Gallic acid analogues with antibreast cancer and antioxidant action: synthesis and pharmacological assessment

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Abstract: Breast cancer is one of the important public health problems today and recent treatments have not been found to be very effective for advanced-stage metastatic disease of the breast. In the present study ten 3,4,5- trihydroxybenzohydrazone derivatives (AR 01- AR 10) were synthesized by two different methods viz. reflux and stirring. It was observed that compounds synthesized by stirring method acquire good yield and require less time in comparison to reflux method. Further cytotoxicity activity performed on two breast cancer cell lines viz. MDA-MB-468 and MCF-7 revealed moderate activity in all compounds at 40 μg/mL and 80 μg/mL which has the highest in samples AR 01 and AR 10 for both cell lines. Considerably all compounds have shown potent antioxidant activity at 50 μg/mL and above concentrations. Tumor in mice treated with compound AR 01 was found to be smaller in comparison to control and AR 10. Findings revealed that compound having electron donating group showed more potent activity against breast cancer cell lines both in vitro and in vivo. Cytotoxicity activity also corelates with QSAR study and showed that compounds having donating group showed positive contribution towards the toxicity. These findings suggest that gallic acid derivatives were potent cytotoxic against breast cancer cell lines and may provide potent therapeutic effects against breast cancer.

Keywords: *Gallic acid derivatives; cytotoxicity; antioxidant; in vivo; QSAR*

1. INTRODUCTION

Polyphenols constitute an important class of chemopreventive agents because of its ROS and RNS quenching and preventing nature [1]. The underlying mechanism for the quenching behaviour of polyphenolic compounds is the characteristic of benzene rings because of both their acidity (ability to donate protons) and their delocalized π-electrons (ability to transfer electrons while remaining relatively stable) [2]. Gallic acid (3,4,5-trihydroxybenzoic acid) is a well-known naturally occurring compound. It is present as hydrolysable tannins in various natural products with various biological activities these are antioxidant, anticarcinogenic, antimutagenic, antibacterial, antifungal, antiviral, neuroprotective, anti-inflammatory, induces apoptosis of tumor cells, direct inhibition of several enzyme activities [3-14].

In recent years there is much attention in the development of synthetic gallic acid derivatives with description of their pharmacological and biological activities. Various pharmacological activities have been evaluated of gallic acid derivatives including antioxidant, anticancer and neuroprotective activities. Indanone derivatives of gallic acid was found to be cytotoxic against various human cancer cell lines viz. KB403 (oral and mouth cancer cells), WRL68 (liver cancer cells), CaCO2 (colon cancer cells), HepG2 (liver cells) and MCF7 (hormone-dependent breast cancer cells) [15-17]. Gallic hydrazones containing an indole moiety exhibited antioxidant and cytotoxicity activities against human colon cancer cell line (HCT-116) and estrogen dependent human breast cancer cell line (MCF-7) [18]. One of the reviews reported the antitumoral properties of alkyl esters of gallic acid against various tumor cell lines [19].

Various mechanisms have been reported for anticancer activity of gallic acid. Some studies revealed that apoptosis is one of the reasons of inducing cancer cell death without harming normal cells [12, 20, 21]. Subramanian et al., reviewed that anticancer activity derivatives of gallic acid is related to generation of reactive oxygen species, regulation of apoptotic and anti-apoptotic proteins, suppression of oncogenes and regulation of cell cycle by arresting it [22]. One of the reviews re-

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ported that high antioxidant activity with ability to inhibit lipid peroxidation and metal ion chelation is a responsible cause of anticancer activity of gallic acid derivatives [23]. Effect of gallic acid derivatives on drug metabolizing enzyme through inhibition of cytochrome P450 activation of indirectly acting mutagens and/or by scavenging of metabolically generated mutagenic electrophiles is one of the causes as anticancer agent [24].

Computer-aided drug discovery/design methods have played powerful tools in the development of therapeutically important small molecules for the study of structure-activity relationships (SAR) [25]. Recently many Quantitative Structure Activity Relationship (QSAR) techniques have been introduced, among them the Free-Wilson analysis (2D QSAR) is a simple and convenient method that is suitable for analyzing compounds with the same parent structure having small set of compounds [26]. The method can distinguish the different contributions of each substituent to each position, and can offer useful information about the mode of action for selected compounds. Beside the QSAR study; in silico study comprises of docking study. Molecular docking is used for predicting the preferred orientation of ligands with large biomolecules, predicting the strength of the bonding forces and finding the best geometrical arrangements [27].

Further they were evaluated for *in vitro* cytotoxicity study on two breast cancer cell lines *viz.* estrogen receptor positive (MCF-7) and estrogen receptor negative (MDA-MB-468) and *in vivo study* on CH3/Jax mice tumor model. Free Wilson (2D QSAR) was analyzed through Valstat software for cytotoxicity study on both breast cancer cell lines. Additionally, different antioxidant activities (DPPH, scavenging of superoxide and iron chelating) of synthesized compounds were investigated.

2. MATERIALS AND METHODS

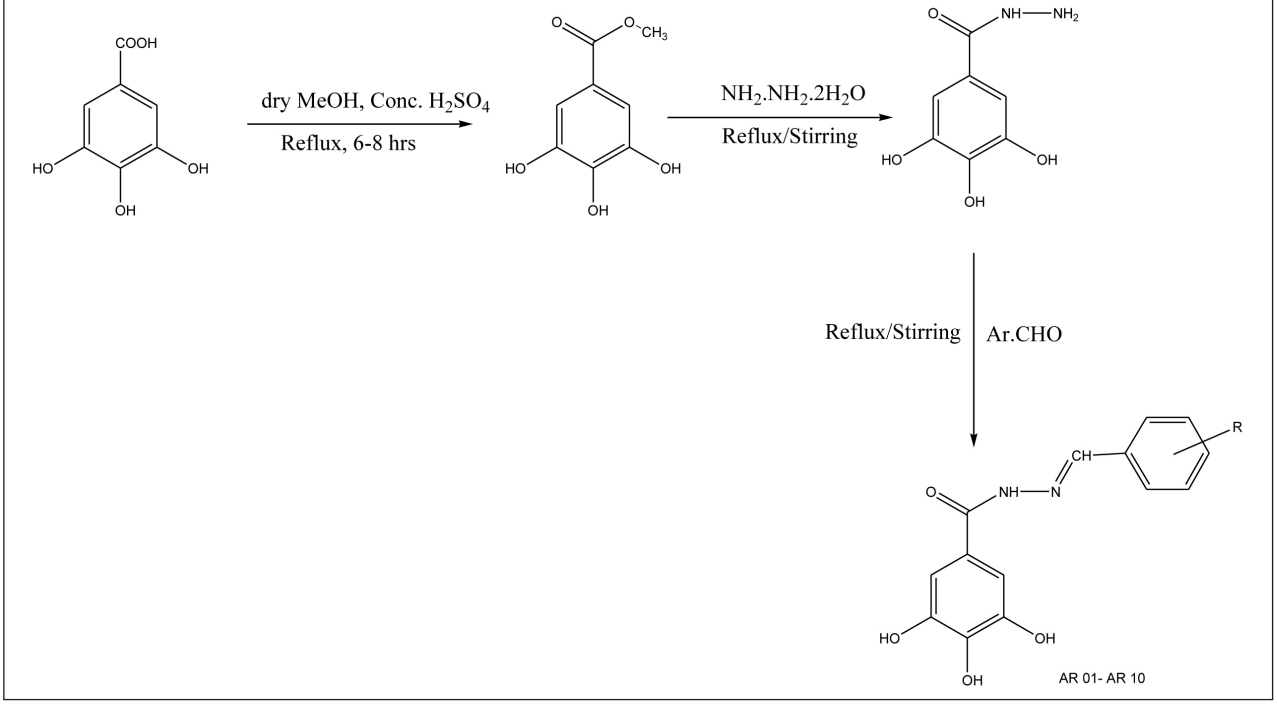
2.1. Chemicals

Synthetic materials and reagents were purchased from Sigma Chemicals Co., St. Louis, USA. All solvents were procured from Merck, Mumbai, India. All chemicals used were of analytical grade.

2.2 Synthesis method (Scheme 1)

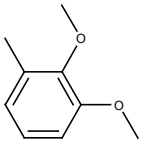
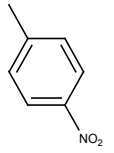
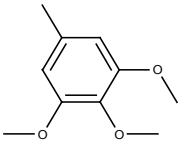
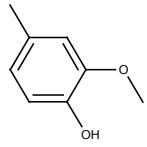
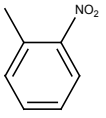
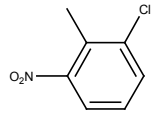
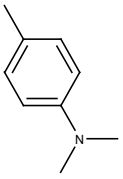
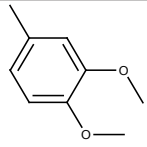
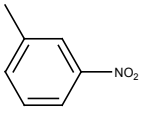
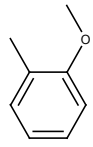
Step I: Synthesis of methyl 3,4,5-trihydroxybenzoate

The selected compounds were synthesized by 3 steps reaction. Structures of compounds with their code are depicted in Table I.



Scheme 1 *Synthesis of 3,4,5-Trihydroxybenzohydrazones (AR 01- AR-10)*

Table I *Structure of synthesized compounds with their IUPAC name*

Compounds No.	*IUPAC name*	*Substituent (R)*	*Compounds No.*	*IUPAC name*	*Substituent (R)*
AR 01	3,4,5-trihydroxy-N'-[(1E)-(2,3-dimethoxyphenyl)methylidene]-benzohydrazide		**AR 06**	3,4,5-trihydroxy-N'-[(1E)-(4-nitrophenyl) methylidene]benzohydrazide	
AR 02	3,4,5-trihydroxy-N'-[(1E)-(3,4,5-trimethoxyphenyl)methylidene]benzohydrazide		**AR 07**	3,4,5-trihydroxy-N'-[(1E)-(4-hydroxy-3-methoxyphenyl) methylidene]benzohydrazide	
AR 03	3,4,5-trihydroxy-N'-[(1E)-(2-nitrophenyl) methylidene]benzohydrazide		**AR 08**	3,4,5-trihydroxy-N'-[(1E)-(2-chloro-5-nitrophenyl)methylidene] benzohydrazide	
AR 04	3,4,5-trihydroxy-N'-[(1E)-[4-(dimethylamino) phenyl]methylidene] benzohydrazide		**AR 09**	3,4,5-trihydroxy-N'-[(1E)-(3,4-dimethoxyphenyl)methylidene] benzohydrazide	
AR 05	3,4,5-trihydroxy-N'-[(1E)-(3-nitrophenyl) methylidene]benzohydrazide		**AR 10**	3,4,5-trihydroxy-N'-[(1E)-(3-methoxyphenyl)methylidene] benzohydrazide	

A mixture of gallic acid (6.0 g, 10.8 mM), dry MeOH (120 mL) and Conc. H_2SO_4 (1.20 mL) in a round bottom (RB) flask were heated under reflux for 6-8 h. After completion of the reaction, the solvent was evaporated under pressure in vacuum and extracted with ethyl acetate followed by washing with sufficient amount of water. The organic layer was concentrated in vacuum giving the crude methyl gallate. Recrystallization was done in hot water to obtain pure methyl gallate.

In the second and third step 3,4,5-trihydroxybenzohydrazide and 3,4,5-trihydroxybenzohydrazone derivatives respectively were synthesized by two methodology *viz.* reflux (*method A*) and stirring (method B). In method A reflux was used whereas, in method B only stirring was performed to synthesiz the same compounds.

Step II: Synthesis of 3,4,5-trihydroxybenzohydrazide

Method A

Methyl 3,4,5-trihydroxybenzoate (9.2 g, 50 mmol) and hydrazine hydrate (45 mL) were taken in RB flask and stirred under room temperature until mixed completely. Ethanol (250 mL) was added to the mixture and was stirred under reflux for 6-8 h and then kept overnight under room temperature. The separated white solid was collected on a Buchner funnel filtered, washed with ethanol and dried over silica gel.

Method B

The synthesis of 3,4,5-trihydroxybenzohydrazide was tried under stirring conditions at room temperature methyl 3,4,5-trihydroxybenzoate (9.2 g, 50 mM) and hydrazine hydrate (45 ml) were taken in 100 mL conical flask and 40 mL of 50 % ethanol was added and the reaction mixture was allowed to stir for 1.5-2 h. The separated white precipitate was collected on a Buchner funnel filtered, dried and recrystallized from 50 % ethanol and dioxane mixture.

Step III: Synthesis of 3,4 5- trihydroxybenzohydrazone derivatives

Method A

3,4,5-trihydroxybenzohydrazide (0.8 mmol) was refluxed with various aromatic aldehydes (0.8 mmol) in 20 mL of 50% ethanol and one drop of glacial acetic acid for about 1-1.5 h. The reaction mixture was poured into excess crushed ice. The solid separated was filtered, washed with water and recrystallized from suitable solvent.

Method B

The synthesis of various 3,4,5-trihydroxybenzohydrazone was tried under stirring conditions at room temperature. 3,4,5-trihydroxybenzohydrazide (0.8 mmol) was allowed to stir with various aromatic aldehydes (0.8 mmol) in 20 mL of 50 % ethanol and one drop of glacial acetic acid for about 40-50 min. The reaction mixture was poured into excess crushed ice. The solid separated was filtered, washed with water and recrystallized from suitable solvent.

2.3 In vitro cytotoxicity assay

The in vitro cytotoxicity of the extract was determined using sulforhodamine-B (SRB) on estrogen receptor positive (MCF-7) and estrogen receptor negative (MDA-MB-468) breast cancer cell lines as described previously (Dhingra et al., 2016). Briefly, cell lines were pre-incubated in Dulbecco's Modified Eagle Medium (DMEM) for 24 h at 37 °C in 5% v/v CO_2 and compounds were added and incubated for another 48 h in four different concentrations (10 μg/mL, 20 μg/mL, 40 μg/mL and 80 μg/mL). After incubation, suforhodamine B was added and plates were incubated at room temperature for 30 min. Finally, the optical densitywas recorded on ELISA reader at wavelength of 540 nm using 690 nm as reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells.

$$\% \text{ Growth} = [A_1 / A_0] \text{ X } 100$$

where A_1 is the average absorbance of the test well and A_0 is the average absorbance of the control well.

2.4 In vivo activity of potent cytotoxic synthesized compounds

2.4.1 Maximum tolerated dose (MTD) assessment in C3H/Jax mice

For each drug *C3H/Jax mice* were randomly divided into groups (6/group) and received a single i.p. injection of compounds in DMSO at dose levels specified in the results. Three doses of compounds were given on every third day to the group of mice with the initial dose of 150 mg/kg. The dose was increased on the basis of the mortality of mouse/mice. With the single mortality of the mouse the MTD was decided.

2.4.2 In vivo study

The animal use and care protocol was approved by the Anti-Cancer Drug screening facility (ACDSF) at ACTREC, Tata Memorial Centre, Navi Mumbai. The animals tumors were divided into various treatment groups and a control group (6 mice/group). The untreated control group received the vehicle only. Compounds were dissolved in the vehicle, and were given i.p. at dose of 50 mg/kg thrice a week for four weeks. Relative tumor volume, tumor/control from relative tumor volume, animal body weights (in g), survival of number of mice were measured for the next 30 days. The body weight data obtained was then converted to percentage body weight changes. The length and width of tumors were measured and the volume (v) was calculated using the formula $v = (\text{width})^2 \times (\text{length}/2)$ [14]. Relative tumor volume was calculated by the following formula:

Relative Tumor Volume (RTV) = Tumor volume on day of measurement/Tumor volume on day 1. Percentage tumor growth was calculated as T/C by the following formula:

$$T/C = (T_n - T_o / C_n - C_o) \times 100$$

$$\text{If } (T_n - T_o) < 0, \text{ then } T/C = (T_n - T_o)/T_o \times 100$$

$C_o(C_n)$: Tumor weight of day 0 (day n) in the control group

$T_o(T_n)$: Tumor weight of day 0 (day n) in the treated group

2.5 QSAR Study

Free Wilson approach has been applied for QSAR study. Different functional groups on substituted aldehydes were used for the preparation of the matrix. Free and Wilson derived a mathematical model that describes presence and absence of certain structural features i.e., functional group at different position (ortho, meta and para) were represented by 1 and 0 respectively with biological activity values (Table II).

$$\text{Log } 1/C = \sum ai + \mu$$

The values of a_i in equation are the biological activity groups contributing of the substituents X1, X2.....Xi in the different positions p of compound and μ is the biological activity values of the refer-

Table II *Free-Wilson structural matrix for the compounds*

Compounds No.	*ortho*				*para*			*meta*				
	H	*OCH_3*	*NO_2*	*Cl*	*H*	*OCH_3*	*NO_2*	*H*	*OCH_3*	*$N(CH_3)_2$*	*NO_2*	*OH*
AR 01	1	1	0	0	1	1	0	1	0	0	0	0
AR 02	1	0	0	0	0	1	0	0	1	0	0	0
AR 03	1	0	1	0	1	0	0	1	0	0	0	0
AR 04	1	0	0	0	1	0	0	0	0	1	0	0
AR 05	1	0	0	0	1	0	1	1	0	0	0	0
AR 06	1	0	0	0	1	0	0	0	0	0	1	0
AR 07	1	0	0	0	1	1	0	0	0	0	0	1
AR 08	1	0	0	1	1	0	1	1	0	0	0	0
AR 09	1	0	0	0	1	1	0	0	1	0	0	0
AR 10	1	0	0	0	1	1	0	1	0	0	0	0

ence compound, most often the unsubstituted parent structure of a series.

2.6 Antioxidant activities

2.6.1. DPPH radical scavenging activity

The DPPH radical-scavenging activity was determined using the method proposed by Dhingra et al., (2016). Briefly, DPPH solution (100 μM) was added to 1 ml of polyphenol extracts with 1 mL of methanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 10 min. The decrease in absorbance of the resulting solution was monitored at 517 nm at 10 min. Butylated hydroxytoluene (BHT) was used as standard control [28]. The percent of DPPH discoloration of the sample was calculated according to the equation:

$$\% \text{ Scavenging [DPPH]} = [(A_0\text{-}A_1) / A_0] \text{ X100}$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the samples or standard.

2.6.2. ABTS assay

For ABTS assay, the procedure followed was taken from that of Arnao et al. (2001) with some modifications. Compounds (1 mL) were allowed to react with 1 mL of the $ABTS^{+}$ solution for 2 hrs. in dark condition [29]. Then the absorbance was taken at 734 nm using the spectrophotometer. The percent of ABTS radicals of the sample was calculated according to the equation:

$$\% \text{ inhibition [ABTS]} = [(A_0\text{-}A_1) / A_0] \text{ X } 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the samples and standard. The results were expressed as IC_{50} (μg/mL).

2.6.3. Scavenging of superoxide

The effect of the extract on superoxide anion radicals was estimated according to the method described previously [28]. The reaction mixture contained 1 mL each of riboflavin (3.3×10 mol L^{-1}), methionine (0.01 mol L^{-1}), NBT (4.6×10 mol L^{-1}) each. After adding 1 mL of sample of different concentrations, the reaction mixture was illuminated at 4000 lx and 25 °C for 30 min. BHT was used as standard. The absorbance of the reaction mixture was measured at 560 nm with a spectrophotometer and the scavenging percentage was calculated according to the following formula:

$$\% \text{ Scavenging} = [(A_0\text{-}A_1) / A_0] \text{ X } 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample/standard.

3. RESULTS

3.1. Synthesis study

Synthesis of 3,4,5-trihydroxybenzohydrazide (step II)

According to literature the synthesis of 3, 4, 5-trihydroxybenzohydrazide was carried out by refluxing methyl 3,4,5-trihydroxybenzoate and hydrazine hydrate in ethanol under reflux for 6-8 h. In an attempt to increase the yield and to save the time we performed the same reaction under stirring condition at room temperature for only 1.5-2 h and the yield was increased from 52 to 74 % (Table III).

Table III *Physical and analytical data of the synthesized intermediates and derivatives*

Steps/Compounds No.	*Molecular Formula*	*M.P. (°C)*	*Yield (%)*		*R_f Value*	*Solvent for recrystallization*
			A	B		
I	$C_8H_8O_5$	201-203	56	-	0.52*	Hot Water
II	$C_7H_8N_2O_4$	167-169	52	74	0.61*	Methanol
AR 01	$C_{16}H_{16}N_20_6$	169-171	76	87	0.78*	Ethyl acetate + Chloroform
AR 02	$C_{17}H_{18}N_2O_7$	171-173	76	88	0.73*	Ethyl acetate + Chloroform
AR 03	$C_{14}H_{11}N_3O_6$	170-171	77	86	0.51**	Ethyl acetate
AR 04	$C_{16}H_{17}N_3O_4$	176-177	74	83	0.68*	Ethyl acetate + Chloroform
AR 05	$C_{14}H_{11}N_3O_6$	171-172	69	78	0.76**	Ethyl acetate
AR 06	$C_{14}H_{11}N_3O_6$	171-172	78	87	0.80**	Ethyl acetate
AR 07	$C_{15}H_{14}N_2O_6$	171-173	77	92	0.75*	Ethyl acetate + Chloroform
AR 08	$C_{14}H_{10}ClN_3O_6$	168-169	82	88	0.62**	Ethyl acetate
AR 09	$C_{16}H_{16}N_2O_6$	168-169	77	86	0.81*	Ethyl acetate + Chloroform
AR 10	$C_{15}H_{14}N_2O_5$	171-173	82	87	0.48*	Ethyl acetate + Chloroform

*A, reflux condition; B, stirring condition; Rf, retardation factor; *Solvent system, Pet ether: Acetone; ** Solvent system CHCl3: MeOH*

Synthesis of 3,4,5-trihydroxybenzohydrazone derivatives (III)

The synthesis of compounds AR 01-10 was carried out by refluxing for 1.5-2 h. The yields obtained were in the range of 69-82 %. The same reaction was performed under stirring condition 40-50 min. The reaction was successful and the products were compared with the earlier products. The yield obtained was in the range of 78-92%. Hence the modified procedure was found to better one in respect of saving time and yield (Table III).

3.2. In vitro cytotoxicity study

For MCF-7 cells at the lowest concentrations tested (10 and 20 μg/mL) none of the compounds showed activity whereas at 40 μg/mL out of 10 synthesized compounds three compounds *viz.* AR 01, AR 03 and AR 10 showed cytostatic effect and showed <50 % of growth in comparison to growth. At the highest concentration tested (80 μg/mL) 7 compounds viz. AR 01, AR 02, AR 03, AR 07, AR 08, AR 09 and AR 10 showed potent activity and showed < 50 % control growth whereas, at the same concentration AR 03 showed cytocidal effect. Among all the compounds, AR 05 and AR 06 were found to be least active (Figure 1a). Growth inhibition of 50 % (GI_{50}) of cells with drug concentration resulting in a 50% reduction in the net protein increase was observed for all the compounds. AR 03 and AR 10 was found to show lowest GI_{50} values of 33.7 and 34.8 μg/mL respectively whereas, other

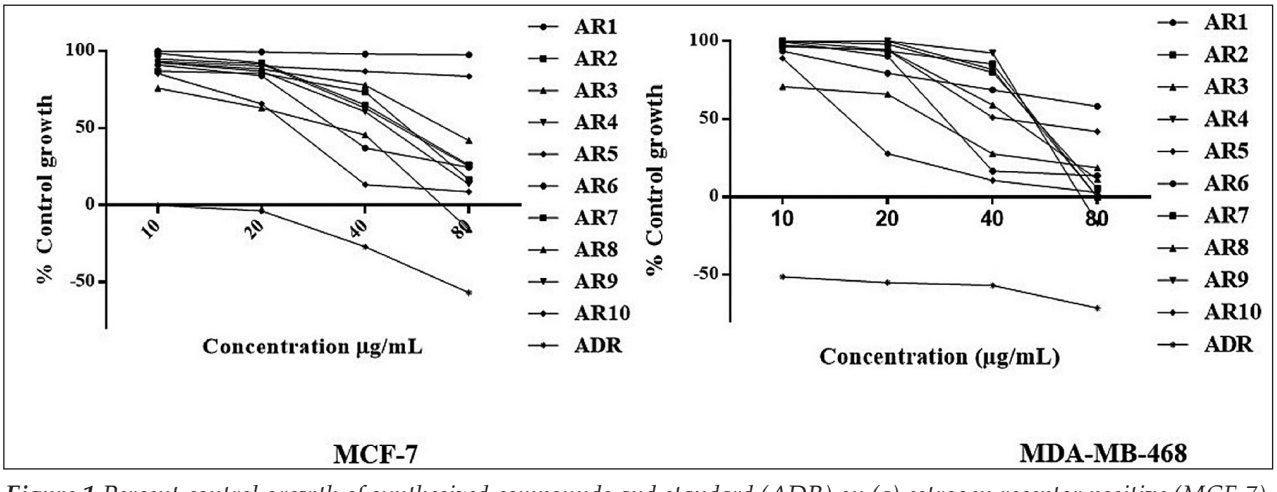


Figure 1 *Percent control growth of synthesized compounds and standard (ADR) on (a) estrogen receptor positive (MCF-7), (b) estrogen receptor positive negative (MDA-MB-468) cell lines.*

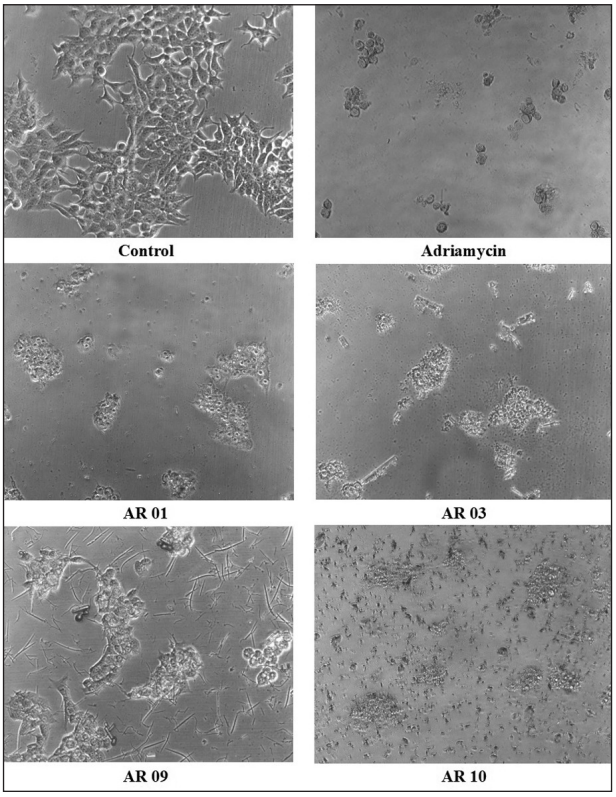


Figure 2 *Cell morphology of MCF-7 cell line.*

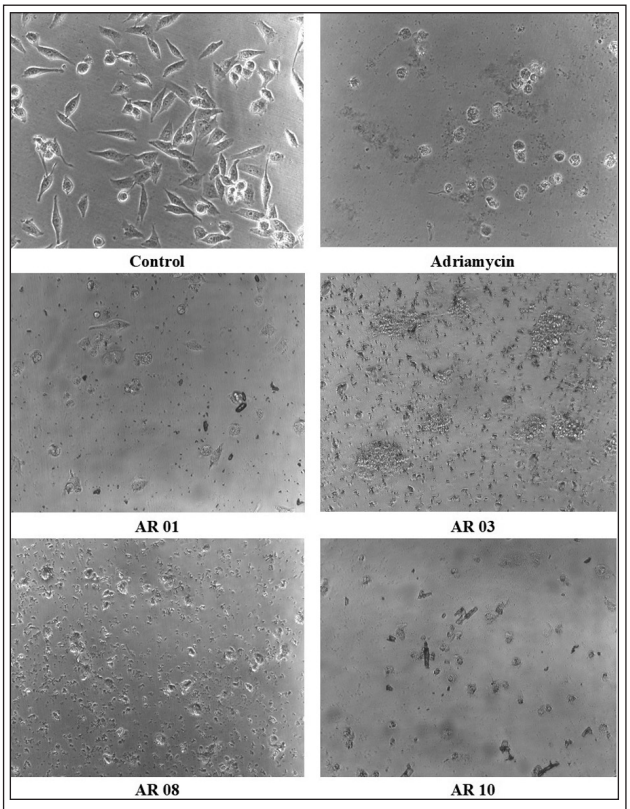


Figure 3 *Cell morphology of MDA-MB-468 cell line*

compounds gave the value in range of 48.0 – 73.0 μg/mL. AR 05 and AR 06 showed GI_{50} of > 80 μg/mL). Most cytotoxic compounds which change the cell morphology of MCF-7 cells is shown in Figure 2 AR 10 causes the cytocidal effect and reduces the number of the cells. AR 10 was found to change the morphology at the best followed by AR 01, AR 03 and AR 09.

At the highest concentration tested (80 μg/mL) all the compounds showed 50 % control growth of MDA-MB-468 cells and thus are cytostatic in nature. AR-09 was found to be the most potent at the highest concentration tested and showed cytocidal effect. At 40 μg/mL, AR 01, AR 02, AR 05 and AR 10 were found to be cytostatic, whereas, at 20 μg/mL only AR 10 was found to be potent and showed cytostatic effect. AR 06 was found to be least active among all, compounds towards MDA-MB-468 cells (Figure 1b). AR 10 was found with lowest GI_{50} value followed by AR 08 with values of 27.7 and 37.8 μg/mL respectively. AR 10, AR 01 and AR 08 were found to be most potent in respect of IC_{50} with lowest values of 32.91, 40.66 and 40.95 μg/mL respectively whereas, other compounds showed moderate activity with values in range of > 40 and ≤ 50 μg/mL. Similar to MCF-7, AR 10 was found to be most potent and changes the morphology of MDA-MB-468 cells followed by AR 01, AR 08 and AR 03 (Figure 3).

Table IV *Maximum Tolerated Dose evaluated for the most potent cytotoxic compounds*

Drug	*Maximum dose used*	*Number of animals dead / Total*	*% death*	*Toxicity Criteria*	*Further dose for MTD*
AR-01	150 mg/Kg	0/6	0	Well tolerated	300 mg/kg
AR-01	300 mg/Kg	0/6	0	Well tolerated	450 mg/kg
AR-01	450 mg/Kg	0/5	0	Well tolerated	600 mg/kg
AR-01	**600 mg/kg**	1/6	16.7		**Dose Selected**
AR-10	150 mg/Kg	0/6	0	Well tolerated	300 mg/kg
AR-10	300 mg/Kg	0/6	0	Well tolerated	450 mg/kg
AR-10	450 mg/Kg	0/6	0	Well tolerated	600 mg/kg
AR-10	**600 mg/Kg**	1/6	16.7		**Dose Selected**

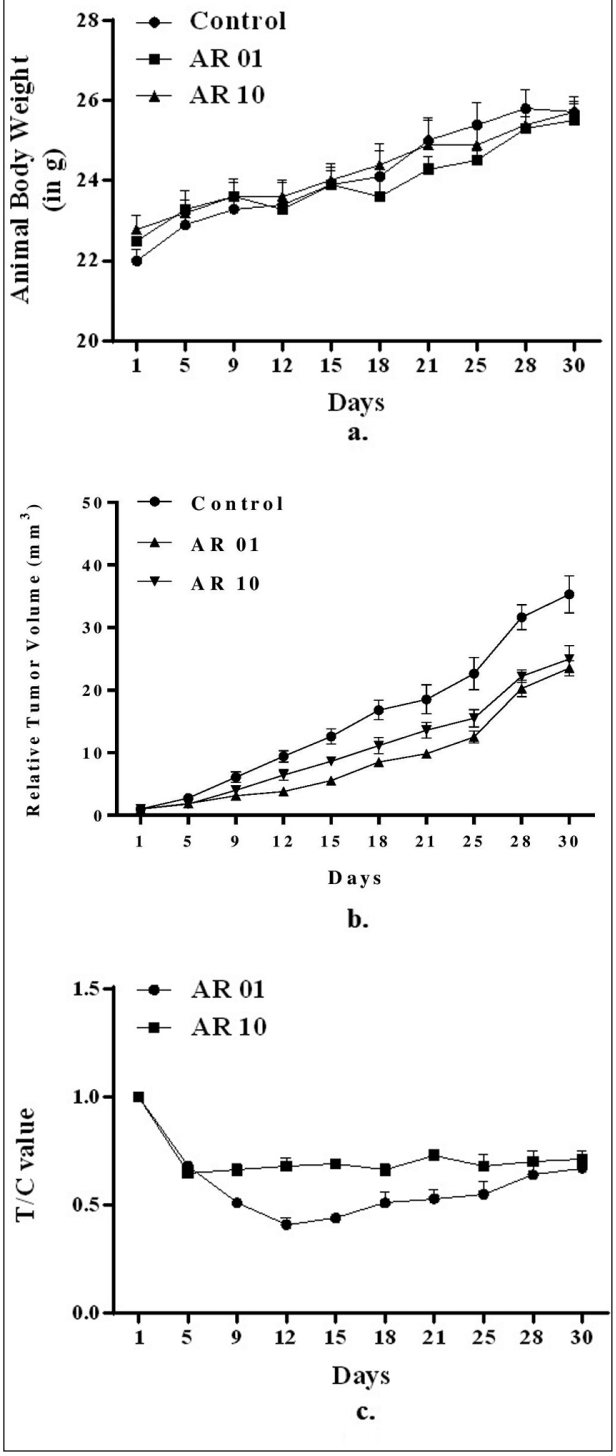


Figure 4 *In vivo study of AR 01 and AR 10 for 30 days (a) change in animal body weight, (b) change in relative tumor volume and (c) change in tumor/control value*

3.3. In vivo study

Maximum tolerable dose (MTD) of compounds AR 01 and AR 10 in mice was found to be 600 mg/kg (Table IV). No obvious evidence of toxicity was observed in treated animals by comparing the body weight as there was no change in body weight of mice for both the compounds (Figure 4a). Tumor volumes of all the mice for control, AR 01 and AR 10 were observed and mean relative tumor was calculated (Figure 4b).

Relative tumor volume of compounds in comparison to control is shown in Figure 4b. It was observed that on 18th, 21st and 25th day there was significant difference ($p<0.05$) between the relative tumor volume of control and AR 01 whereas, on 28th and 30th day there was highly significant ($p<0.01$) difference between the tumor volume of control and AR 01 and significant ($p<0.05$) difference between control and AR 10. Relative tumor volume on 28th day for control, AR 01 and AR 10 was 31.66, 20.29 and 22.23 respectively. However, on 30th day it was 35.31, 23.53 and 24.98 for control, AR 01 and AR 10 respectively (Figure 4b).

Tumor by control (T/C) ratio was measured and it was observed that AR 01 showed less T/C value in comparison to AR 10. From 9th to 25th day there was reduction in T/C value of AR 01 in comparison to AR 10 whereas, for 28th and 30th day there was no change in T/C value for both compounds (Figure 4c). Tumor in treated vs. by control was expressed as T/C ratio was less in AR 01 as compared to AR 10. From 9th to 25th day there was reduction in T/C value of AR 01 in comparison to AR 10, whereas, for 28th and 30th day there was no change in T/C value for both the compounds (Figure 7). Tumor in mice treated with compound AR 01 was smaller in comparison to control and AR 10.

3.4. QSAR study (Free-Wilson approach)

QSAR study was performed for cytotoxicity activity for both cell lines. Two best models were selected out of 10 different generated models. The best QSAR model built using multiple linear regression (MLR) method is represented by the following equation:

For MCF-7

Model 1:

BA= 4.333 - m-NO_2 (0.418) - p-NO_2 (1.5280)
(n = 10, r = 0.968, r^2 = 0.936, *variance* = 0.021, *SD* = 0.143, F = 51.771, FIT = 739.582, q^2 = 0.822) (1)

Model 2:

BA= 4.1385 + m-OCH_3 (0.1829) - p-NO2 (1.3335)
(n = 10, r = 0.922, r^2 = 0.849, *variance* = 0.048, *SD* = 0.221, F = 19.779, FIT = 282.570, q^2 = 0.752) (2)

For MDA-MB-468

Model 1:

BA= 4.30575 + m-OCH_3 (0.06245) - p-NO_2 (0.22875) (n = 10, r = 0.798, r^2 = 0.638, *variance* = 0.006, *SD* = 0.076, F = 6.154, FIT = 87.914, q^2 = 0.462) (3)

Model 2:

BA= 4.35386 - m-NO_2 0.0604 - p-NO_2 0.276857 (n = 10, r = 0.781, r^2 = 0.611, *variance* = 0.006, *SD* = 0.078, F = 5.488, FIT = 78.394, q^2 = 0.407) (4)

where, n is the number of observations, r is the correlation coefficient, r^2 is the squared correlation coefficient, *SD* is the standard error of estimate, p is the statistical significance with Fisher's statistic F, q^2 is cross-validated square correlation coefficient.

Model generated for MCF-7 cells cytotoxicity was statistically highly significant in comparison to MDA-MB-468 cells. The high correlation coefficient r (0.96 and 0.9217) indicates the susceptibility of descriptors to form the above model (1 and 2). Squared correlation coefficient (r^2) of 0.94 and 0.85 explains 94% and 85% variance in biological activity of the tested compounds. It also indicates the statistical significance >99.9% with F values (51.778 and 19.779). Cross-validated square correlation coefficient (q^2) by LOO technique was 0.82 and 0.75 which showed a good internal predictive ability of the model 1 and 2 respectively.

From the contribution values for both MCF-7 and MDA-MB-468 activity it is clear that the electron-donating group on the phenyl ring had posi-

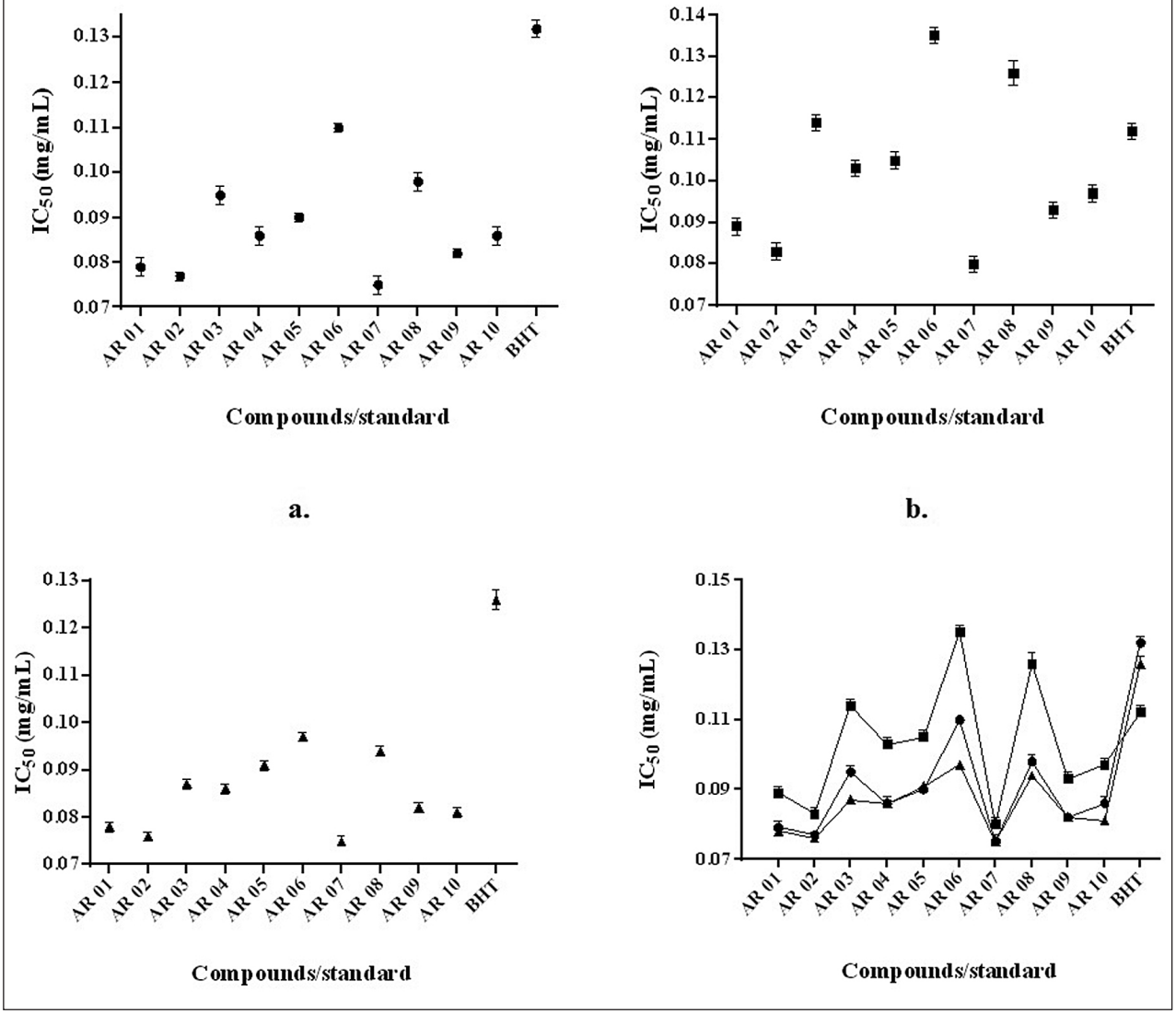


Figure 5 *IC_{50} values of different antioxidant activities (a) DPPH scavenging, (b) ABTS scavenging, (c) Scavenging of superoxide and (d) comparison of antioxidant activities.*

Table V *Experimental and predicted values from QSAR models for MCF-7 and MDA-MB-468 cell lines activity*

	MCF-7		*MDA-MB-468*	
Compounds No.	*Experimental* $pI_{C5}0$	*Predicted* pIC_{50}	*Experimental* pIC_{50}	*Predicted* pIC_{50}
AR 01	4.324	4.333	4.391	4.368
AR 02	4.284	4.333	4.314	4.368
AR 03	4.466	4.333	4.321	4.306
AR 04	4.258	4.333	4.314	4.305
AR 05	3.693	3.915	4.199	4.305
AR 06	2.804	2.805	4.077	4.077
AR 07	4.246	4.333	4.296	4.368
AR 08	4.138	3.915	4.388	4.305
AR 09	4.317	4.333	4.356	4.368
AR 10	4.436	4.333	4.482	4.368

tive contribution and electron withdrawing group had negative contribution toward the activity. Compounds having nitro group at ortho position contribute positively toward the activity. The experimental and predicted values for both the activities are given in Table V.

3.5. Antioxidant activities

3.5.1. DPPH scavenging

DPPH scavenging capacities of synthesized compounds have been expressed in IC_{50} values (Figure 6a). All the compounds showed potent activity in comparison to standard BHT and showed IC_{50} value n range of 0.075-0.110 mg/mL. AR 07 was found to be most potent followed by AR 02 and AR 01 with IC_{50} value of 0.075 ± 0.002, 0.077 ± 0.001 and 0.079 ± 0.002 mg/mL. The values of IC_{50} for compounds AR 09, AR 10, AR 04, AR 05, AR 03, AR 08, AR 06 and BHT were 0.082 ± 0.001, 0.086 ± 0.002, 0.086 ± 0.002, 0.090 ± 0.001, 0.095 ± 0.002, 0.098 ± 0.002, 0.110 ± 0.001 and 0.132 ± 0.002 mg/mL respectively. The DPPH scavenging of compounds in respect of their IC_{50} values were in the order of AR 07 > AR 02 > AR 01 > AR 09 > AR 10 ~ AR 04 > AR 05> AR 03 >AR 08 > AR 06 > BHT.

At the highest concentration (200 µg/mL) AR 07, AR 02, AR 01 and AR 09 scavenged > 90% of DPPH. AR 10, AR 04, AR 05 and AR 03 scavenged > 80 % and < 90% whereas; AR 08, AR 06 and BHT scavenged > 70 % and < 80% of DPPH (Figure 6a).

3.5.2. ABTS scavenging

The IC_{50} values of all the compounds are depicted in Figure 5b. The order of IC_{50} values of all the synthesized compounds is AR 07 > AR 02 > AR 01 > AR 09 > AR 10 > AR 04 > AR 05 > BHT AR 03 >AR 08 > AR 06. The IC_{50} values of all compounds and standard are in the range of 0.080-0.135 mg/mL. AR 07 exhibited ABTS scavenging activity followed by AR 02 and AR 01 with IC_{50} value of 0.080 ± 0.002, 0.083 ± 0.002 and 0.089 ± 0.002 mg/mL respectively. AR 06 showed lowed ABTS scavenging activity with IC_{50} value of 0.126 ± 0.003 mg/mL.

At the highest concentration (200 µg/mL), AR 07 and AR 02 scavenged > 90 % of ABTS, AR 01, AR 09, AR 10 scavenged > 80 % and < 90 % whereas, AR 04, AR 05, AR 03 and BHT scavenged > 70 % and < 80 % of ABTS. AR 08 and AR 06 showed moderate activity with scavenging of > 50 % and < 70 % of ABTS (Figure 6b).

3.5.3. Scavenging of superoxide

All the compounds significantly scavenged the superoxide radical in dose-dependent manner. All compounds showed potent superoxide scavenging activity in comparison to standard BHT. Similar to the previous antioxidant activities, AR 07 exhibited highest superoxide scavenging with IC_{50} value of 0.075 ± 0.001 mg/mL followed by, AR 02 and AR 01 with IC_{50} value of 0.076 ± 0.001 and 0.078 ± 0.001 mg/mL respectively. The scavenging of compounds in respect of their IC_{50} values were in the order of AR 07 > AR 02 > AR 01 > AR 10 > AR 09 > AR 04 > AR 03> AR 05 >AR 08 > AR 06 > BHT (Figure 5c). Correlation of IC_{50} values of all antioxidant activities is depicted in Figure 5d.

At the highest concentration (200 µg/mL) AR 07, AR 02, AR 01 AR 10 and AR 09 scavenged >

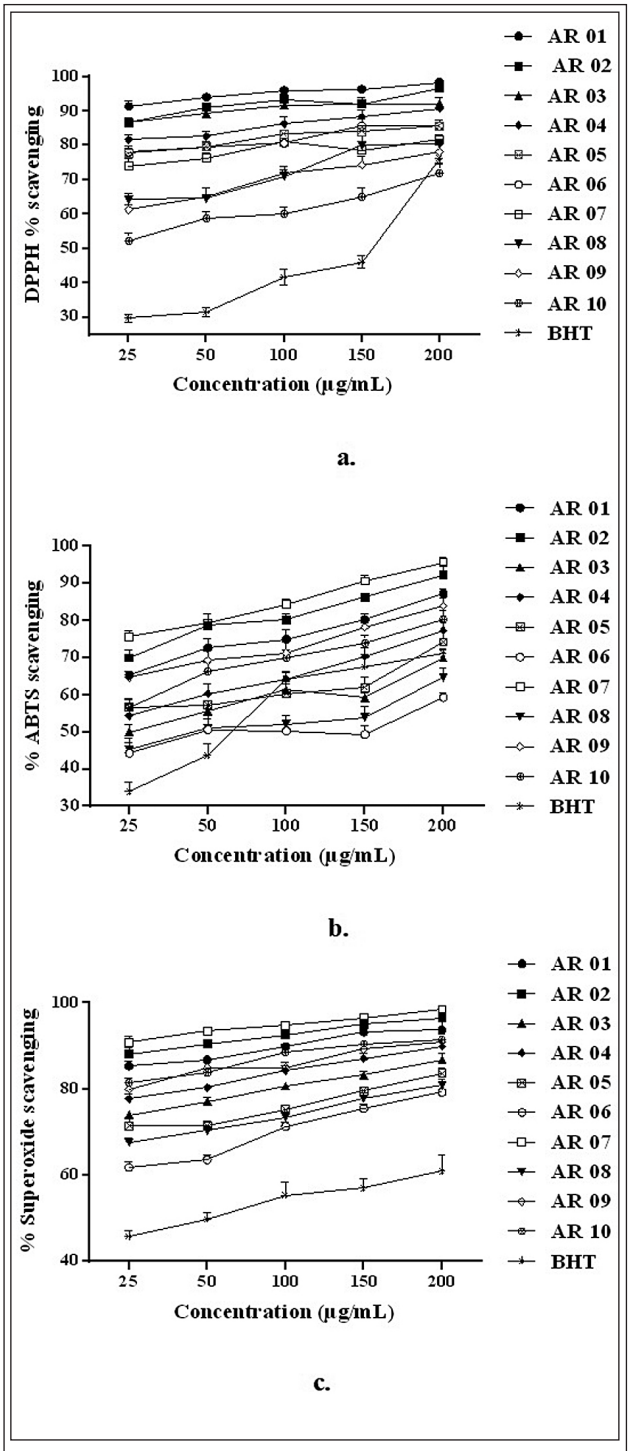


Figure 6 *Percentage scavenging of free radicals by synthesized compounds at five different concentrations a) DPPH scavenging (b) ABTS scavenging (c) Scavenging of superoxide*

90% of superoxide whereas, AR 04, AR 03, AR 05 AR 06 and AR 08 scavenged > 75 % and < 90 % superoxide, BHT showed lowest activity with scavenging of 60 % of superoxide radicals (Figure 6c).

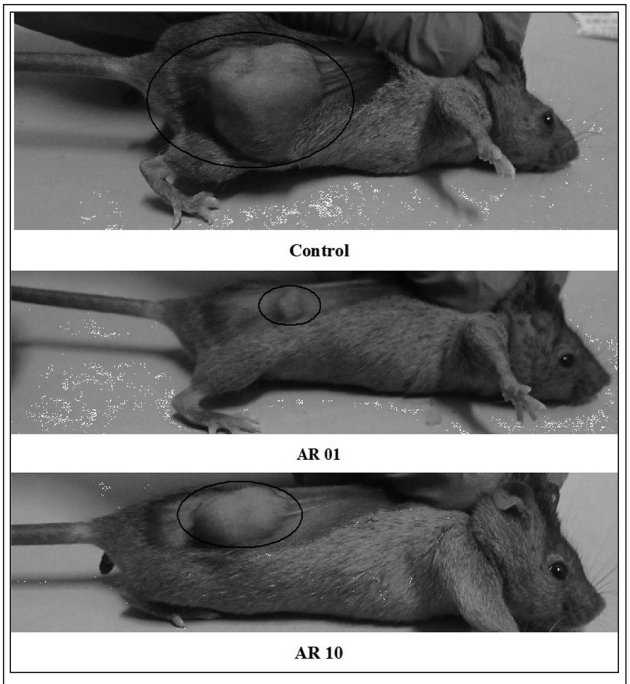


Figure 7 *Implanted tumor tissue in control, AR 01 and AR 10.*

4. DISCUSSION

Gallic acid, a natural phytochemical, present in various fruits, vegetables and nuts. It is known to possess various biological activities such as anti-tumor, antioxidant and antinflammatory cardio-protective, neuroprotective and anti-ageing [30, 31]. It has been reported that antiumor activity of gallic acid is associated with the anti-oxidative stress nature [32]. Because of its natural origin and various beneficial activities, the interest in the synthesized derivatives of gallic acid is still there. Studies have reported that derivatives of gallic acid such as schiff base, indanone, hydrazones derivatives and azo exhibited anticancer, antioxidant ability and neuroprotective effect, scavenging of free radicals, induction of apoptosis of cancer cells, etc. [16, 18, 33].

Out of the two methods (reflux and stirring) to synthesize 3,4,5-trihydroxybenzohydrazone derivatives, stirring method was found to be more effective with respect of yield, time and energy. In the reaction, methyl 3,4,5-trihydroxybenzoate underwent a nucleophilic substitution reaction with hydrazine hydrate to result in the synthesis of 3,4,5-trihydroxybenzohydrazide. The possible mechanism that the acid hydrazide underwent Schiff reaction with various aromatic aldehydes to yield various Schiff bases. This reaction first of all requires protonation of carbonyl oxygen of aldehyde by acetic acid. The nitrogen of free amino

group of acid hydrazide attacks the electron deficient carbonyl carbon of protonated aldehyde to form an intermediate known as a carbinolamine. This carbinolamine is again protonated by acetic acid, followed by deprotonation and dehydration to yield the Schiff bases.

There are several studies investigating the cytotoxicity effects of gallic acid derivatives on various cancer types. Alkyl esters of gallic acid are effective against various cancer cell lines HL60RG (human promyelocytic leukemia), P388-D1 (mouse lymphoid neoplasm), HeLa (human epithelial carcinoma), dRLh-84 (rat hepatoma), PLC/PRF/5 (human hepatoma), and KB (human epidermoid carcinoma) cells and these compounds exhibited low toxicity in vivo and a relative selectivity to tumor cells, exhibiting potential antitumor activity [19, 34]. Jara et al. reported that alkyl gallate triphenylphosphonium lipophilic cations showed selective cytotoxicity mouse mammary adenocarcinoma TA3/Ha cell line. Gallic acid based indanone derivatives exhibited potential cytotoxicity against various human cancer cell lines viz. cell lines *i.e.*, KB403 (oral and mouth cancer cells), WRL68 (liver cancer cells), CaCO2 (colon cancer cells), HepG2 (liver cells) and MCF7 (hormone-dependent breast cancer cells) [16,35]. Different mechanisms have been proposed for the anticancer activity of gallic acid derivatives. One of the papers reported that hydrophobic moiety of alkyl gallic acid derivatives seem to contribute greatly to the activity, presumably by increasing affinity for cell membranes and permeability [35]. Jara et al. (2014) reported that mitochondrial membrane potential might be one of the mechanisms underlying the anticancer activity. In vivo activity of alkyl gallate triphenylphosphonium cation at the dose of 10 mg of on CAF1-Jax mice showed resulted in a survival rate of mice without significant differences compared with the control group [35]. Van et al. (1986) reported that gallic acid is non-toxic, even when administered at 120 mg/kg/day in rats [37].

In the given study gallic acid derivatives were found to be cytotoxic against breast cancer cell lines. *In vivo* activity of our compounds showed that at 600 mg/kg of dose they were not toxic. Majority of the electron donating groups is found to be more potent against breast cancer cell lines and thus intracellular antioxidant activity of the compounds might be the reason of their cytotoxicity. Compounds which comprise of methoxy group are found to be more potent as compared to compounds containing nitro group. Previously it has been reported that compounds having hydroxyl and methoxy group inhibits more growth of ovarian cell than compounds having nitro group. Compounds having methoxy group at meta position are found to be more cytotoxic than compounds having methoxy at para position whereas, in case of nitro group substituted compounds ortho nitro is considered to be more potent than meta and para nitro substituted compounds. Similar results were observed for colon cancer too [38]. Our results concord with the given studies. For MCF-7 cell line compounds having nitro at para position and methoxy at meta position were found to be most potent with least IC_{50} value whereas for MDA-MB-468 compounds methoxy at meta position and di methoxy at ortho and meta position were more effective. Compounds having nitro at meta and para position were found to be least active with highest IC_{50} value for both MCF-7 and MDA-MB-468 cell lines.

The correlation between various descriptors with biological activity is the most important means of structure–activity relationship (SAR) study. Equation is generated with minimum number of descriptors to obtain best fit. By interpreting the resulting descriptors from the equations generated, it is possible to gain some insight into factors that are likely to govern the cytotoxic activity. In the given study free Wilson approach was performed which incorporates the contributions made by various structural fragments to the overall biological activity [39-41].

In the given study all the donating group *viz.* p-OH, o-OCH_3, m-OCH_3, p-OCH_3 and p-$N(CH_3)_2$ on phenyl ring had positive contribution to toxicity. Majority of the compounds are positioned at 3^{rd}, 4^{th} or 5^{th} position and thus -O will be easy available to form hydrogen bond with binding site. It is clear from the equation that o-OCH_3 showed very low contribution towards cytotoxicity activity. Group o-NO_2 showing positive contribution towards cytotoxicity activity for both cell lines, could be due to "penetrating effect" which causes a great change on the electronic cloud on -OH group [42].

The DPPH and ABTS radical-scavenging assays are tow common assays to access antioxidant activities. Both assays work on the principle of redox functioned proton ion for unstable free radicals and thus stabilize the harmful free radicals in the human body [43]. Mechanism behind both the activities is the reduction of unstable free radicals by hydrogen-donating antioxidants to a stable

free radical [44-46]. Mechanism behind superoxide scavenging is that, superoxide anion, being weak oxidant, produces dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress. It is known that donating group, particularly hydroxyl group plays one of the major reasons for the antioxidant activity [47]. The ortho and para hydroxyl substitution is commonly regarded as important for the radical scavenging activities. In our study compound AR 07 possessed highest antioxidant activity due to the presence of hydroxyl group at para position and one methoxy group at meta position. It is reported that presence of hydroxyl group in place of methoxy group is prone to make compound more antioxidant and thus compound AR 02 (three methoxy group) exhibited less antioxidant activity than AR 07. Compounds AR 05, AR 06 and AR 08 are found to be with least antioxidative activity due to the presence of electron withdrawing group (-NO_2). In the given study compounds with electron donating group are found to be potent antioxidant in nature.

Conclusion

A total of ten gallic acid analogues were synthesized and were screened for cytotoxicity on breast cancer cell lines and various antioxidant activities. It was observed that compounds synthesized by stirring method acquires more yield and requires less time. All synthesized compounds showed potent antioxidant activity at 50 μg/mL and above whereas, compound AR 01 and AR 10 showed potent cytotoxic activity at 40 μg/mL and 80 μg/mL for both cancer cell lines. Further cytotoxicity activity with QSAR study showed that compounds having donating group showed positive contribution towards the toxicity. Development of these cytotoxic agents against breast cancer cell lines with significant antioxidant property might be useful for anticancer drug development in the future.

Conflict of Interest

There is no conflict of interest among authors.

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