to negative control and the efficacy was comparable to standard **(Fig.6.)**

4.2.3 The effect of 7a and 7b on Hematological parameters in EAC inoculated mice

In EAC inoculated negative control, the reduction in total RBC, hemoglobin (Hb) content and increase in total WBC count were significant when compared with normal mice. DADS (50 mg/kg i.p) significantly reversed and normalized the EAC induced hematological changes. 7a and 7b at both doses (10 and 25 mg/kg) significantly reversed the elevated WBC count and increase in RBC and Hb content was significant when compared to negative control **(Fig.7.)**.

4.8.4 Effect of 7a and 7b on Mean Survival time and % increase in life span of EAC inoculated mice

Effective decrease in mean survival time of EAC inoculated negative control mice was found (15.0±0.516) days, standard DADS at 50mg/kg showed an increase in MST (22.8±0.872) when compared to negative control, treatment with 7a at 25mg/kg effectively enhanced the MST (28.8±0.654) when compared to negative control mice. While treatment with 7b at 10mg/kg prolonged the MST (22.3±0.333) when compared to negative control. Treatments with 7a at 25mg/kg showed significant increase in % increase in life span (93.10 %) **(Fig. 8,9)**.

4.8.5 Effect of 7a and 7b on Serum Enzyme levels

In EAC inoculated negative control mice, enhancement in ALP, SGOT and SGPT were significantly high when compared with normal. DADS (50 mg/kg i.p) significantly reversed the elevation in EAC induced serum level changes. Treatment with 7a and 7b at both doses (10 and 25 mg/kg) significantly reversed the elevated serum enzymes level. Higher dose of test compounds was highly effective when compared to lower dose (**Fig.10**).

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4.8.6 Effect of 7a and 7b on Liver antioxidant markers

In EAC treated negative control mice, the decrease in SOD, GSH, CAT and increase in LPO were significantly high when compared with normal. DADS (50mg/kg i.p) significantly increased the decline in EAC induced liver endogenous enzymes while significantly reduced the elevated level of LPO. Treatment with 7a and 7b at both doses (10 and 25 mg/kg) significantly reversed the declined liver enzymes level (**Fig.11**).

4.8.7 *In-vivo* mechanistic studies of EAC tumor cells (DNA fragmentation assay and fluorescent imaging of EAC cells following treatment with 7a and 7b

Analysis of DNA isolated from EAC cell of animals treated with 7a , 7b and DADS showed fragmented DNA predominantly in 7a treated animals compared to 7b treated animals indicating the induction of apoptosis further For accurate determination of live/dead nucleated cell concentration in heterogeneous samples dual-fluorescence staining of EAC cells was performed using the dyes acridine orange/ethidium bromide. 7a and 7b (10 &25 mg/kg) treated EAC cells displayed shrinkage as observed under varying magnifications of fluorescent imaging (**Fig.12**,**13**). The standard DADS also showed similar morphology when examined.

4.8.8 Structure Activity Relationship (SAR)

Structure activity relationship (SAR) study indicated that the different substitution on flavone (4*H*-chromen-4-one) ring, exerted varied effect on anti-breast cancer activities. The anti-breast cancer activity of the newly synthesized compounds revealed that 1,3,4-thiadiazole substitution at C-3 position of 4*H*-chromen-4-one is more potent compared to the imidazo[2,1-*b*][1,3,4]thiadiazole (Compound **7a**, **7b** and **8a**, **8b**). From the data shown in **Table 1** and **2**, it is clear that the presence of 4-methoxy phenyl at 2nd position of 4*H*-chromen-4-one ring of 1,3,4-thiadiazole series enhances the anti-breast cancer activity (Compound **7b**) as compare to the plane phenyl substitution (**7a**). The possible enhancement in the anti-breast cancer activity can be further accomplished by slender variation in the ring substituents and/or extensive additional fictionalization warrants further investigations.

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5. CONCLUSION

Conclusively, a new series of flavone analogs **7(a-g)** and **8(a-n)** have been successfully synthesized from 2-hydroxy acetophenone and substituted benzaldehydes. The yields of flavone derivatives were found to be ranging between 35-70%. The structure of compounds was characterized and confirmed by IR, ^{1}H-NMR, ^{13}C-NMR, and Mass spectral studies. In initial screening, analogs **7a [**3-(5-amino-1,3,4-thiadiazol-2-yl)methoxy)-2-phenyl-4*H*-chromen-4-one] and **7b** [3-(5-amino-1,3,4-thiadiazol-2-yl)methoxy)-2-(4-methoxyphenyl)-4*H*-chromen-4-one] was found to be effective against estrogen receptor negative cell line (MDA-MB 453) followed by their evaluation in five dose assay. For confirmation of nuclear fragmentation, DNA laddering assay was performed. Compound **7b** at 150 and 300μM showed a characteristic fragmentation in DNA, whereas **7a** showed an effective degradation in DNA at 300μM when compared to 50 and 150 μM treated concentration. Cell cycle specificity of compound (**7a** and **7b**) on **MDA-MB453 cells** has also been performed and results demostrated that percentage of G2/M phase significantly increased with 150 μM and 50 μM of **7b** and at 50 μM of **7a** compared to control. *In-vivo* EAC model study demonstrated that the treatment with **7a** and **7b** at 10 mg/kg and 25 mg/kg significantly reduced the % increase in the body weight, significantly reversed the elevated WBC count and increase in RBC and Hb content; considerably inverted the elevated serum enzymes and liver enzyme level. It was concluded that the synthesized flavone derivatives have potential to act as an anticancer agents and the activity of various compounds varied according to the substituent attached. These preliminary encouraging results of biological screening of the tested compounds could offer an excellent framework in this field that may lead to discovery of potent antitumor agent.

6. Experimental

6.1. Materials and Methods

The chemicals employed in the synthetic work i.e. 5-methoxy 2'-hydroxy acetophenone was purchased from Sigma-Aldrich while all other chemicals i.e. thiosemicarbazide, 2'-hydroxy acetophenone, other benzaldehydes, 2- chloro acetic acid, Bromine, H_2O_2 and $POCl_3$ etc. were purchased from Spectrochem. All the solvents were used after distillation. Most of the solvents and chemicals used were of LR or

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Analytical grade. The purity of the compounds was confirmed by thin layer chromatography using precoated TLC plates and solvent systems like, Chloroform: Methanol: Formic acid (7:2.5:0.5), Chloroform: Methanol (8:2) and their different ratios. The spots were visualized under ultraviolet lamp and by using of Iodine chamber. ^{1}H NMR and ^{13}C NMR spectra of synthesized compounds were recorded in DMSO solution Bruker Model (400 MHz) instrument at CSMCRI, Bhavnagar and SAIF, Punjab University, Chemical shifts are reported as parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. Column chromatography separations were progressed on silica gel (200-300 mesh). A series of molecules were synthesized, molecules (flavones) were dissolved in dimethyl Sulphoxide (DMSO) to a stock concentration of 100 mM. Working standard for *in-vitro* study was made up in serum containing media. The final concentration of DMSO in cell culture did not exceed 0.1 %.

6.1.1 General procedure for the synthesis of 2-(3,4-disubstitutedphenyl)-3-hydroxy-6-substituted-4H-chromen-4-one 5(a-g) and 2-((6-substituted-4-oxo-2-phenyl-4H-chromen-3-yl)oxy)acetic acid 6(a-g)

It was synthesized as per the method given by Gupta et *al* [46].

6.1.2 General procedure for the synthesis of 3-((5-amino-1,3,4-thiadiazol-2-yl)methoxy)-6-substituted-2-(3,4-substitutedphenyl)-4H-chromen-4-one 7(a-g)

A mixture of flavone acid **6** (**a-g**) (0.01 mol), thiosemicarbazide (0.01 mol) and $POCl_3$ (10 mol) are refluxed at 75 ℃ for 30 min. After cooling down to room temperature, sufficient water was added. The reaction mixture was then reflux at 75℃ for 3 hrs. After cooling reaction mixture was basified to pH 8 by drop wise addition of 50% NaOH solution under stirring. The precipitate was filtered and recrystallized with ethanol.

***6.1.2.1 3-((5-amino-1,3,4-thiadiazol-2-yl)methoxy)-2-phenyl-4H-chromen-4-one* (7a)**

Yield 69%, mp 237-239℃, IR (KBr) V_{max} 3400 (NH strech), 2922 (CH aro. strech), 2851 (CH ali. strech), 1642 (C=O) cm^{-1}, ^{1}H NMR(DMSO-d_6) δ ppm: 6.52-8.21 (m, 9H, Ar-H), 6.12 (s, 2H, NH_2), 5.54 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ ppm: 172.12, 169.34, 163.12, 159.29, 156.22, 148.12, 138.92, 136.22, 135.22, 134.12, 132.22, 130.82, 128.27, 127.45, 125.48, 123.44, 122.46, 121.33, 120.37, 118.13, 116.23, 66.39, 55.38; HRMS (ESI) m/z calcd for $C_{18}H_{13}N_3O_3S$: 351.0678; found: 351.0683.

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6.1.2.2 3-((5-amino-1,3,4-thiadiazol-2-yl)methoxy)-2-(4-methoxyphenyl)-4H-chromen-4-one(7b)

Yield 39%, mp 245-247℃, IR (KBr) V_{max} 3338 (NH strech), 3141 (CH aro. strech), 2918 (CH ali. strech), 1642.69 (C=O) cm^{-1}, ^{1}H NMR(DMSO-d_6) δ ppm: 6.38-8.23 (m, 8H, Ar-H), 6.11 (s, 2H, NH_2), 5.13(s, 2H, CH_2), 3.92(s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 175.02, 169.20, 163.26, 161.28, 156.49, 154.72, 145.89, 142.42, 130.48, 128.48, 125.38, 123.24, 122.46, 121.77, 118.61, 116.42, 114.62, 65.35, 56.81 ; HRMS (ESI) m/z calcd for C19H15N3O4S: 381.0783; found: 381.0789.

6.1.2.3 3-((5-amino-1,3,4-thiadiazol-2-yl)methoxy)-2-(3,4-dimethoxyphenyl)-4H-chromen-4-one(7c)

Yield 47%, mp 248-251℃, IR (KBr) V_{max} 3339 (NH strech), 3142 (CH aro. strech), 2917 (CH ali. strech), 1668 (C=O) cm^{-1}, ^{1}H NMR(DMSO-d_6) δ ppm: 6.58-8.34 (m, 8H, Ar-H), 6.09 (s, 2H, NH_2), 5.15(s, 2H, CH_2), 3.92(s, 3H, OCH_3), 3.82(s, 3H, OCH_3) ; ^{13}C NMR (DMSO-d_6) δ ppm: 176.12, 167.78, 166.82, 161.28, 158.97, 156.82, 152.27, 139.37, 135.92, 125.27, 123.12, 122.13, 121.12, 120.21, 118.78, 114.16, 112.17, 65.28, 56.28, 56.01; HRMS (ESI) m/z calcd for $C_{20}H_{17}N_3O_5S$: 411.0889; found: 411.0895.

6.1.2.4 3-((5-amino-1,3,4-thiadiazol-2-yl)methoxy)-6-methoxy-2-phenyl-4H-chromen-4-one(7d)

Yield 48%, mp 244-246℃, IR (KBr) V_{max} 3321 (NH strech), 3111 (CH aro. strech), 2932 (CH ali. strech), 1662 (C=O) cm^{-1}, ^{1}H NMR(DMSO-d_6) δ ppm: 6.62-8.10 (m, 8H, Ar-H), 6.14 (s, 2H, NH_2), 5.25(s, 2H, CH_2), 3.76 (s, 3H, OCH_3) ; ^{13}C NMR (DMSO-d_6) δ ppm: 174.18, 169.18, 164.17, 159.91, 156.23, 149.51, 140.12, 132.13, 130.16, 128.12, 127.12, 124.32, 122.12, 120.55, 111.62, 64.23, 55.81; HRMS (ESI) m/z calcd for $C_{19}H_{15}N_3O_4S$: 381.0783; found: 381.0788.

6.1.2.5 3-((5-amino-1,3,4-thiadiazol-2-yl)methoxy)-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one (7e)

Yield 63%, mp 235-237℃, IR (KBr) V_{max} 3348 (NH strech), 3028 (CH aro. strech), 2934 (CH ali. strech), 1683 (C=O) cm^{-1}, ^{1}H NMR(DMSO-d_6) δ ppm: 6.82-8.12 (m, 7H, Ar-H), 6.15 (s, 2H, NH_2), 5.01(s, 2H, CH_2), 3.71(s, 3H, OCH_3), 3.92(s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 178.12, 168.23, 167.12, 161.23, 159.112, 156.43, 150.51, 139.12, 128.28, 128.01, 124.12, 122.34, 122.01, 120.12, 114.12, 112.12, 110.12 ,64.26, 56.81, 55.82; HRMS (ESI) m/z calcd for $C_{20}H_{17}N_3O_5S$: 411.0889; found: 411.0881.

6.1.2.6 3-((5-amino-1,3,4-thiadiazol-2-yl)methoxy)-2-(3-methoxyphenyl)-4H-chromen-4-one (7f)

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Yield56%, mp 239-242℃, IR (KBr) V_{max} 3328 (NH strech), 3011 (CH aro. strech), 2934 (CH ali. strech), 1676 (C=O) cm^{-1}, ^{1}H NMR(DMSO-d_6) δ ppm: 6.82-8.38 (m, 8H, Ar-H), 6.10 (s, 2H, NH_2), 5.16(s, 2H, CH_2), 3.91(s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 172.12, 169.12, 165.12, 162.12, 159.12, 158.12, 140.12, 138.12, 130.12, 129.16, 125.58, 123.84, 121.47, 120.22, 116.51, 113.55, 112.57, 64.25, 55.18 ; HRMS (ESI) m/z calcd for $C_{19}H_{15}N_3O_4S$: 381.0783; found: 381.0792.

6.1.2.7 3-((5-amino-1,3,4-thiadiazol-2-yl)methoxy)-6-methoxy-2-(3-methoxyphenyl)-4H-chromen-4-one (7g)

Yield 59%, mp 244-246℃, IR (KBr) V_{max} 3312 (NH strech), 3021 (CH aro. strech), 2973 (CH ali. strech), 1681 (C=O) cm^{-1}, ^{1}H NMR(DMSO-d_6) δ ppm: 6.72-8.29 (m, 7H, Ar-H), 6.11 (s, 2H, NH_2), 5.06(s, 2H, CH_2), 3.81(s, 3H, OCH_3), 3.91(s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 173.12,168.12,165.12,162.12,159.38,158.93,150.12,140.38,130.43,129.12,126.34,124.12,124.72, 122.43,116.23,114.23,110.61, 63.52,55.92,55.18; HRMS (ESI) m/z calcd for $C_{20}H_{17}N_3O_5S$: 411.0889; found: 411.0895.

6.1.3 General procedure for the synthesis of 2-(3,4-disubstitutedphenyl)-6-substituted-3-((6-(2,3,4-trisubstitutedphenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-4H-chromen-4-one 8(a-n)

A mixture of equimolar quantities of flavone thiadiazole **7(a-g)** (0.01 mol) and 2-bromo-1-(substituted-aryl) ethanone (0.01 mol) was refluxed in dry ethanol for 20hr. The excess of solvent was distilled off and the solid hydrobromide that separated was collected by filtration, suspended in water and neutralized by 10% sodium carbonate solution to get free base. It was filtered, washed with water, dried and crystallized from ethanol.

6.1.3.1 3-((6-phenylimidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-phenyl-4H-chromen-4-one(8a)

Yield 57%, mp 252-254℃, IR (KBr) V_{max} 3048 (CH aro. strech), 2913 (CH ali. strech), 1691 (C=O) cm^{-1}, ^{1}H NMR(DMSO-d_6) δ ppm: 6.28-8.29 (m,14H, Ar-H), 8.41 (s,1H, CH-Imidazole), 5.18 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ ppm: 178.12, 169.12, 158.12, 148.12, 138.12, 158.12, 148.21, 142.21, 138.12, 134.12, 132.12, 130.12, 129.12, 128.12, 126.12,, 125.12, 124.12, 122.12, 121.12, 120.12, 118.11, 66.91; HRMS (ESI) m/z calcd for $C_{26}H_{17}N_3O_3S$: 451.0991; found: 451.0997.

6.1.3.2 3-((6-phenylimidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-(4-methoxyphenyl)-4H-chromen-4-one (8b)

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Yield 63%, mp 259-261℃, IR (KBr) V_{max} 3056 (CH aro. strech), 2910 (CH ali. strech), 1684 (C=O) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.46-8.36 (m,13H, Ar-H), 8.44 (s,1H, CH-Imidazole), 5.12 (m, 2H, CH_2), 3.91 (s,3H, OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 172.12, 169.12, 162.12, 158.38, 156.12, 148.12, 142.12, 138.12, 136.12, 134.10, 130.12, 129.12, 128.71, 126.51, 124.18, 122.41, 122.16, 120.31, 120.17, 118.11, 116.12, 64.19, 55.18; HRMS (ESI) m/z calcd for $C_{27}H_{19}N_3O_4S$: 481.1096; found: 481.1091.

6.1.3.3 3-((6-(3-methoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-phenyl-4H-chromen-4-one (8c)

Yield 31%, mp 252℃, IR (KBr) V_{max} 3034 (CH aro. strech), 2921 (CH ali. strech), 1689 (C=O) cm^{-1}, ^1H NMR (DMSO-d_6) δ ppm: 6.36-8.23 (m,13H, Ar-H), 8.32 (s,1H, CH-Imidazole), 5.10 (m, 2H, CH_2), 3.89 (s,3H, OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 175.01, 169.10, 166.11, 159.91, 156.21, 148.11, 136.91, 135.12, 134.12, 134.01, 130.31, 129.21, 128.16, 127.19, 126.19, 125.81, 123.14, 122.23, 121.67, 119.78, 116.31, 114.33, 113.66, 64.92, 55.28; HRMS (ESI) m/z calcd for $C_{27}H_{19}N_3O_4S$: 481.1096; found: 481.1090.

6.1.3.4 3-((6-(3-methoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-(4-methoxyphenyl)-4H-chromen-4-one (8d)

Yield 34%, mp 248-250℃, IR (KBr) V_{max} 3012 (CH aro. strech), 2934 (CH ali. strech), 1678 (C=O) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.36-8.23 (m,12H, Ar-H), 8.32 (s,1H, CH-Imidazole), 5.14 (m, 2H, CH_2), 3.91 (s,3H, OCH_3), 3.81 (s,3H, OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 174.23, 169.23, 164.48, 162.91, 159.91, 158.12, 148.13, 136.92, 134.22, 133.22, 132.03, 130.22, 128.49, 126.82, 123.45,, 122.26, 121.33, 120.72, 119.82, 116.12, 114.23, 114.12, 112.16, 64.92, 56.22, 55.08; HRMS (ESI) m/z calcd for $C_{28}H_{21}N_3O_5S$: 511.1202; found: 511.1206.

6.1.3.5 3-((6-(2-methoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-phenyl-4H-chromen-4-one (8e)

Yield 54%, mp 251-253℃, IR (KBr) V_{max} 3048 (CH aro. strech), 2923 (CH ali. strech), 1681 (C=O) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.48-8.10 (m,13H, Ar-H), 8.28 (s,1H, CH-Imidazole), 5.04 (m, 2H, CH_2), 3.92 (s,3H, OCH_3) ; ^{13}C NMR (DMSO-d_6) δ ppm: 176.01, 169.02, 159.92, 156.31, 155.22, 148.23, 142.91, 138.22, 136.22, 132.11, 130.33, 129.37, 128.36, 127.39, 126.29, 125.38, 123.44, 122.43, 121.67, 120.35,

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119.39, 118.14, 114.13, 65.92, 56.23; HRMS (ESI) m/z calcd for $C_{27}H_{19}N_3O_4S$: 481.1096; found: 481.1090.

6.1.3.6 3-((6-(2-methoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-(4-methoxyphenyl)-4H-chromen-4-one (8f)

Yield 42%, mp 247-250℃, IR (KBr) V_{max} 3012 (CH aro. strech), 2921 (CH ali. strech), 1686 (C=O) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.28-8.16 (m,12H, Ar-H), 8.35 (s,1H, CH-Imidazole), 5.09 (m, 2H, CH_2), 3.96 (s,3H, OCH_3), 3.86 (s,3H, OCH_3) ; ^{13}C NMR (DMSO-d_6) δ ppm: 175.23, 169.02, 160.12, 159.28, 158.93, 157.33, 156.32, 148.13, 138.92, 136.32, 135.42, 133.15, 120.27, 128.48, 127.48, 126.48, 123.44, 120.33, 120.39, 118.13, 116.22, 114.23, 112.13, 65.92, 56.21, 55.08; HRMS (ESI) m/z calcd for $C_{28}H_{21}N_3O_5S$: 511.1202; found: 511.1208.

6.1.3.7 3-((6-(2,4-dimethoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-phenyl-4H-chromen-4-one (8g)

Yield 44%, mp 245-246℃, IR (KBr) V_{max} 3053 (CH aro. strech), 2921 (CH ali. strech), 1682 (C=O) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.38-8.36 (m,12H, Ar-H), 8.41 (s,1H, CH-Imidazole), 5.16 (m, 2H, CH_2), 3.91 (s,3H, OCH_3), 3.85 (s,3H, OCH_3) δ ppm: 176.23, 171.02, 165.62, 162.23, 159.92, 158.22, 148.12, 138.29, 136.22, 135.23, 132.25, 130.43, 128.56, 128.36, 126.92, 125.83, 123.34, 122.43, 121.47, 118.13, 116.23, 114.23, 112.13, 110.12, 66.92, 56. 38, 55.12; HRMS (ESI) m/z calcd for $C_{28}H_{21}N_3O_5S$: 511.1202; found: 511.1208.

6.1.3.8 3-((6-(2,4-dimethoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-(4methoxyphenyl)-4H-chromen-4-one (8h)

Yield 42%, mp 244-248℃, IR (KBr) V_{max} 3063 (CH aro. strech), 2942 (CH ali. strech), 1672 (C=O) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.18-8.06 (m,11H, Ar-H), 8.21 (s,1H, CH-Imidazole), 4.98 (m, 2H, CH_2), 3.92 (s,3H, OCH_3), 3.84 (s,3H, OCH_3), 3.81 (s,3H, OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 178.10, 169.23, 167.82, 164.81, 162.23, 160.13, 159.33, 156.42, 149.14, 142.92, 138.22, 136.22, 134.35, 130.28, 128.28, 126.42, 124.36, 122.33, 120.27, 119.18, 118.24, 114.22, 112.23, 111.12, 110.82, 65.39, 56.12, 55.88, 55.10; HRMS (ESI) m/z calcd for $C_{29}H_{23}N_3O_6S$: 541.1308; found: 541.1302.

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6.1.3.9 3-((6-(4-chlorophenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-phenyl-4H-chromen-4-one (8i)

Yield 75%, mp 267-269℃, IR (KBr) V_{max} 3024 (CH aro. strech), 2912 (CH ali. strech), 1684 (C=O), 712 (C-Cl) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.12-8.21 (m,13H, Ar-H), 8.41 (s,1H, CH-Imidazole), 4.99 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ ppm: 178.23, 169.23, 158.92, 156.22, 148.12, 142.91, 138.12, 136.21, 134.13, 132.11, 130.23, 129.33, 128.49, 128.16, 127.92, 127.29, 125.28, 123.24, 122.13, 121.17, 118.12, 66.19; HRMS (ESI) m/z calcd for $C_{26}H_{16}ClN_3O_3S$: 485.0601; found: 485.0607.

6.1.3.10 3-((6-(4-chlorophenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-(4-methoxyphenyl)-4H-chromen-4-one (8j)

Yield 68%, mp 266-268℃, IR (KBr) V_{max} 3034 (CH aro. strech), 2910 (CH ali. strech), 1681 (C=O), 701 (C-Cl) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.19-8.26 (m,12H, Ar-H), 8.38 (s,1H, CH-Imidazole), 5.09 (m, 2H, CH_2), 3.84 (s,3H, OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 172.60, 162.23, 160.38, 154.41, 152.31, 145.52, 138.12, 133.33, 130.68, 129.33, 124.69,124.31, 123.54, 121.30, 118.18, 114.43, 113.91, 63.55, 55.27; HRMS (ESI) m/z calcd for $C_{27}H_{18}ClN_3O_4S$: 515.0707; found: 515.0701.

6.1.3.11 3-((6-(4-fluorophenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-phenyl-4H-chromen-4-one (8k)

Yield 39%, mp 268-272℃, IR (KBr) V_{max} 3012 (CH aro. strech), 2923 (CH ali. strech), 1684 (C=O), 1201 (C-F) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.12-8.21 (m,13H, Ar-H), 8.41 (s,1H, CH-Imidazole), 4.99 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ ppm: 176.12, 169.12, 161.27, 159.91, 156.22, 149.12, 141.92, 138.22, 136.21, 132.31 130.61, 129.16, 128.92, 126.19, 125.26, 124.28, 122.43, 120.23, 119.27, 118.21, 116.20, 64.92; HRMS (ESI) m/z calcd for $C_{26}H_{16}FN_3O_3S$: 469.0896; found: 469.0891.

6.1.3.12 3-((6-(4-fluorophenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-(4-methoxyphenyl)-4H-chromen-4-one (8l)

Yield 45%, mp 218-220℃, IR (KBr) V_{max} 3064 (CH aro. strech), 2963 (CH ali. strech), 1689 (C=O), 1223 (C-F) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.23-8.32 (m,12H, Ar-H), 8.38 (s,1H, CH-Imidazole), 5.12 (m, 2H, CH_2), 3.91 (s,3H, OCH_3) ; ^{13}C NMR (DMSO-d_6) δ ppm: 178.12, 167.23, 164.92, 162.83, 160.92, 158.22, 148.21, 142.19, 138.22, 136.32, 130.12, 129.26, 128.16, 126.18, 124.14, 122.33, 121.26, 120.71,

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118.21, 116.03, 114.32, 64.92, 55.18; HRMS (ESI) m/z calcd for $C_{27}H_{18}FN_3O_4S$: 499.1002; found: 499.1009.

6.1.3.13 3-((6-(4-methoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-phenyl-4H-chromen-4-one (8m)

Yield 34%, mp 249-253℃, IR (KBr) V_{max} 3084 (CH aro. strech), 2985 (CH ali. strech), 1684 (C=O) cm^{-1}, ^1H NMR(DMSO-d_6) δ ppm: 6.13-8.32 (m,13H, Ar-H), 8.34 (s,1H, CH-Imidazole), 5.15 (m, 2H, CH_2), 3.81 (s,3H, OCH_3); 13C NMR (DMSO-d6) δ ppm: 178.12, 168.01, 165.62, 158.92, 156.22, 148.12, 138.92, 136.22, 135.22, 130.23, 129.36, 128.25, 127.29, 126.93, 125.28, 124.32, 122.43, 120.23, 119.73, 118.13, 116.83, 65.39, 55.38; HRMS (ESI) m/z calcd for $C_{27}H_{19}N_3O_4S$: 481.1096; found: 481.1090.

6.1.3.14 3-((6-(4-methoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazol-2-yl)methoxy)-2-(4-methoxyphenyl)-4H-chromen-4-one (8n)

Yield 48%, mp 259-262 ºC, IR (KBr) Vmax 3042 (CH aro. strech), 2978 (CH ali. strech), 1678 (C=O)cm-1, 1H NMR(DMSO-d6) δ ppm: 6.23-8.23 (m,12H, Ar-H), 8.30 (s,1H, CH-Imidazole), 5.05 (m, 2H, CH_2), 3.86 (s,3H, OCH_3), 3.92 (s,3H, OCH_3) ; ^{13}C NMR (DMSO-d6) δ ppm: 178.12, 169.12, 164.61, 160.18, 159.91, 158.12, 152.11, 142.91, 138.12, 136.21, 130.18, 128.51, 126.81, 125.31, 123.14, 122.36, 120.31, 119.71, 118.12, 116.81, 114.21, 65.91, 55.84, 55.02; HRMS (ESI) m/z calcd for $C_{28}H_{21}N_3O_5S$: 511.1202; found: 511.1209.

6.2 *In vitro* anticancer activity

6.2.1 Cell lines

The human br*e*ast cancer cell line MDA-MB 453 was obtained from the National center of cell sciences (NCCS), Pune, India. MDA-MB231 cells were cultured in DMEM medium (Gibco) supplemented with 10% foetal bovine serum (FBS) and 100 IU/mL penicillin. All cells were cultured at 37ºC with 5% CO_2.

6.2.2 Sulphorhodamine-B Assay

The cytotoxic potential of test compounds on growth of breast cancer cells (MDA-MB 453) was assessed by SRB assay. A fixed number of cells (5000), taken in a volume of 100µL were added to each well of a flat-bottomed 96-well plate, and kept in CO_2 incubator for adherence of the cells for 48 h. The test compounds were dissolved in DMSO (0.1%) and further diluted with the medium. After 48 h, cells were

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treated with 100 µL of two different concentrations (250 and 500 µM) of test compounds at different time points (24, 48 and 72h). Cells in the control wells received the same volume of medium containing 0.1% DMSO. Di-allyl di Sulphide (DADS) was used as a positive control [47]. After incubation cells were fixed with 10% TCA and stained with 0.4% of SRB for 30 min, bound SRB was solubilized in 10 mM Tris base solution and the absorbance was measured at 490 nm in a Bio-Rad plate reader. Percentage inhibition was calculated using formula:

$$\textbf{Percentage Inhibition} = \frac{C - T}{C} \times 100$$

C= Absorbance of DMSO vehicle control treated cells

T= Absorbance of compound treated cells

SRB assay for optimization of dose with the selected compounds by five dose assay

The promising compounds (7a and 7b) identified from the preliminary screening were further subjected to five dose assay by SRB assay at an extended time points like 24,48 and 72hr on MDA-MB 453 cells to determine the time dependent activity of compounds and to optimize the dose for further assay, IC_{50} was determined.

6.2.3 DNA Fragmentation Assay

Optimum number of MDA-MB 453 cells (1×10^6) was incubated for 48h in DMEM media supplemented with 10% FBS. After 48h incubation, media was aspirated and treated with different concentrations (50, 150 and 300 µM) of the compounds (7a and 7b) for 72h. After treatment media containing cells were subjected to centrifugation. The adherent cells in the flasks as well as cell pellets in the centrifuge tube were washed with PBS and 25µl of RNAse solution was added to the centrifuge tube kept at 57ºC for 1h. Cell lysate containing DNA sample was loaded into each well of the agarose gel and electrophoresis was carried at 60 V, 400mA for 90 min. Images were captured using gel documentation system.

6.2.4 Flow Cytometry Analysis

A fixed number of MDA-MB 453 cells (1×10^6) were treated with different concentrations of **7a** and **7b** (50 and 150 µM). After treatment, cells were harvested and washed twice with PBS and re-suspended in 70% EtOH while vortexing and stored until analysis. At the time of analysis, cells were washed with PBS

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to remove alcohol and incubated with FACS reagent containing RNAse (100 µg/ml), Triton X (0.1%) and trisodium citrate (0.1%) for 75 min at 37 ºC. After incubation cells were stained with propidium iodide (2 mg/ml) for 15 min in ice-cold dark condition, cells were then mixed thoroughly and subjected to flow cytometer (BD FACS Calibur, Mountain View, USA) for acquisition. The relative DNA content per cell was obtained by measuring the fluorescence of PI that bound stoichiometrically to DNA. The DNA histograms were analyzed using cell Proquest software, the experiment was conducted in triplicates and Di-Allyl Di Sulphide (DADS) was used as standard.

6.2.5 Determination of Maximum tolerable dose of the selected compounds (7a and 7b)

Acute toxicity of selected compounds was done according to OECD guidelines 425, Swiss albino mice of either sex was selected. Mice were deprived of the food 4h prior to administration of test compounds and till 2h after administration. On the day Zero, test compounds were administered to animals at a dose of 2000mg/kg and 550mg/kg and animals were observed for death for 4, 6 and 8h upto 14 days following administration of compounds, MTD was calculated.

6.3 *In Vivo* Study

In-vivo study Ehrlich's ascites carcinoma (EAC) cells were originally obtained from Amala cancer Research Centre, Thrissur, India, were maintained and propagated as ascites tumor in swiss albino mice by serial intra-peritoneal transplantation at animal quarantine house, JSS College of pharmacy, Mysore, India.

6.3.1 Animals

Eight to ten week old swiss albino mice weighing between 25 and 30 g were procured form central animal facility, JSS medical college, animals were maintained under the controlled conditions of temperature (23 ± 30ºC), humidity (50 ± 5%), light and dark respectively. The animals were provided with sterile food and water ad libitium. Six animals were housed in each polypropylene cage containing paddy husk as bedding. Animal care and handling was done according to guidelines issued by CPCSEA, Government of India. Experiments were performed after obtaining ethical clearance from IAEC [IAEC approval no: 154/2014].

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6.3.2 Anticancer activity of selected compounds (7a and 7b) against EAC inoculated mice

EAC cells obtained from the peritoneal cavity of an EAC bearing mouse, after 15 days of tumor transplantation. Aspirated EAC cells were diluted in PBS and fixed number of viable EAC cells (2.5×10^6 cells/mouse) was injected intraperitoneally into each mouse. After 24h of tumor inoculation the animals were randomly divided into following groups of six mice each and treated with test compounds (7a and 7b) at different concentration based on MTD.

Group I : Normal

Group II : EAC Cells + DMSO i.p for 7 days

Group III : EAC Cells + DADS (50mg/kg) i.p for 7 days

Group IV : EAC Cells + 7a-14 (25mg/kg) i.p for 7 days

Group V : EAC Cells + 7a-14 (10mg/kg) i.p for 7 days

Group VI : EAC Cells + 7b-4 (25mg/kg) i.p for 7 days

Group VII : EAC Cells + 7b-4 (10mg/kg) i.p for 7 days

The test compounds (7a and 7b) were administered for seven days, i.p, alternatively starting from day 1 of tumor inoculation. Di-Allyl Di Sulphide (DADS) at dose of 50 mg/kg was administered i.p. which served as standard drug. Body weight was assessed every third day after tumor inoculation to assess the increase in tumor growth. The animals were monitored for 15 days and various parameters were evaluated. *i.e.* haematological parameters (RBC, WBC, Hb content), viable tumor cell count was done to check the number of viable cell among the treated group, mortality was assessed to calculate Mean survival time [MST] and percentage increase in life span using formula.

Cell count = (No. of cells x Dilution) / (Area x thickness of liquid film)

Percentage increase in body wt = (body weight on respective day - body weight on day 0)*100 / Body weight on day 0

% ILS = [(MST Test – MST Con)] * 100/ MST (Con.)

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6.3.3 Biochemical Tests of EAC inoculated mice

On completion of treatment Serum alanine transaminase (ALT), aspartate transaminase (AST) and ALP activities were determined using a commercial kit (Merck), liver homogenate glutathione (GSH) was determined by the method of Alapati *et al* [48]. Malondialdehyde (MDA) was determined in liver tissue homogenate superoxide dismutase (SOD) and catalase (CAT) activity was assayed spectrophotometrically [48].

6.3.4 DNA Fragmentation assay on isolated EAC cells

DNA fragmentation assay on isolated EAC cells was done by non-enzymatic method, adequate volume of EAC cells was aspirated and diluted with PBS and centrifuged for 3 min at 3000 rpm, supernatent was discarded and obtained pellets was washed with PBS, 100µl of 10% SDS & TE mixture was added to pellets and incubated for 30 min, after incubation 1.6 ml of 8 M potassium acetate was added and centrifuged at 7000 rpm for 1hr.The obtained supernatent was separated out and to that organic propionate mixture of (phenol: chloroform: isoamyl alcohol) was added, to above mixture 2 vol of ice cold ethanol was added and centrifuged at 10000 rpm for 30 min . Cell lysate containing DNA was dissolved into 50µL of Tris EDTA (TE) buffer and sample was loaded into each well of the agarose gel and electrophoresis was carried at 60 V, 400 mA for 90 min. Images were captured using gel documentation system.

6.3.5 Fluorescent imaging of EAC cells using dual staining

Dual staining with ethidium bromide and acridine orange was performed. Aspirated EAC cells suspended in PBS (25µL) from each group was mixed with 1µL AO/EB for 15 min prior to microscopic evaluation. Thin smear of stained cells was prepared on microscopic slides, Images were captured using fluorescent microscope.

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Table 1. Preliminary screening of compounds 7(a-g) and 8(a-n) on MDA-MB 453 cells (Breast Cancer Cell Line) at different time points.

Compounds	Percentage (%) cytotoxicity of MDA-MB453 cells at different time points					
	24h		48h		72h	
	500µM	250µM	500µM	250µM	500µM	250µM
7a	**45.44±0.23**	**27.0±1.3**	**39.7±0.67**	**45.4±0.76**	**56.5±1.1**	**44.05±1.23**
7b	**48.8±1.34**	**33.3±0.72**	**57.5±0.12**	**37.3±0.23**	**63.1±0.56**	**29.90±0.12**
7c	-16.7±0.9	-14.2±0.87	-12.7±0.09	-12.9±0.12	-11.3±0.98	-10.90±0.78
7d	-8.9±2.321	-4.91±1.32	-28.9±0.98	-9.09±0.1	-9.6±3.2	-10.2±0.98
7e	-14.0±0.34	-10.6±0.87	-24.6±0.31	-12.9±1.23	-12.7±0.45	-19.09±0.34
7f	-4.23±1.22	-7.9±1.23	-10.29±0.78	-9.0±2.1	-6.7±0.23	-10.09±1.98
7g	-6.6±1.034	-1.3±0.99	-2.3±1.2	-9.0±2.1	-7.6±0.34	-10.098±1.09
8a	-5.4±1.23	-0.3±0.01	-8.9±1.3	-3.3±3.21	-9.4±1.29	-3.098±1.07
8b	-8.7 ± 1.89	-1.3±0.0.87	-1.9±0.08	-8.8±3.89	-6.2±0.29	-6.098±2.09
8c	29.3±0.98	11.0±3.2	13.1±1.42	10.0±0.9	3.7±1.23	12.90±1.06
8d	0±0.01	-7.1±1.23	2.3±1.23	-8.0±1.23	-8.1±1.08	-6.78±1.05
8e	-3.23±1.48	-4.5±1.89	-9.38±0.99	-7.0±1.8	-4.6±0.48	-9.12±2.12
8f	-23.2±0.33	-25±0.98	-43.7±2.1	-43±3.21	-29.4±1.09	-23±1.08
8g	-17.8±0.98	-16±0.87	-19.0±0.98	-23±2.43	-30.2±1.02	-32±1.09
8h	-67.2±1.45	-34.8±1.2	-46.8±1.2	-13.0±1.34	-31.4±1.2	-56.96±1.12
8i	-6.9±2.56	-6.91±1.68	-22.8±0.91	-8.01±0.8	-6.2±4.8	-9.23±0.62
8j	-11.7±3.1	-9.27±0.76	-10.1±0.12	-4.5±2.3	-12.3±1.8	-9.90±0.91
8k	-12.8±0.5	-12.2±0.89	-11.7±0.19	-11.9±0.16	-12.3±0.94	-11.90±0.98
8l	-12.8±0.92	-11±0.27	-11.0±0.12	-21±1.43	-12.2±12.02	-21±2.09
8m	-12.7±2.1	-6.27±0.98	-13.1±0.09	-6.5±1.3	-10.3±1.2	-8.90±0.89
8n	-19.6±0.98	-5.2±1.34	-34.8±1.2	-6.7±1.32	-33±1.23	-9.08±0.67

All the values are expressed as mean ± SEM of triplicates compared to control

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Table 2. Five Dose assays of selected compounds in MDA-MB453 cells by SRB assay

Compounds	MDA MB 453 IC_{50}(µM) (Breast Cancer Cell Line)		
	24h	48h	72h
7a	478±24.1[a]	185±2.20[a]	240±25.3[a]
7b	578±7.77[a]	167±83.4[a]	315±66.9
DADS	929.2±1.05	724.2±11.05	324.2±32.40

All values are expressed as mean ± SEM, n = 3. Inhibitory concentration 50 (IC_{50}) was calculated by plotting % cytotoxicity vs. concentration using linear regression. Three independent experiments were carried out. Data was analyzed by one way ANOVA followed by post Dunnet's test; a = p<0.05, as compared to standard Di-Allyl Di Sulphide (DADS)

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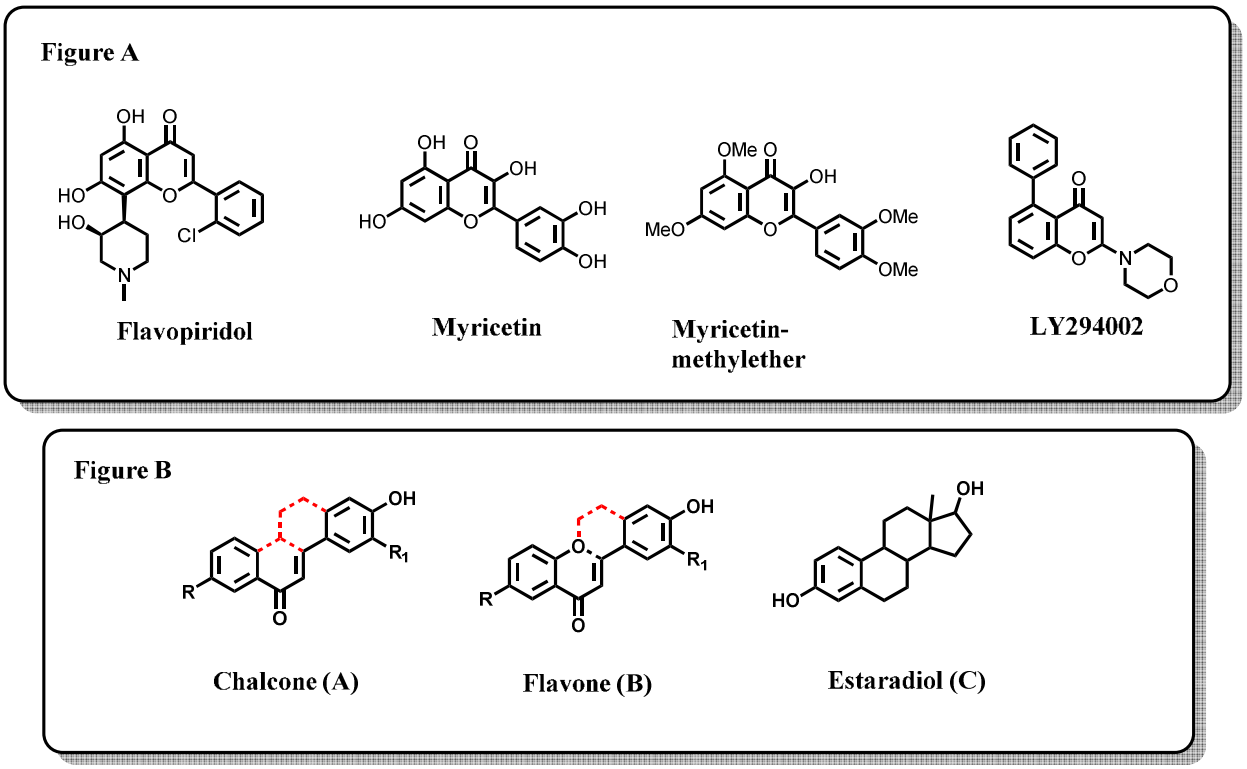

Fig. 1. A) Anticancer Flavonoid leads B) Structural similarities between Chalcone (A), Flavone (B) and Estradiol (C)

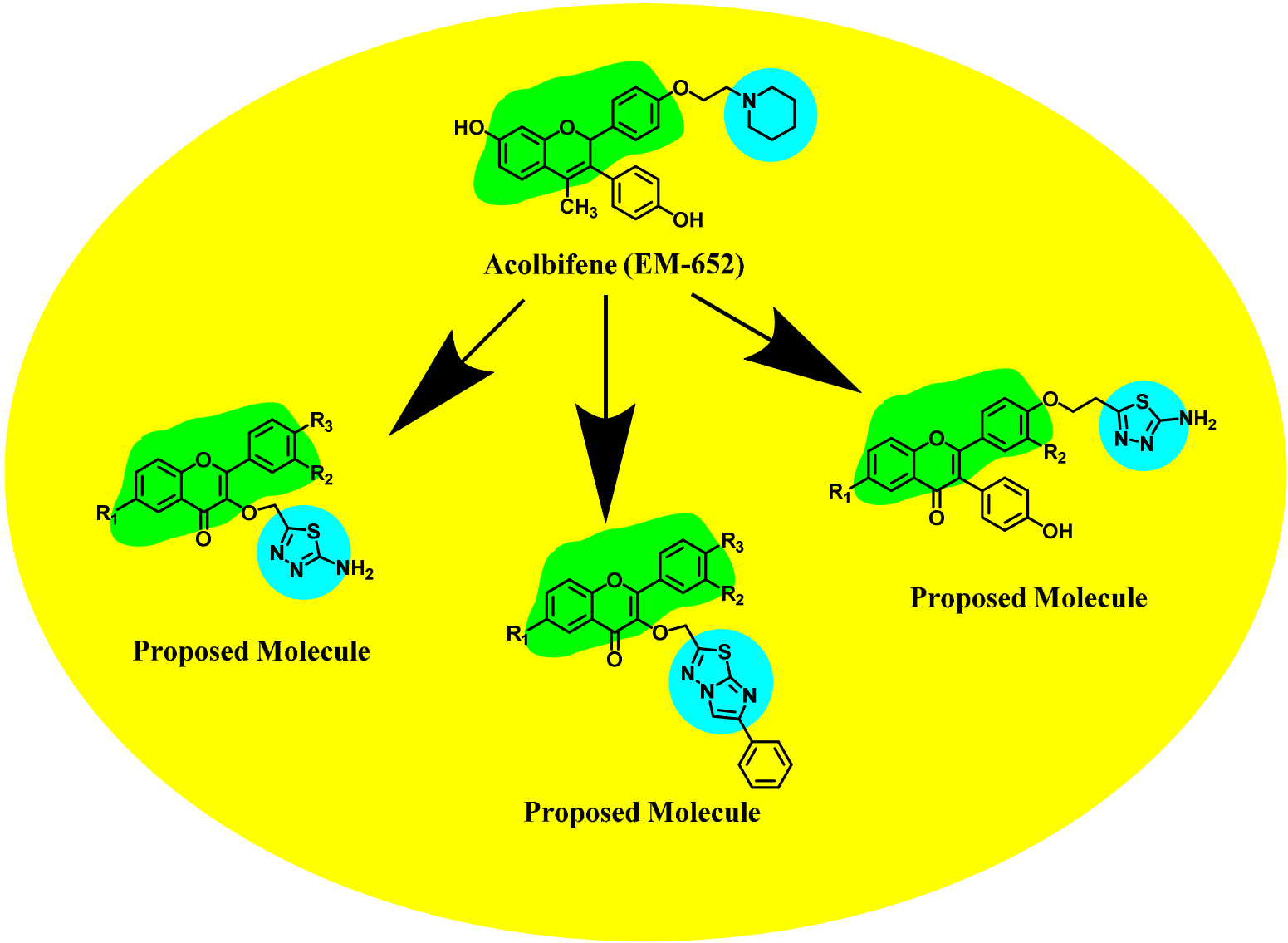

Fig. 2. Structural comparison between acolbifene and proposed molecule.

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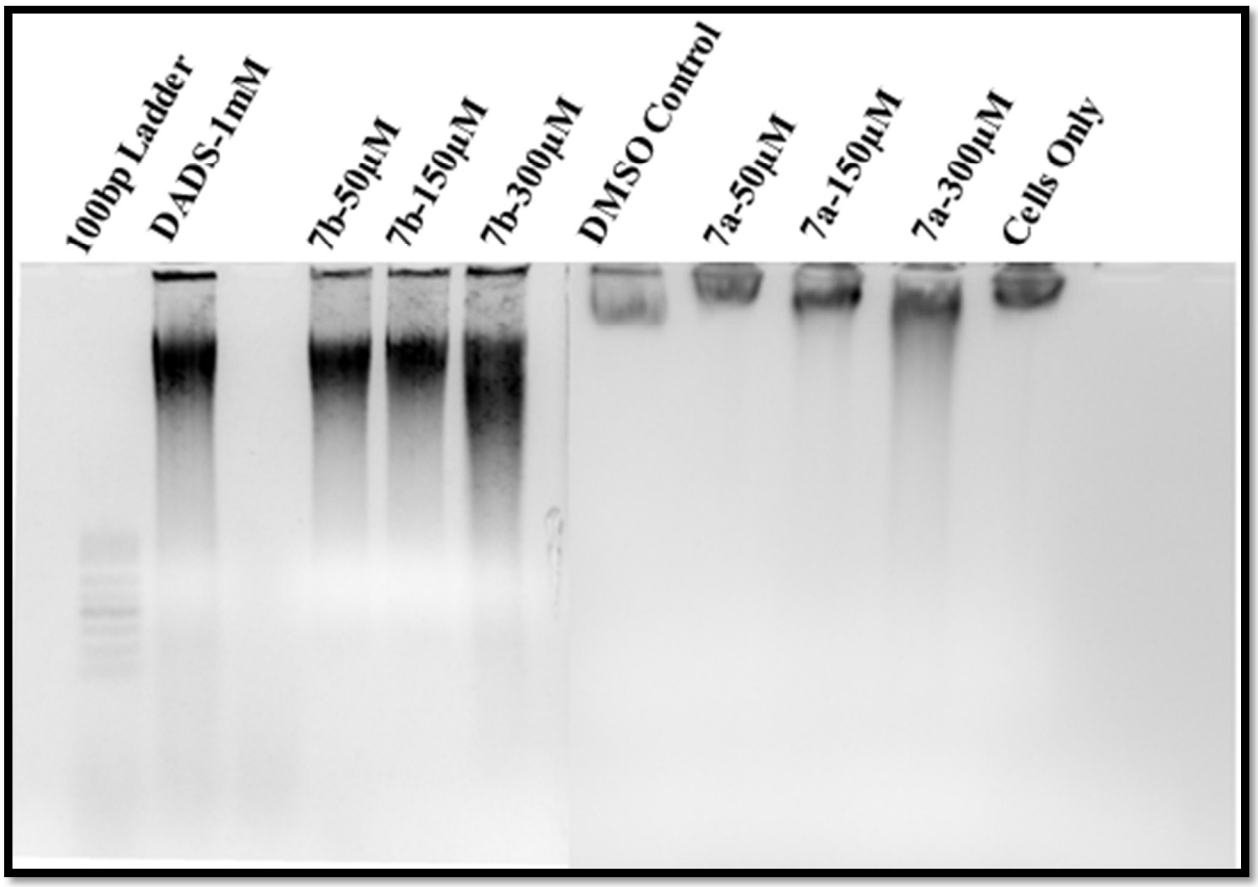

Fig. 3. Agarose gel electrophoresis nucleosomal DNA fragmentation of MDA-MB453 cells induced by selected compounds 7a and 7b at 72h.

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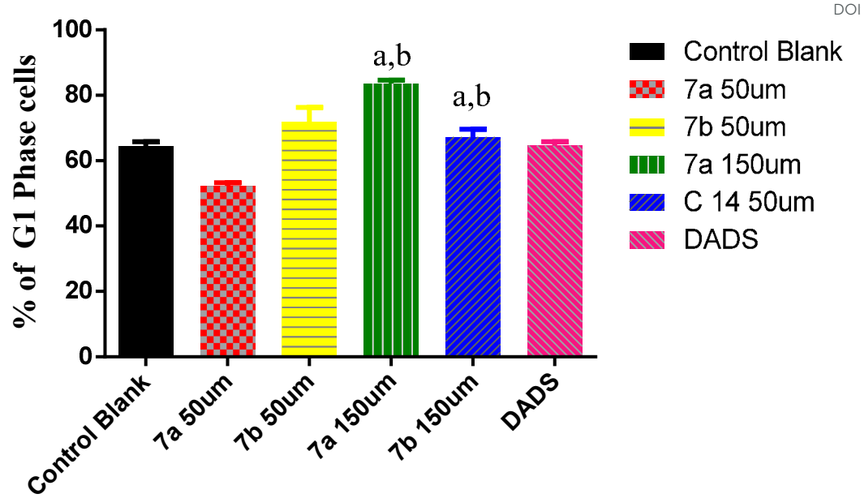

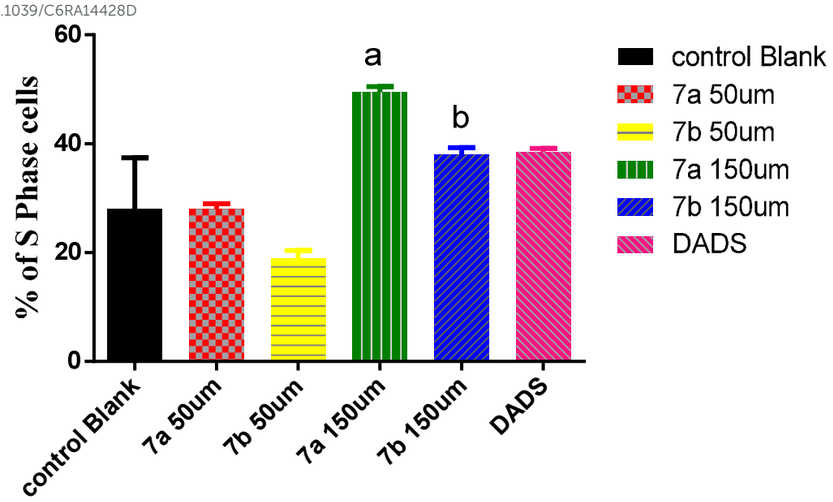

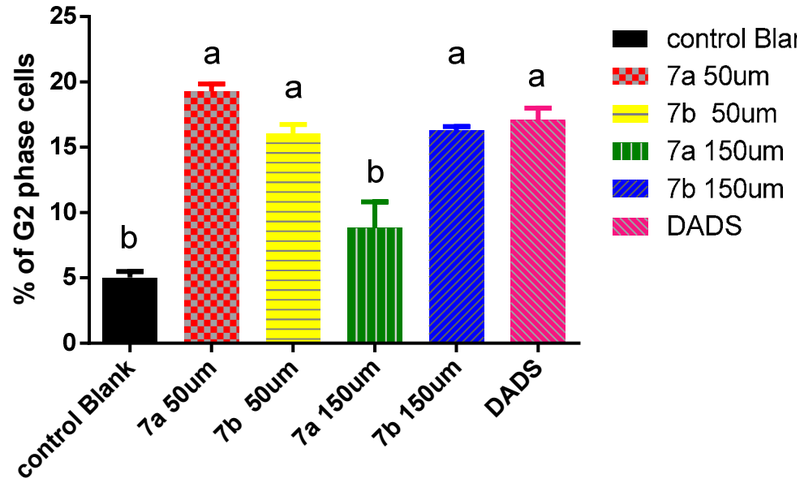

Fig.4. Influence of 7a and 7b on the cell cycle progression after 48 h treatment on MDA-MB453 cells

All values are expressed as mean±SEM, n=3. [a]P <0.05 when compared to control and [b]P<0.05 when compared to DADS (1mM). The data was analyzed by one way ANOVA followed by followed by Dunnett's multiple comparison post hoctest.

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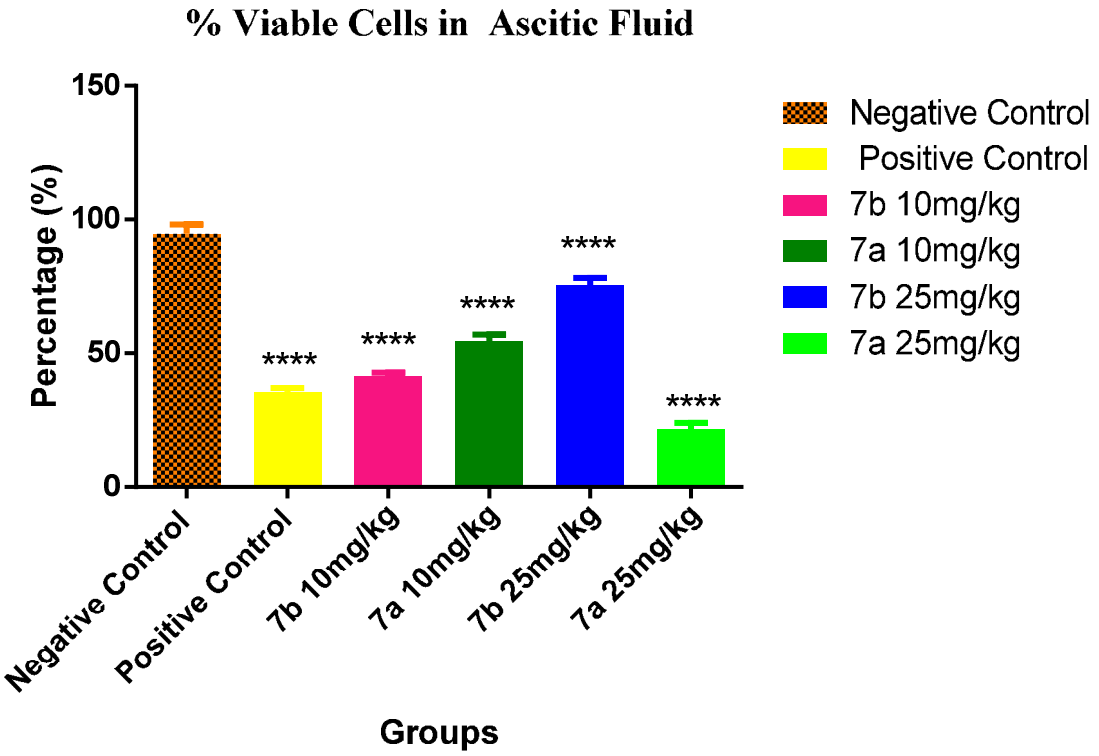

Fig 5. Effect of Selected compounds on % viability of tumor cells

All the values are mean ± SEM of six mice, *p<0.05 compared to negative control. The data was analyzed by one way ANOVA followed by Dunnett's multiple comparison post hoc test.

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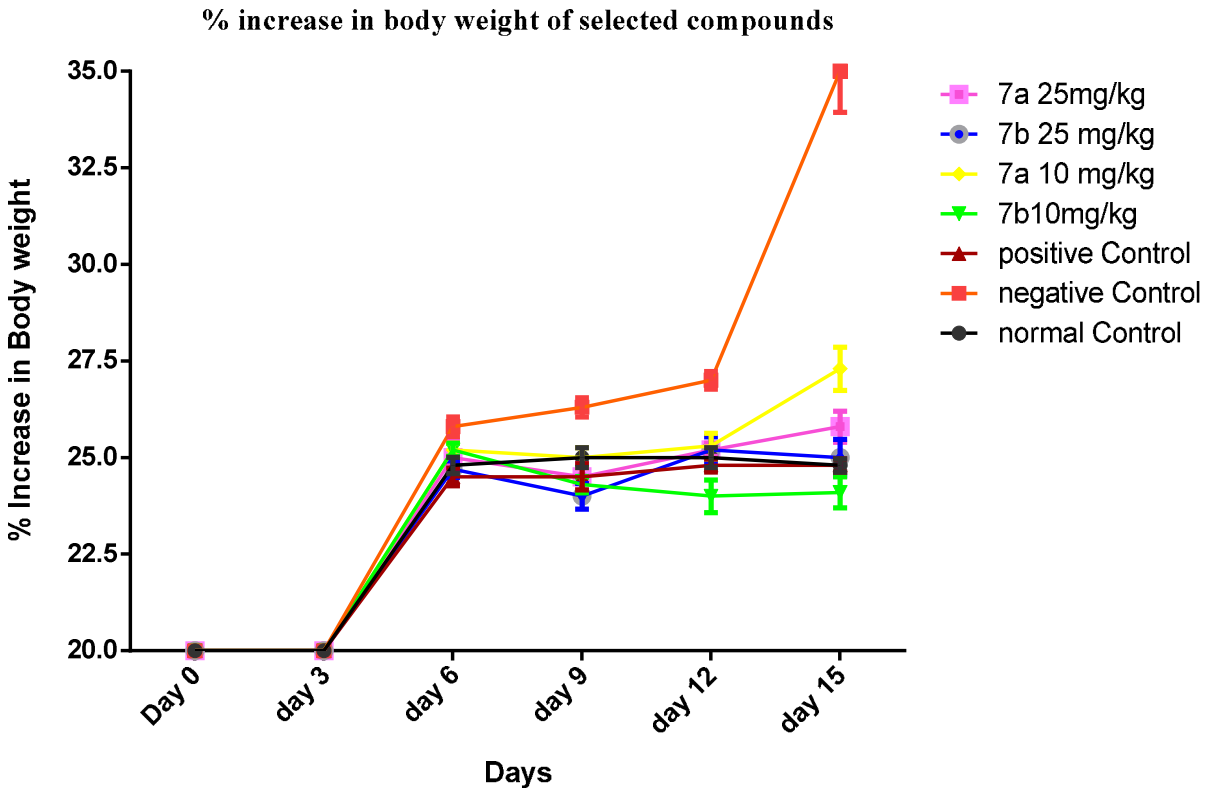

Fig. 6. The effect of selected compounds on body weight of EAC inoculated animals

All the values are mean ± SEM of six mice. The data was analyzed by one way ANOVA followed by followed by Dunnett's multiple comparison post hoc test.

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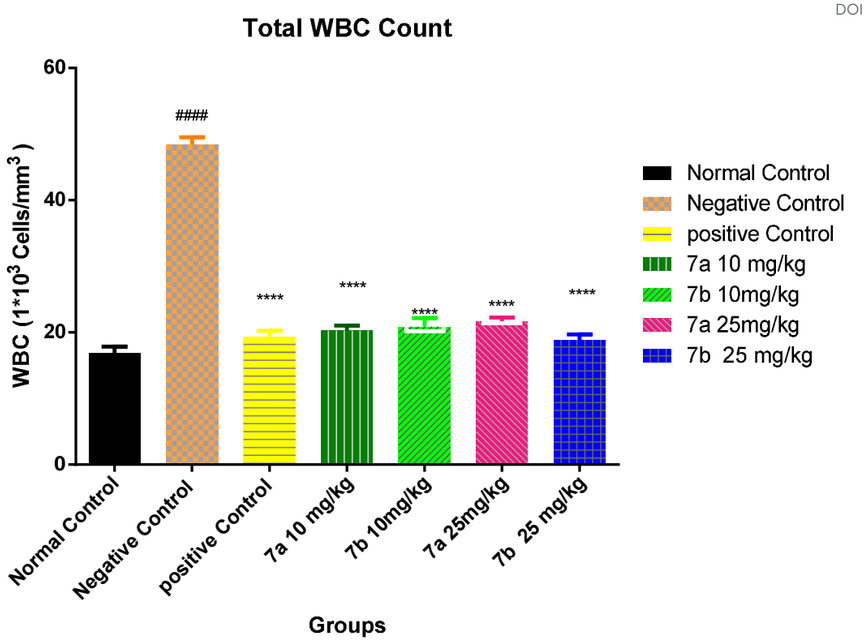

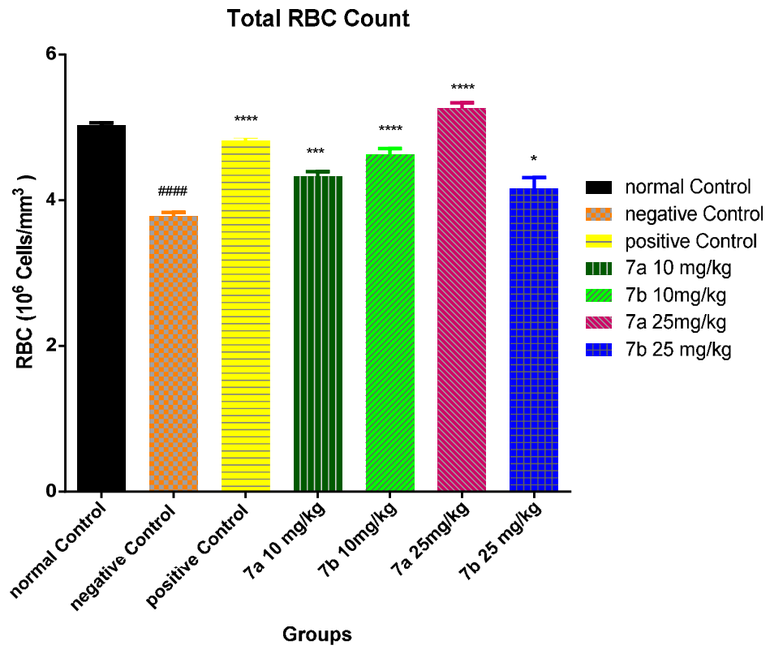

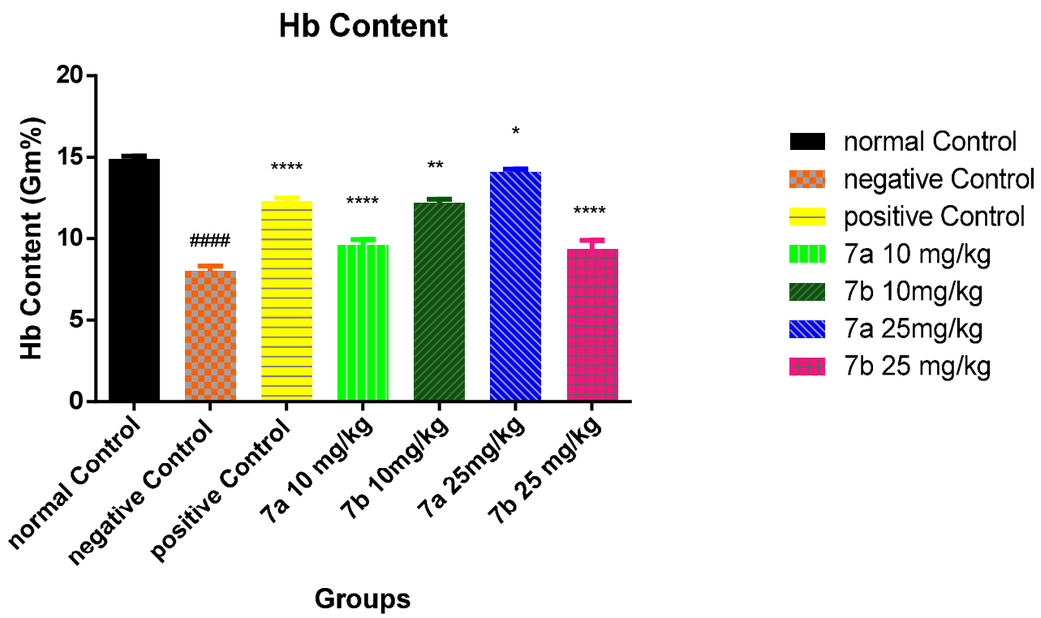

Fig. 7. Effect of 7a and 7b on haematological parameters in EAC inoculated mice

All the values are mean ± SEM of six mice, #p < 0.05 compared to normal, *p<0.05 compared to negative control. The data was analyzed by one way ANOVA followed by Dunnett's multiple comparison post hoc test.

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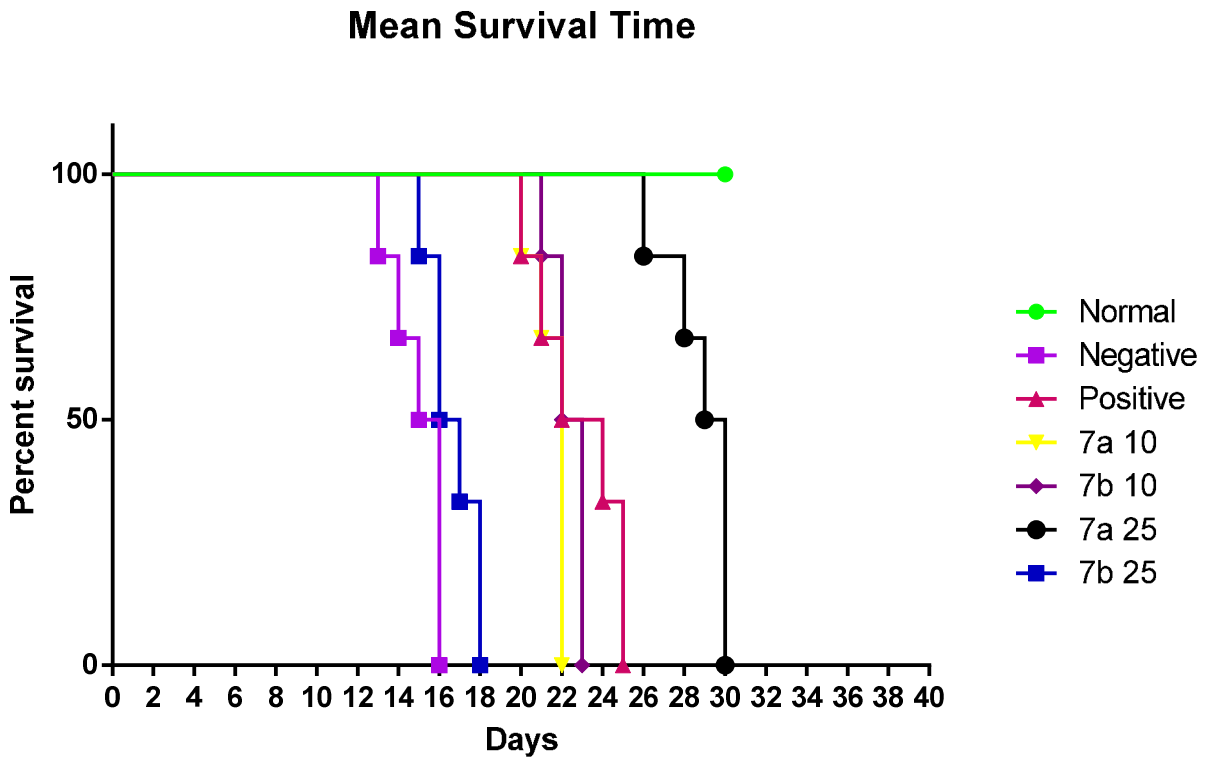

Fig.8. Effect of selected compounds on Kaplan Meier's Estimate of EAC bearing mice

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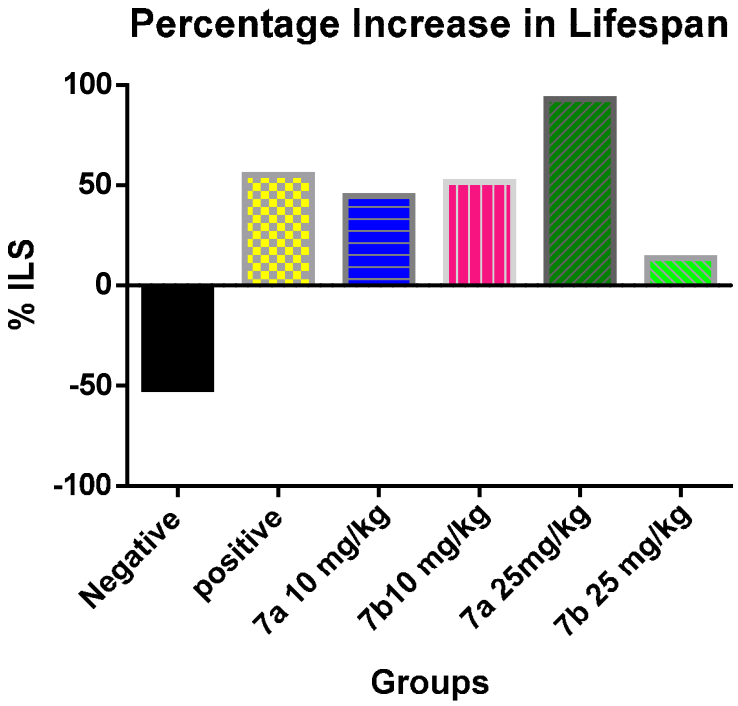

Fig.9. Effect of 7a and 7b on % increase in life span in EAC inoculated mice.

All the values are mean ± SEM of six mice, #p < 0.05 compared to normal, *p<0.05 compared to negative control. The data was analyzed by one way ANOVA followed by Dunnett's multiple comparison post hoc test.

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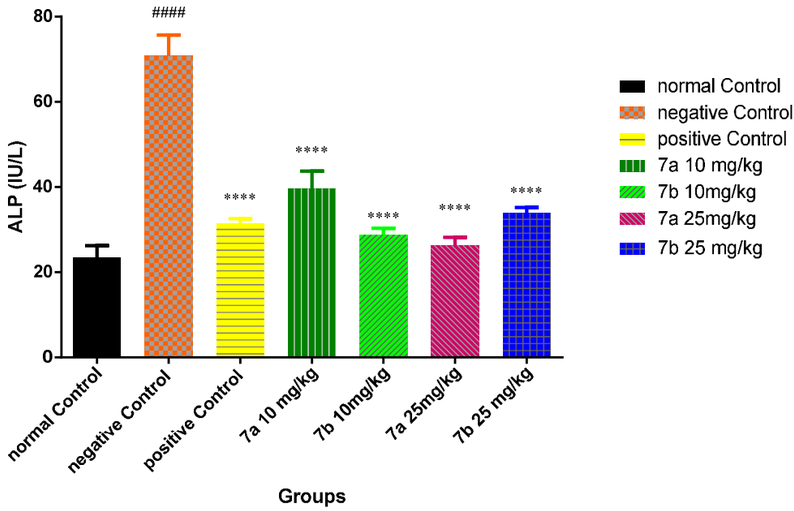

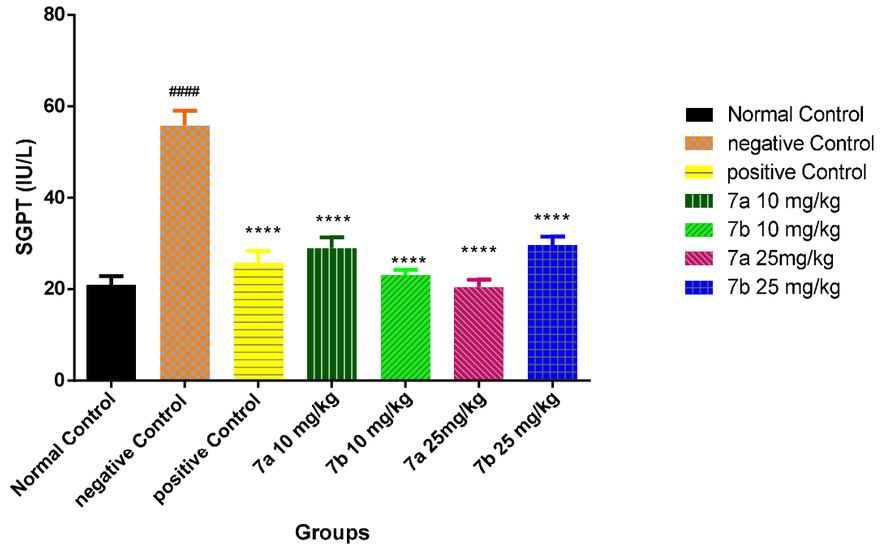

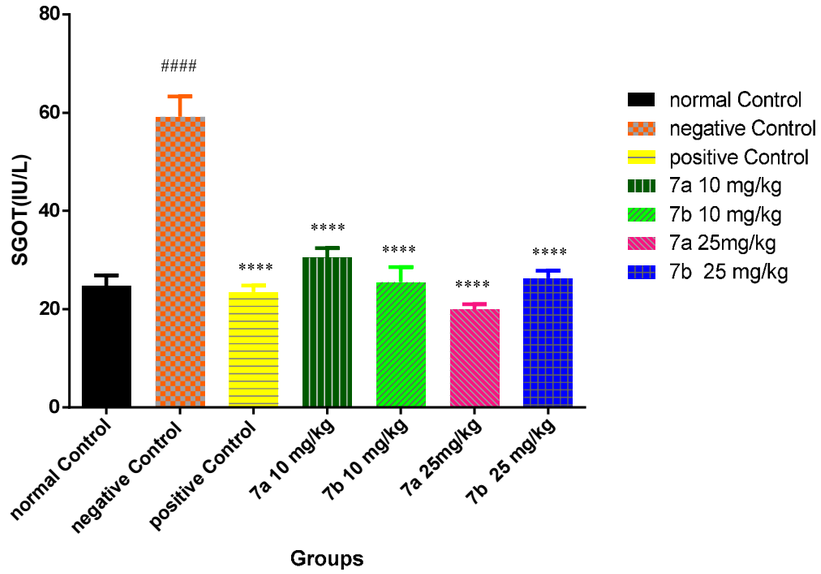

Fig.10. Effect of 7a and 7b on EAC inoculated mice serum enzymes level

All the values are mean ± SEM of six mice, #p < 0.05 compared to normal, *p<0.05 compared to negative control. The data was analyzed by one way ANOVA followed by Dunnett's multiple comparison post hoc test.

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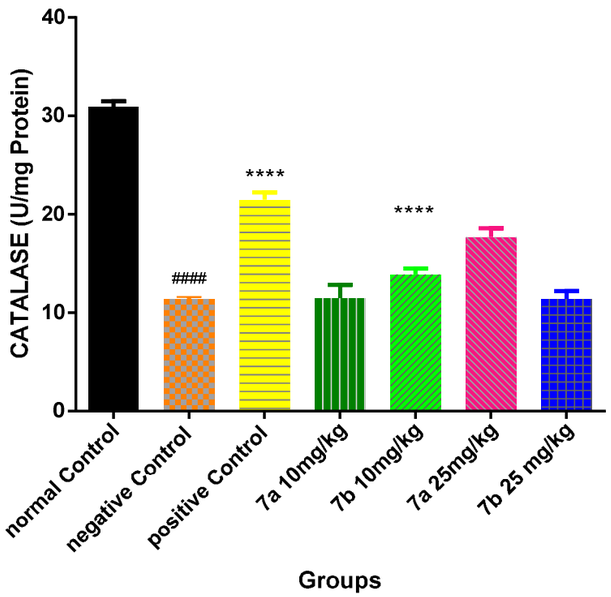

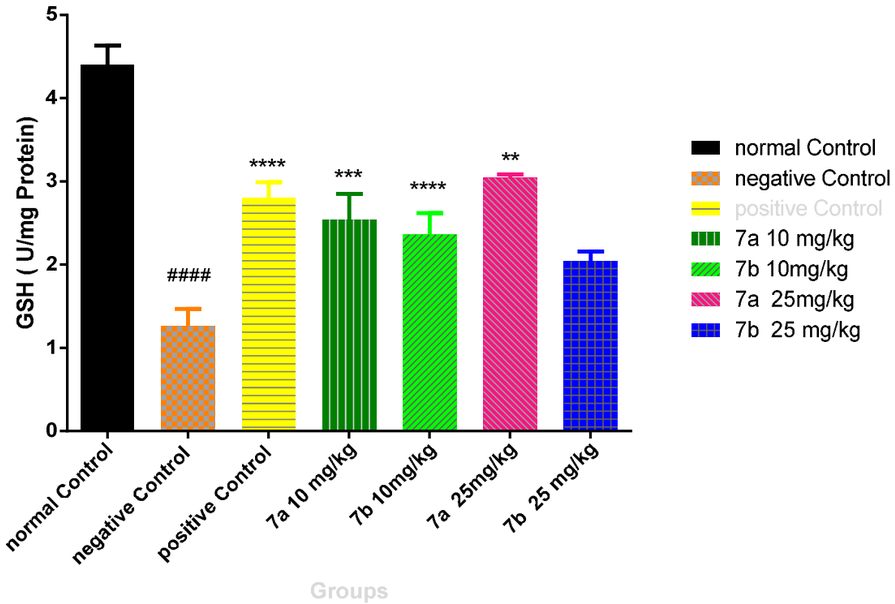

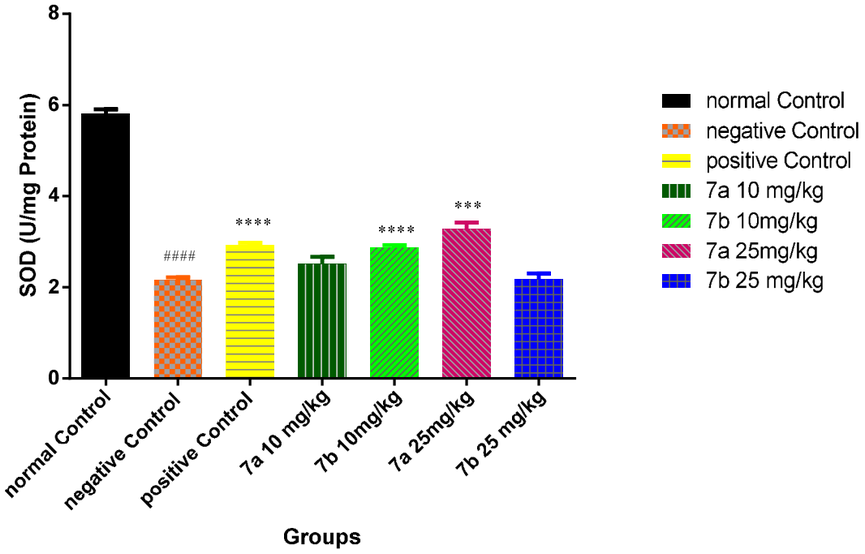

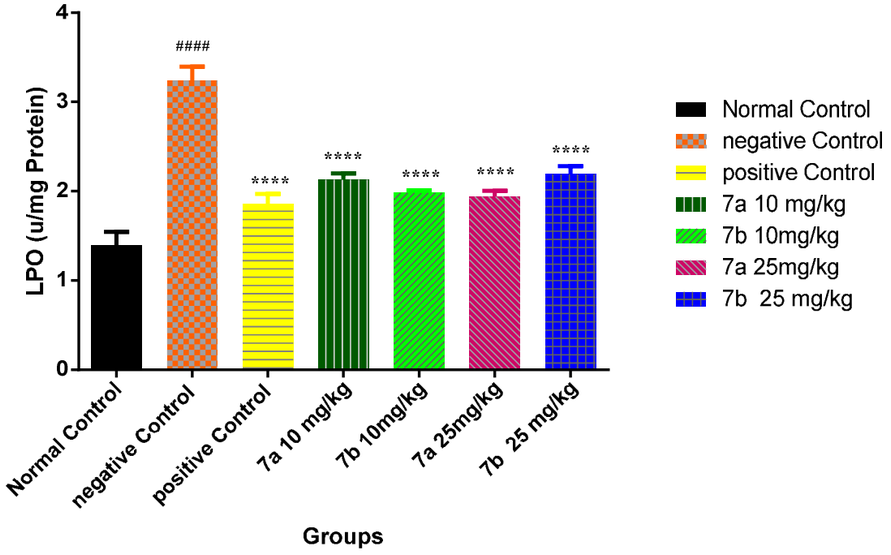

Fig. 11. Effect of 7a and 7b on liver antioxidant markers

All the values are mean ± SEM of six mice, #p < 0.05 compared to normal, *p<0.05 compared to negative control. The data was analyzed by one way ANOVA followed by followed by Dunnett's multiple comparison post hoc test.

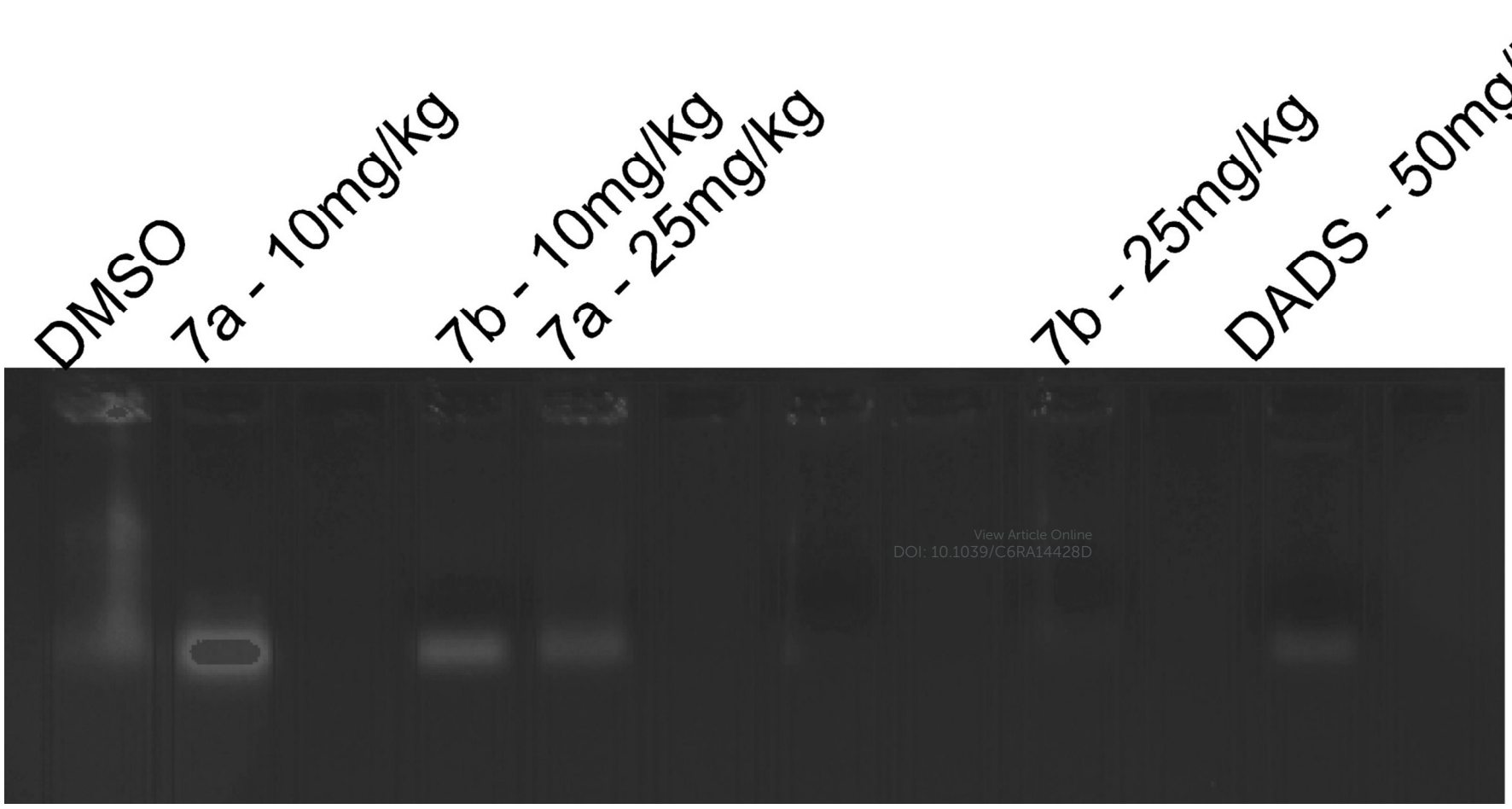

Fig. 12. In-vivo DNA fragmentation assay of EAC cells following treatment with 7a and 7b

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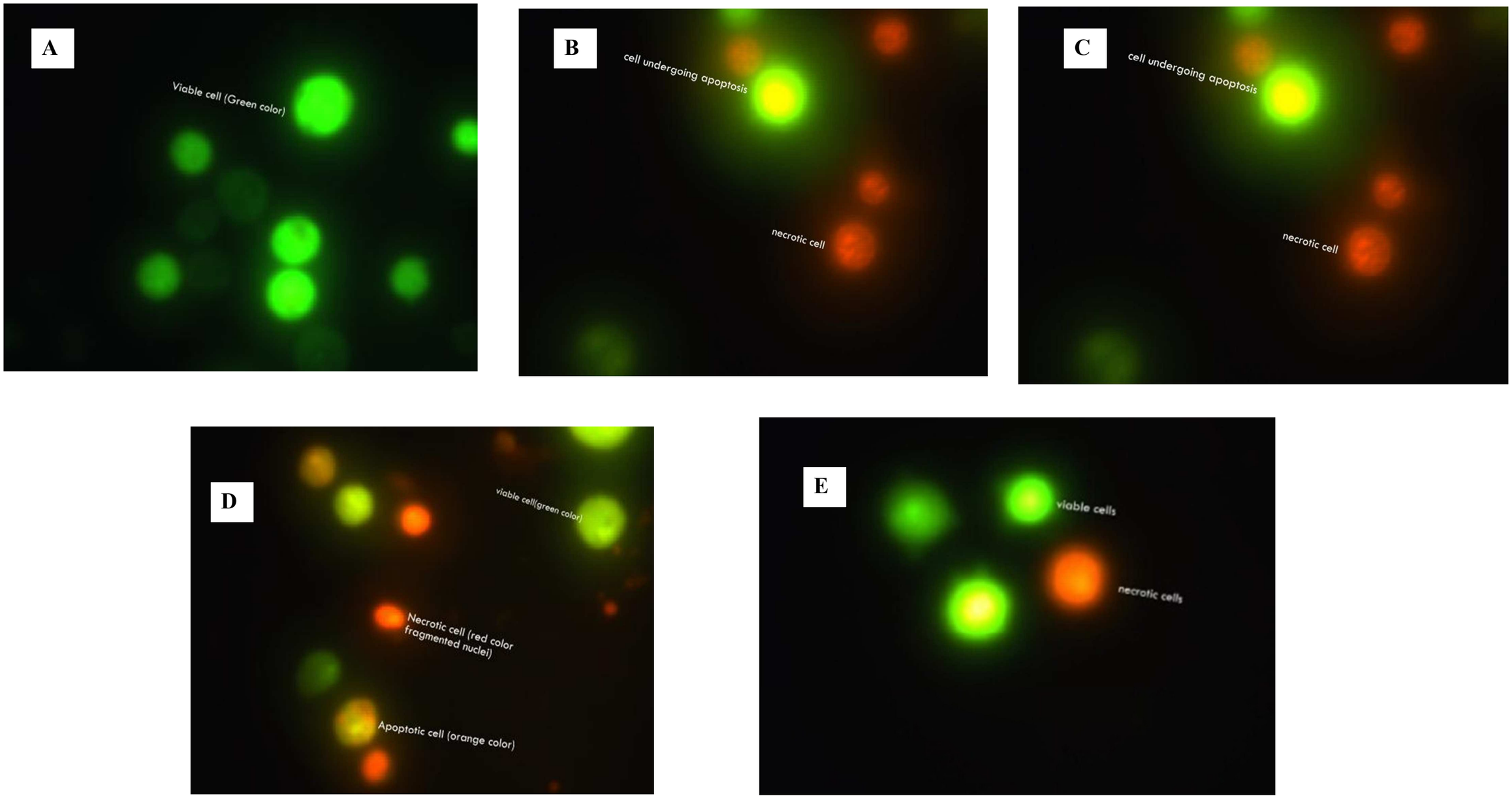

Fig.13. The fluorescent images were captured using fluorescent microscope after dual staining with AO/EB, A- control cell, B- 7a 10mg/kg, C- 7b 10 mg/kg, D-7a 25 mg/kg, E- 7b 25 mg/kg.

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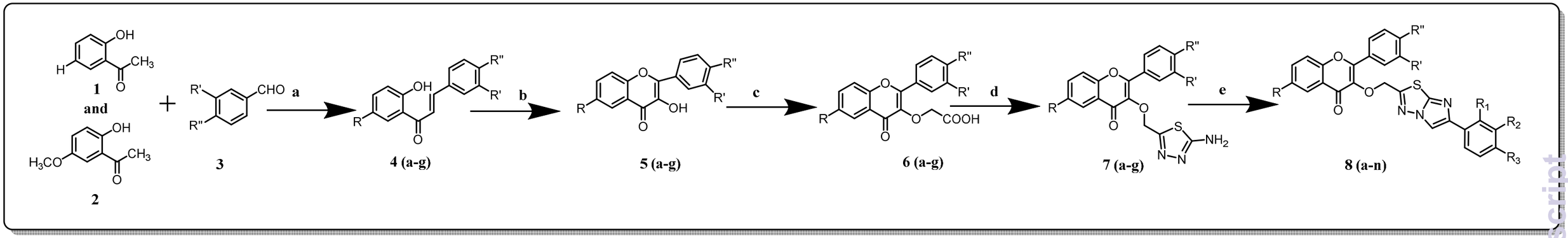
Scheme 1.



Scheme 1. **a**) NaOH, EtOH; **b**) H_2O_2, EtOH; **c**) $ClCH_2COOH$, K_2CO_3, KI, EtOH; **d**) thiosemicarbazide, H2O, $POCl_3$; **e**) Dry EtOH, Substituted phenacyl bromide, 10% Na_2CO_3

Compound No.	R	R'	R"	R^1	R^2	R^3
7a	H	H	H	-	-	-
7b	H	H	OCH_3	-	-	-
7c	H	OCH_3	OCH_3	-	-	-
7d	OCH_3	H	H	-	-	-
7e	OCH_3	H	OCH_3	-	-	-
7f	H	OCH_3	H	-	-	-
7g	OCH_3	OCH_3	H	-	-	-
8a	H	H	H	H	H	H
8b	H	H	OCH_3	H	H	H
8c	H	H	H	H	OH	H
8d	H	H	OCH_3	H	OH	H
8e	H	H	H	OH	H	H
8f	H	H	OCH_3	OH	H	H
8g	H	H	H	OH	H	OH
8h	H	H	OCH_3	OH	H	OH
8i	H	H	H	H	H	Cl
8j	H	H	OCH_3	H	H	Cl
8k	H	H	H	H	H	F
8l	H	H	OCH_3	H	H	F
8m	H	H	H	H	H	OCH_3
8n	H	H	OCH_3	H	H	OCH_3

GRAPHICAL ABSTRACT

Exploring the Anti-Breast Cancer Potential of Flavonoid Analogs

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