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In vitro cytotoxic, antioxidant, hemolytic and cytoprotective potential of promising ethacrynic acid derivatives

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Abstract: The present study aims to screen some pharmacological activities of three promising ethacrynic acid (**EA**) derivatives (**P3**, **P4**, and **P5**) containing a piperazine moiety. The **EA** derivatives were tested against MDA-MB-468 cancer cell line as a model for the triple-negative breast cancer and MCF7 cancer cell line as a model subtype of luminal breast cancer using the methyl tetrazolium test (MTT). Additionally, the modulation of the glutathione and thioredoxin enzymatic systems, as well as the antioxidant and cytoprotective potential of each compound, were investigated. Moreover, initial *in vitro* safety screening was conducted using human blood cells. As a result, **EA** derivatives showed clear dose-dependent antiproliferative activity in the micromolar range (between 1.13 and 2.51 µM), with high selectivity to cancer cells by orchestrating oxidative stress. Derivative **P3** has the most promising potential for further preclinical investigation, owing to its safety profile and significant cytoprotective and antitumor properties.

Keywords: Ethacrynic acid, breast cancer, antioxidant activity, antioxidant enzymes, reactive oxygen species

1. Introduction

Cancer is a serious public health problem, and breast cancer is the most common cause of cancer-related death in women (Sung *et al.*, 2021). Female breast cancer in women has distinctive histological and biological characteristics, clinical presentations, and therapeutic responses (Barzaman *et al.*, 2020; Testa *et al.*, 2020; Yersal & Barutca, 2014). Triple-negative breast cancer (TNBC) is the most invasive subtype of breast cancers (Bardia *et al.*, 2019), which is characterized by insensitivity to targeted therapies since it has a lack of human epidermal growth factor receptor 2 (HER2-), progesterone

receptor (PR-), and estrogen receptor (ER-) expression. Hence, TNBC has long been a challenging disease to treat (Bouchmaa *et al.,* 2018; Tarantino *et al.,* 2022).

Although intensive therapeutic approaches have been developed, chemotherapeutic drugs remain a crucial approach to combat breast cancer. They are also considered to be the principal pillars of cancer therapy research (Bardia *et al.,* 2019). The current treatment approaches for patients with TNBC are still unsatisfactory and poly-chemotherapy is the standard treatment for early TNBC to assess tumor sensitivity(Tarantino *et al.,* 2022). The major obstacles to achieving effective chemotherapy are acquired resistance (Barzaman et al., 2020), high general systemic toxicity, and metastasis (Pondé *et al.,* 2019; Xu *et al.,* 2021). Accordingly, the advancement of newest or supplemental chemotherapeutic candidates is still considered a tremendously challenging medicinal chemistry field (Ali *et al.,* 2012). Therefore, the development of new, alternative, efficient, and noninvasive antitumor drugs to combat systemic relapse for breast cancer therapy is a priority in biomedicine.

Over the last few decades, multiple distinct mechanisms contributing to drug resistance have been identified. The modulation of antioxidant enzyme expression is one of the common mechanisms underlying a prominent class of chemotherapeutic drugs (Yang *et al.,* 2018). Conspicuously, the largest anticancer drugs typically increase the amount of intracellular reactive oxygen species (ROS) that overcome the reduction ability of cancerous tissue and integrally alter the redox homeostasis of cancer cells (Castaldo *et al.,* 2016). Nevertheless, ROS abundance is suppressed by enzymatic antioxidants (glutathione, peroxiredoxins, catalase (CAT), superoxide dismutase (SOD), etc.) and non-enzymatic antioxidants (vitamins, uric acid, bilirubin, etc.) (Ali *et al.,* 2012; Moussa *et al.*, 2020). The antioxidant systems allow cancer cells to face ROS-induced damage (Bułdak *et al.,* 2014; Shanker *et al.,* 2010). Although ROS induction is a potent therapeutic approach for treating cancer cells, its combination with the inhibition of antioxidant processes could potentially lead to the control of cancer cell resistance (Kim *et al.,* 2019).

Ethacrynic acid (**EA**) is a potent inhibitor of glutathione S-transferases (GSTs) through the cysteine side chain, which reduces resistance to oxidative stress in cancer cells (Dong *et al.,* 2018). The α, β-unsaturated carbonyl unit plays a crucial role in the activities of **EA** and its derivatives (Dong *et al.,* 2018; Mignani *et al.,* 2016). It has been reported that **EA** has been explored as an antitumor agent at high concentrations, as well as a drug that potentiates many chemotherapeutic agents to improve treatment outcomes in chemo-resistant tumors (Aizawa *et al.,* 2003; Awasthi *et al.,* 1996; Bernig *et al.*, 2016). Furthermore, several **EA** analogs have been widely reported as promising antitumor agents and sensitizing tumors in drug combinations against different cancer types (Punganuru *et al.,* 2016; Yu *et al.,* 2023; Zhang *et al.,* 2013).

In our previous study, we developed an efficient series of **EA** derivatives bearing a piperazine moiety with high cytotoxic effects (El Abbouchi *et al.,* 2020; Mignani *et al.,* 2016). We also showed that the **EA** derivatives activated the caspase cascade without altering cell division (Mignani *et al.*, 2016). In addition, they caused caspase-induced apoptosis through mitochondrial dysfunction in HCT116 cells (El Abbouchi *et al.,* 2021). In light of this, the present study, accordingly, aims to test selective active **EA** derivatives toward TNBC. The three **EA** derivatives were investigated for their ability to inhibit TNBC cell proliferation. Therefore, *in vitro* cytotoxicity assay was conducted using MDA-MB-468 and MCF7 cell lines as models for TNBC and (ER+ and PR+) molecular grade subtypes of breast cancer, respectively. The antioxidant and cytoprotective properties were investigated using different *in vitro* methods that utilize different principles of redox reactions. Initial hemocompatibility

and toxicity tests were conducted on non-cancerous human peripheral blood mononuclear cells (PBMCs) and red blood cells (RBCs). Thereafter, the modulation of glutathione and thioredoxin-dependent systems in response to **EA** derivatives was investigated to elucidate the mechanisms underlying their cytotoxic effects.

2. Methodology

The **EA** derivatives (**Figure 1**) were synthesized following our previously reported procedure (El Brahmi *et al.*, 2015; Mignani *et al.*, 2016).

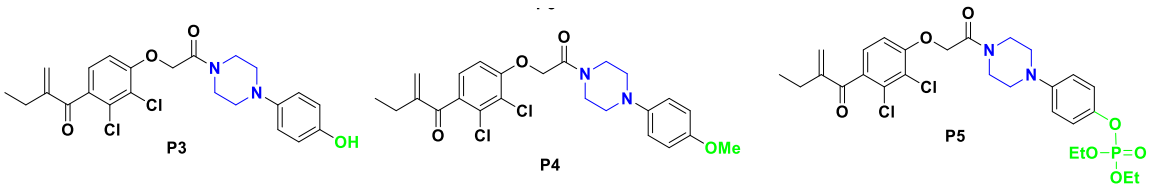

Figure 1. Structures of compounds P3, P4 and P5**.**

2.1 Cell culture

The adenocarcinoma cancer cell lines MDA-MB-468 and MCF-7 were grown in RPMI 1640 culture medium supplemented with heat-inactivated fetal bovine serum (5%), L-glutamine (2%), and penicillin/G-streptomycin (1%). The cell culture was performed in a humidified atmosphere at 37 °C and 5% CO_2.

2.2 Cytotoxicity assay against tumor cells

The *in vitro* antitumor activity of **EA** derivatives on adenocarcinoma cancer cell lines, using the [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2H-tetrazolium bromide] (MTT) assay, was assessed as described by (Ben Mrid *et al.,* 2019) with some modifications. Briefly, the target cells were washed with phosphate buffer solution (PBS, pH 7.4), seeded in 96-well microtiter plates (2×10^4 cells per well for MCF7 and 10^4 cells per well for MDA-MB-468), and incubated overnight before treatment. Cells were treated with 100 µL of culture medium containing different concentrations (0.30–40 µM) of each **EA** derivative. DMSO and paclitaxel were used as negative and positive controls, respectively. The final concentration of DMSO in all cases was less than 0.2%. Following 48h of incubation in a humidified atmosphere at 37 °C and 5% CO_2, 20 µL of MTT solution (5 $mg.mL^{-1}$ in PBS) was added to each well and incubated under the same conditions. After 4h, 150 µL of medium was carefully removed from each well and replaced with 150 µL of acid-isopropanol (0.04 N HCl in isopropanol) to dissolve the Formosan crystals. The optical density was measured at 540 nm using MultisKan EX (Labsystem) microplate reader. Cell viability was determined by dividing the absorbance values of the treated cells by those of untreated cells.

2.3 Cytotoxicity evaluation toward normal cells: PBMCs and RBCs

Blood samples from healthy human donors were collected in sterile EDTA-coated tubes under the supervision of a medical and ethics committee. PBMCs were isolated by Ficoll-Hypaque density centrifugation as described by the manufacturer (Capricorn Scientific). PBMCs were seeded into 96-well microtiter plates at a density of 2×10^4 cells/well. The cytotoxic effect was evaluated under the same conditions, and concentrations as those previously described for tumor cells. Residual red blood

cells (RBCs) were repeatedly rinsed with PBS and suspended in 40 mL of PBS (2%). The RBCs suspension (200 µL) was separately added to 200 µL of PBS containing different concentrations (3.12–50 µM) of **EA** derivatives. Distilled water and PBS were used as the positive (PC) and negative (NC) controls, respectively. The reaction mixture was mixed gently, while being incubated at 37 °C for 6 h, and centrifuged for 5 min at 7000 g. The optical density of the supernatant was recorded at a wavelength of 540 nm. The percentage of hemolysis was calculated using the following formula(S. Li et al., 2017):

$$\text{Hemolysis } \% = \frac{\text{Abs (Sample)} - \text{Abs (NC)}}{\text{Abs (PC)} - \text{Abs (NC)}} \times 100$$

2.4 Antioxidant enzymes activity

2.4.1. Cells extract preparation for enzymatic assays

As described in our previous work (Bouchmaa *et al.,* 2019). Human adenocarcinoma MCF7 and MDA-MB-468 tumor cells were treated with the IC_{50} of **EA** derivatives. After 48 h, cells were harvested and washed with PBS at 1200 g for 10 min. The cells were then lysed in 500 µl of a lysis buffer composed of 1 mM phenylmethanesulfonyl (PMSF), 0.1% Triton X-100, 1.5 $\mu g.mL^{-1}$ aprotinin, 2.5 $\mu g.mL^{-1}$ leupeptin and 2 mM EDTA in Tris-HCl buffer (50 mM, pH 8), under constant agitation for 30 min at 4 °C. After centrifugation at 1600 g for 20 min at 4 °C, the supernatant containing soluble cytosolic proteins was used to determine the enzymatic activity of glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPX), superoxide dismutase (SOD), thioredoxin reductase (TrxR), and isocitrate dehydrogenase (NADP-ICDH) enzymes. The protein concentration in the cell extracts was estimated using the Bradford method (Bio-Rad) with bovine serum albumin as the standard.

2.4.2. Cells extract preparation for enzymatic assays

GST activity was measured using CDNB as a substrate by applying a modified method of Habig (Habig *et al.,* 1974). The reaction mixture was composed of 0.1% Triton X100, 2 mM CDNB, and 5 mM reduced glutathione (GSH), and cells extract in phosphate buffer (100 mM, pH 7.5). The rate of change in absorbance at 340 nm was monitored spectrophotometrically. The CDNB conjugate concentration was calculated using the molar extinction coefficient of 9.6 $mM^{-1}. cm^{-1}$ and GST activity was expressed as nmol of CDNB conjugates formed $min^{-1}. mg^{-1}$ of proteins.

SOD activity was measured using the method described in our previous study (Oulmidi *et al.*, 2021). The assay mixture was composed of 2 µM riboflavin, 75 µM nitro blue tetrazolium (NBT), 0.1 µM EDTA, and 10 mM methionine, and the cells extract in phosphate buffer (50 mM, pH 7.5). The change in the absorbance of the mixture was measured at 560 nm. SOD activity was expressed as $unit.mg^{-1}$ of proteins. One unit of SOD activity corresponds to the quantity of enzyme that inhibits NBT reduction by 50%.

GPx activity was measured using a method described in our previous study (Bouchmaa *et al.,* 2018). Briefly, the reaction mixture contained 250 µM NADPH, 100 µM EDTA, 1 mM sodium azide, 1 mM GSH, 10 $\mu g.mL^{-1}$ GR and the cells extract in potassium phosphate buffer (100 mM, pH 7). After 3 min of incubation at 25 °C, 250 µM of H_2O_2 was added to the reaction mixture. NADPH oxidation was monitored at 340 nm wavelength for 5 min. GPx activity was calculated and expressed as µmol NADPH oxidized $min^{-1}. mg^{-1}$ of proteins, using an extinction coefficient of 6.2 $mM^{-1}. cm^{-1}$.

TrxR activity was estimated following the reduction of DTNB at 412 nm, as described in our previous work (Bouchmaa *et al.*, 2018). The reaction mixture comprised 1 mM EDTA, 250 µM NADPH, and 1 mM DTNB, and the cells extract in 100 mM phosphate buffer (pH 7.6). TrxR activity was expressed as nmol of reduced DTNB min^{-1}. mg^{-1} of proteins, using an extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$.

GR activity was assayed using a modified protocol of Carlberg and Mannervik (Oulmidi *et al.*, 2021). The reaction mixture was composed of 0.2 mM NADPH and 1 mM GSSG in phosphate buffer (100 mM, pH 7.6). After a short incubation period of 3 min at 25 °C, the reaction was initiated by adding cells extract. The rate of NADPH oxidation was monitored at 340 nm wavelength. Using the extinction coefficient of 6.2 mM^{-1}. cm^{-1}, GR activity was calculated and expressed as nmol of NADPH oxidized min^{-1}. mg^{-1} of proteins.

NADP-ICDH activity was determined using a modified procedure of (Bouchmaa *et al.*, 2019). The reaction mixture contained 4 mM isocitric acid, 1 mM $MnCl_2$, 0.2 mM $NADP^+$ and cells extract in phosphate buffer (50 mM, pH 7.5). The reduction of $NADP^+$ was monitored at 340 nm. NADP-ICDH activity was expressed as µmol of reduced NADP min^{-1} mg^{-1} of proteins.

2.5 Antioxidant activities

In this study, various complementary spectrophotometric methods were used to assess the antioxidant ability of **EA** and its derivatives, including the 1,1-Diphenyl-2-picryl hydrazyl (DPPH) assay, thiobarbituric acid-reactive species inhibition (TBARS) assay, iron chelating activity, and reducing power. Butylated hydroxytoluene (BHT), ascorbic acid (AA), EDTA, and rutin were used as the standard antioxidants (Cheng *et al.*, 2007; Ebrahimzadeh *et al.*, 2008; Gęgotek *et al.*, 2019; Zeouk *et al.* 2020).

2.5.1. TBARS Assay

Malondialdehyde (MDA) is a major reactive aldehyde generated by lipid peroxidation. To investigate the effect of **EA** derivatives on MDA production, a TBARS assay was performed using egg yolk as a lipid-rich medium (Upadhyay *et al.*, 2014). Briefly, 250 µL of egg yolk homogenate (10% in distilled water, v/v) was mixed with 150 µL of **EA** derivatives. Next, 100 µL of $FeSO_4$ (17.5 mM) was added. After one hour at 37 °C, 750 µL of acetic acid (20%), 750 µL of thiobarbituric acid (0.8% in 1.1% sodium dodecyl sulfate), and 50 µL of trichloroacetic acid (TCA) (20%) were added to the mixture and heated in a boiling water bath for one hour. The final concentrations of the tested compounds ranged from 0 mM to 2 mM. The control reaction mixture contained 150 µL of distilled water. The blank mixture did not contain $FeSO_4$. After cooling, 1.5 mL of 1-butanol was added to the mixtures and centrifuged for 10 min at 3000 g. The optical density of the organic upper layer was measured at 532 nm wavelength. The results were calculated using the following equation and expressed as the percentage of lipid peroxidation inhibition:

$$\text{Lipid peroxidation inhibition } (\%) = 100\left(1 - \frac{\text{Abs}_t}{\text{Abs}_0}\right),$$

where Abs_0 is the optical density of the control reaction and Abs_t is the optical density in the presence of the test compound.

2.5.2. Free Radical Scavenging Activity

The radical-scavenging activity of **EA** derivatives was evaluated based on their ability to interact with DPPH using the method described by (Yokozawa *et al.*, 1998). The assay was conducted in a 96-

well microtiter plate. Briefly, 100 µL of the appropriate concentrations (0.15–50µM) of **EA** derivatives or ascorbic acid were added to each well separately and in triplicate. DMSO was used as a negative control. Then, 25 µL of DPPH solution (0.2 M in MeOH) was added to each well. The plates were then incubated in the dark at room temperature for 30 min. The optical density was measured at 540 nm and the percentage inhibition was calculated using the following equation:

$$\text{Radical scavenging activity (\%)} = 100\left(1 - \frac{\text{Abs}_1}{\text{Abs}_0}\right),$$

where Abs_0 is the absorbance of the negative control, and Abs_1 is the absorbance of the tested **EA** compounds.

2.5.3. Ferric ion reducing antioxidant power (FRAP)

The FRAP assay is based on the reduction of potassium ferricyanide ($K_3Fe^{+III}[CN]_6$) to potassium ferrocyanide ($[K_4Fe^{+II}(CN)_6]$) in the presence of antioxidants with electron donating abilities (Adjimani & Asare, 2015). The reducing power of **EA** derivatives was measured using the method described by(Canabady-Rochelle *et al.,* 2015) with slight modifications. Briefly, 200 µL of each derivative at different concentrations was added to 500 µL phosphate buffer (200 mM, pH 6.6) and 500 µL potassium ferricyanide (1%). After incubation at 50 °C for 20 min in a water bath, the reaction was stopped by adding 500 µL of 10% TCA to the mixtures. Finally, 500 µL distilled water and 100 µL of ferric chloride (0.1%) were added. Ascorbic acid was used as a reference standard. After 10 min at room temperature, the absorbance at 700 nm was measured, and the concentration at absorbance 0.5 was expressed as IC_{50} value.

2.5.4. Chelating activity

The chelation of ferrous ions by **EA** derivatives was evaluated by measuring the decrease in absorbance of the iron (II)-ferrozine complex at 540 nm (Mladenović *et al.*, 2011). The assay was performed using a 96-well microplate. Briefly, the reaction mixture was composed of 60 µL of $FeCl_2$ (2 mM), 100 µL of the tested compound, and 40 µL of ferrozine (0.8 mM). The mixture was shaken vigorously and incubated for 10 min at room temperature, and the absorbance was measured at 540 nm. EDTA was used as the standard and the control contained only iron and ferrozine. The ability of each derivative to chelate ferrous ions was determined using the following equation.

$$\text{Iron chelation (\%)} = 100\left(1 - \frac{\text{Abs}_1}{\text{Abs}_0}\right),$$

where Abs_0 is the absorbance of the control and Abs_1 is the absorbance of the tested compound.

2.6 Membrane protective activity

Inhibition of oxidative hemolysis was used to assess the membrane protective activity. Briefly, the RBCs suspension was mixed with each compound separately for 30 min, as described above, and hemolysis was initiated with a solution of H_2O_2 (0.2%). The reaction mixture was then incubated at 37 °C for 6 h with slow mixing. Hemolysis was quantified spectrophotometrically at 540 nm based on the hemoglobin content in the supernatant (Buravlev *et al.,* 2019). The estimation of hemolysis percentage was calculated relative to H_2O_2 induced hemolysis.

2.7 Statistical analysis

Raw data were exported to GraphPad Prism 8 and further analyzed using one-way or two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Data are expressed as the mean ± standard deviation of three independent experiments unless otherwise noted. Differences were considered statistically significant at p-value <0.05.

3. Results and Discussion

3.1 Cytotoxicity of EA derivatives against MCF7 and MDA-MB-468 cancer cell lines

The first set of pharmacological activities in this study examined the effect of **EA** derivatives on breast cancer cells proliferation using MTT assay. The treatment of MDA-MB-468 and MCF7 cancer cell lines with **EA** derivatives showed strong cytotoxic effect against both cancer cell lines. **EA** had a weak effect on both cancer cell lines (IC_{50}>20µM), featuring its general lack of antitumor activity (Aizawa *et al.*, 2003; Bryant *et al.*, 2011; T. Li *et al.*, 2012; Mignani *et al.*, 2016). However, the three **EA** derivatives exhibited much more improved and a dose-dependent antiproliferative activity (**Figure 2**) with IC_{50} values ranging between 1.13 µM and 2.51 µM as summarized in **Table 1**.

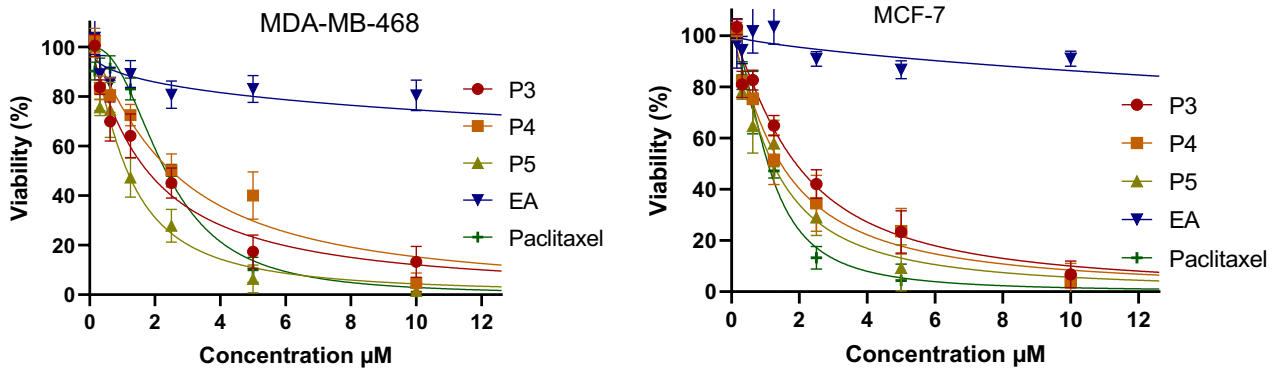

Figure 2. Viability of MDA-MB-486 and MCF7 cancer cell lines treated with different concentrations of **EA** derivatives and Paclitaxel for 48h, evaluated by MTT assay. Data are means of three independent experiments. ± Standard deviation.

Studies have suggested that substituting the carboxylic acid part of **EA** with various heterocycle moieties can lead to the improvement of the antiproliferative activity and cancer cell selectivity (Yu *et al.*, 2023). Additionally, modifying both the carboxylic acid part and the aromatic ring part has been found to significantly enhance the chemical reactivity of the Michael acceptor in **EA** analogs (El Abbouchi *et al.*, 2023). Furthermore, our results show that the insertion of a piperazine moiety onto the carboxylic function has demonstrated effective inhibition of cell growth. As such, it is not surprising that piperazine is widely used as a preferred structure in the synthesis of a variety of biologically active compounds (Durand & Szostak, 2021; Karaytuğ *et al.*, 2023). Structurally, piperazine has a 1,4 link between its two azote atoms. As hydrogen bond donors and acceptors, these two nitrogen atoms improve the pharmacological and pharmacokinetic profiles of drugs containing piperazine by tuning receptor interactions and modulating the drug lipid–water partition coefficient, acid–base equilibrium constant, and bioavailability (Durand & Szostak, 2021; Elliott, 2011). However, our results suggest that the size and nature of the substitution at the piperazine nucleus ring does not significantly influence the cytotoxic activity of **EA** derivatives in either cancer cell line. Interestingly, our investigation was further confirmed a possible specificity of **P3** and **P5** derivatives on TNBC tumor cells compared to the conventional drug "Paclitaxel" used in this study.

Table 1. IC_{50} values for antiproliferative activity of **EA** derivatives against MDA-MB-468 and MCF7 cancer cell lines and PBMCs.

Compound	IC_{50} (µM)			Safety ratio	
	MDA-MB-468	MCF7	PBMCs		
P3	1.78 ± 0.318[a]	1.90 ±0.073 [a]	16.6 ± 4.70 [b]	9.32+	8.73‡
P4	2.51 ±0.273 [a]	1.49 ± 0.314 [a]	11.1 ± 2.33 [c]	4.42+	7.45‡
P5	1.13 ±0.224 [a]	1.25 ± 0.303 [a]	10.05 ± 2.30 [c]	8.89+	8.04‡
Paclitaxel	2.36 ±0.063 [a]	1 .07 ± 0.149 [a]	1.93 ± 0.196 [a]	0.82+	1.8‡

Values are mean of three independent experiments ± Standard deviation. Different lowercase letters indicate significant difference according to two-way ANOVA and Tukey's multiple comparison tests *($p < 0.05$)*.

+IC_{50} (PBMCs)/IC_{50} (MDA-MB-468)

‡ IC_{50} (PBMCs)/IC_{50} (MCF7)

3.2 Cytotoxicity evaluation against normal cells (PBMCs and RBCs)

Most chemotherapeutic agents are administered intravenously (Corrie, 2008). After intravenous treatment, blood cells are the first normal cell population to be affected, which results in significant immune deficiencies, hemolytic anemia, and increased side effects (Ben Mrid *et al.,* 2019; Mameri *et al., 2021; Rodgers et al., 2012)*. As a result, anticancer drug candidates must exhibit minimal or no cytotoxicity on blood cells. Therefore, the *in vitro* cytotoxic activity of **EA** derivatives on human PBMCs and RBCs was evaluated to determine their eventual interactions with blood components. Interestingly, all **EA** derivatives showed low cytotoxicity in human PBMCs, with IC_{50} values higher than 10µM (**Table 1**). Based on the safety ratios (PBMCs/cancer cells), compound **P3** exhibited the highest selectivity for MDA-MB-468 cells, with a safety ratio of 9.32, followed by MCF7 cells, with a safety ratio of 8.73 (**Table 1**). It should be noted that all **EA** analogs exhibited higher selectivity than paclitaxel, which exhibited the lowest safety ratio (0.82 and 1.8 in MCF7 and MDA-MB-468 cancer cells, respectively). By the same token, the hemolytic activity of **EA** derivatives was observed and compared to the chemotherapeutic drug "Paclitaxel". As shown in **figure 3**, while paclitaxel induced complete hemolysis at 50µM, the hemolytic activity of **EA** derivatives did not exceed 3% at the same concentration.

3.3 Analysis of antioxidant enzyme balance in MDA-MB-468 and MCF7 tumor cell lines treated with EA derivatives

Multiple factors are known to contribute to cancer cell death, including immune-mediated destruction (Zhang *et al.,* 2019), growth factor deprivation (Porębska *et al.*, 2019), and diverse metabolic stresses (Altman & Rathmell, 2012). Reactive oxygen species (ROS) are important metabolic stress that limits the survival of cancer cells (Tasdogan *et al.,* 2021). In the field of chemotherapy, redox homeostasis disruption has been actively studied because cancer cell death can be induced by increased oxidative stress levels (Kim *et al.*, 2019). SOD, CAT, glutathione, and peroxiredoxin systems are the main intracellular antioxidant systems for the maintenance of oxidative stress homeostasis (Kruk *et al.,* 2019). SOD catalyzes the dismutation of superoxide anion radicals (O2·) to hydrogen peroxide (H_2O_2), which is further transformed to H_2O by catalase and peroxidases (GPx and Prx). The GSH system directly removes ROS in the presence of GSH, GPx, and GST. This

reaction produces glutathione disulfide (GSSH), the oxidized form of glutathione, which can also be reduced by GR.

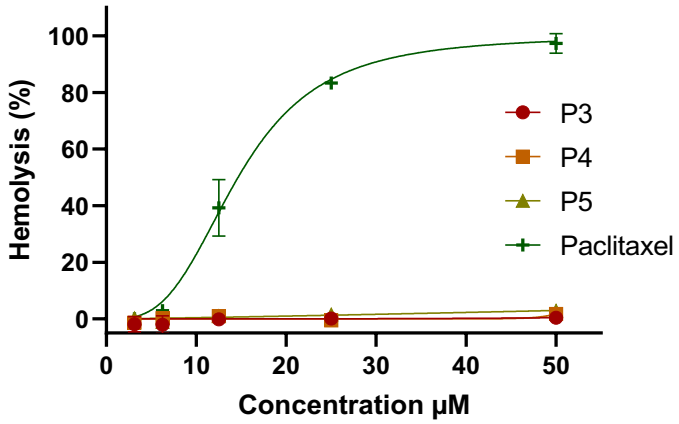

Figure 3. Induced hemolysis in human RBCs treated with **EA** derivatives and Paclitaxel for 6 hours, Values are means of three independent experiments ± Standard deviation.

The thioredoxins (Trx) system maintains proteins in the reduced state in the presence of Trx and TrxR. Both the GSH and Trx systems require NADPH as an electron donor to maintain their activity (Jiang *et al.,* 2018). In this study, further investigation was performed to examine variations in the antioxidant enzyme balance in both molecular grade breast cancer cell lines, treated for 48h with the corresponding IC_{50} concentrations of **EA** derivatives. As shown in **figure 4**, both cancer cell lines showed significantly increased SOD, GPx, and GST activities, and an increase in ICDH in MDA-MB-468 cancer cells. In addition, the maximum increase was observed in tumor cells treated with **P4** derivative. The increase in the activity of antioxidant enzymes can be considered as a normal response of cancer cells to the overproduction of ROS. However, GR and TrxR activities decreased compared to non-treated cells (NT). A slightly significant decrease in ICDH activity was observed in MDA-MB-468 cancer cells. These results suggest that **EA** derivatives disrupt redox homeostasis by targeting the Trx and GSH antioxidant systems.

3.4. Antioxidant activities

Lipid peroxidation is one of the major molecular pathways involved in the oxidative damage to cell structures and toxicity (Repetto *et al.,* 2012). As antioxidants, effective substances either directly prevent lipid peroxidation by neutralizing lipid radicals and preventing propagation processes, or indirectly neutralize one of the initiating agents of lipid peroxidation (photons, ROS, or transition metal ions) (Félix *et al.,* 2020). The antioxidant capacities of **EA** and its derivatives were measured using different *in vitro* assays, and the results are shown in **figure 5**. Thus, the introduction of 4-hedroxyphenyl-piperazine into the structure of **EA** resulted in compound **P3**. This compound showed good antioxidant activity in TBARS and DPPH assays (**Figure 5 B&C**) with IC_{50} values of 46.2 µM and 4.24 µM, respectively. Substitution of the hydroxyl group of **P3** with a methoxy (**P4**) or diethyl phosphate (**P5**) moiety resulted in the loss of radical scavenging and lipid peroxidation activities. In the FRAP assay, antioxidants reduced Fe^{3+} to Fe^{2+} by donating electrons. **Figure 5D** shows that all **EA** derivatives had a dose-dependent reducing power, with the highest activity being that of **P3** with an IC_{50} of 21.5 µM.

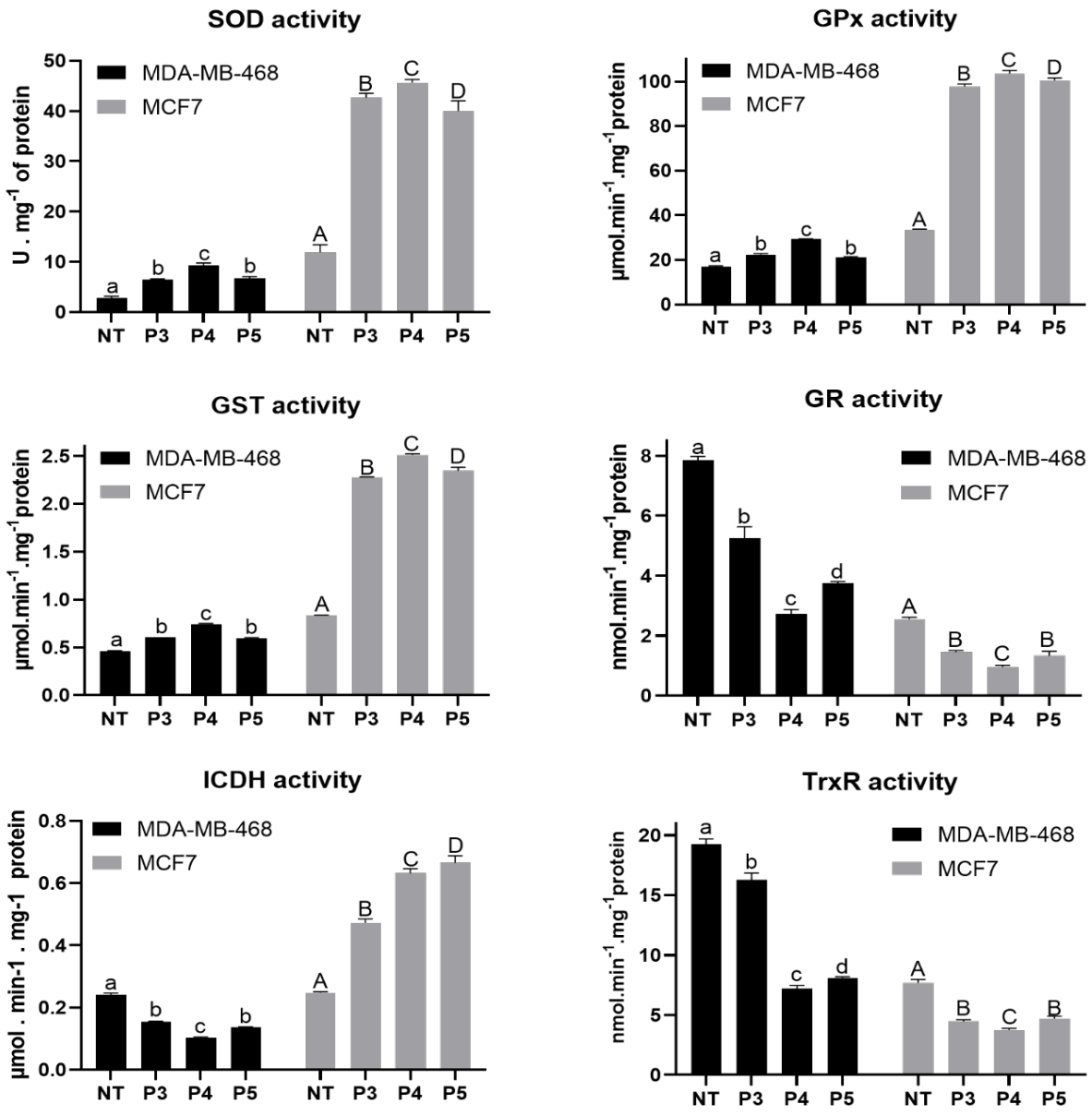

Figure 4. Antioxidant enzymatic activities (GR, ICDH, GST, TrxR, GPx and SOD) in MCF7 and MDA-MB-468 cancer cell lines after 48h treatment with IC_{50} of **EA** derivatives. Values are means of at least four replicates ± standard deviation. NT (negative control). Different letters indicate significant differences among treatments (*p*-Value <0.05).

P4 and **P5** displayed moderate reducing powers, with IC_{50} values of 53 and 52 µM, respectively. However, we observed no improvement in the iron-chelating activity of **EA** derivatives (**Figure 5A**). Overall, it can be stated that the presence of a variety of functional groups as linkers to the piperazine ring has significant effects on the antioxidant properties of the resulting molecules. Hydrogen and electron-donating functional groups influence the antioxidant properties of **EA** derivatives. According to these findings, the phenolic hydroxyl group is more crucial for the antioxidant properties than the methoxy and diethyl phosphate groups. The phenolic hydroxyl group is known to be an efficient free-radical scavenger; the hydrogen from this group is given to the free radicals, and the phenoxyl radical formed can be stabilized by resonance (Bendary et al., 2013; Prihantini et al., 2015).

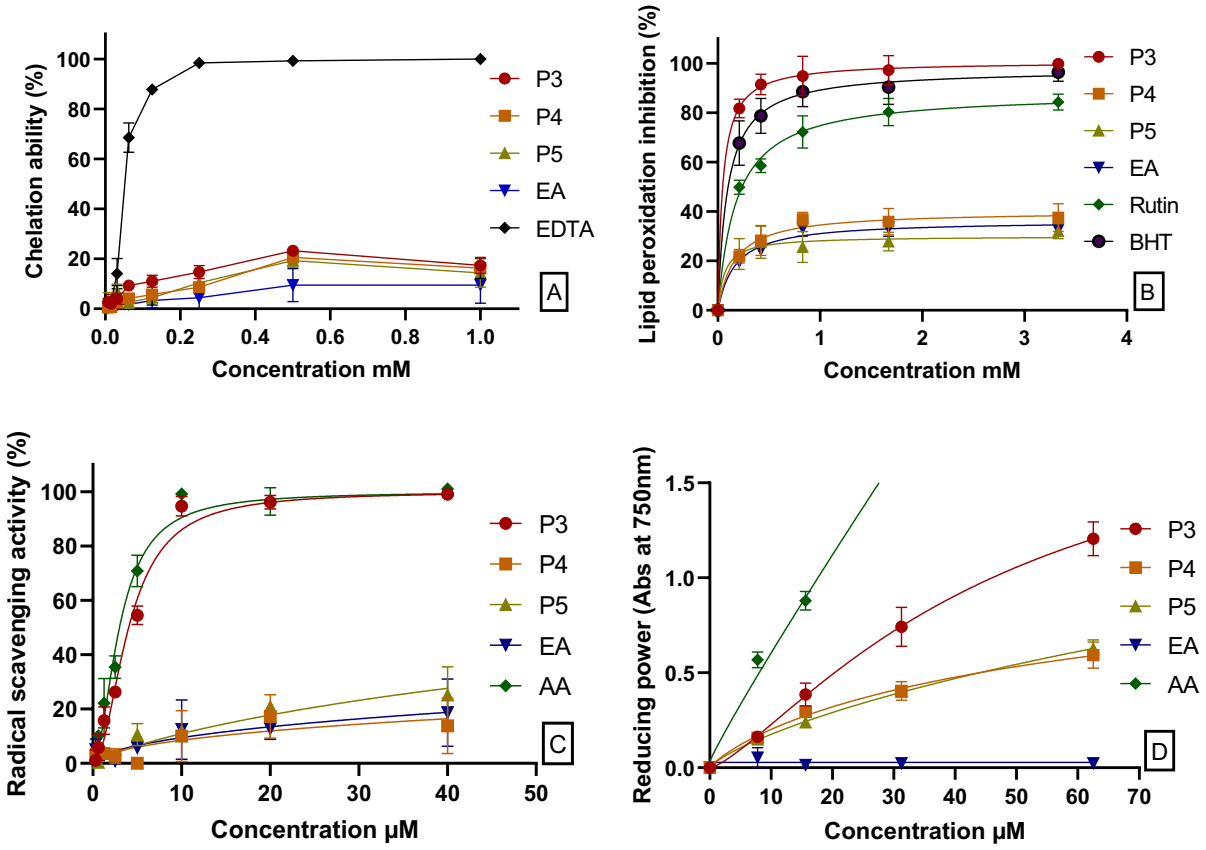

Figure 5. Antioxidative capacity of **EA** derivatives measured at different concentrations by the following assays: (A) Iron Chelating Activity; (B) TBARS assay; (C) DPPH Assay and (D) FRAP assay. Values are means of three independent experiments ± standard deviation.

3.5 Membrane protective activity

The ion flow and mechanical properties of RBCs are regulated by transmembrane proteins, which can influence a cell's response to toxic compounds. In addition, the high amount of polyunsaturated fatty acids in the cell membrane and auto-oxidation of hemoglobin makes RBCs sensitive to ROS from both endogenous and external sources (Mameri *et al.,* 2021; Mohanty *et al.,* 2014). In the presence of free radicals, RBCs may undergo lipid peroxidation, changes in cell morphology, and even hemolysis (Mameri et al., 2021). As **EA** derivatives showed considerable antioxidant activity, especially **P3**, they were subjected to membrane protective activity evaluation against free radical-initiated hemolysis of human RBCs. Evaluation of the membrane protective activity demonstrated that pretreatment of RBCs with different concentrations of **EA** derivatives (1.5-25 µM) had significant protective activity (**Figure 6**). All compounds exhibited dose-dependent protection of RBCs under H_2O_2-oxidative stress (**Figure 6**). As summarized in **table** 2, high activity was exhibited by **P3** (IC_{50} <1.5 µM) which completely prevented the hemolysis of RBCs while **P4** and **P5** showed a significant cytoprotective activity with IC_{50s} 9.1 ± 1.6 µM and 22 ± 5.3 µM, respectively. The results showed that the antioxidant activities of **EA** derivatives were strongly correlated with their anti-hemolytic activities.

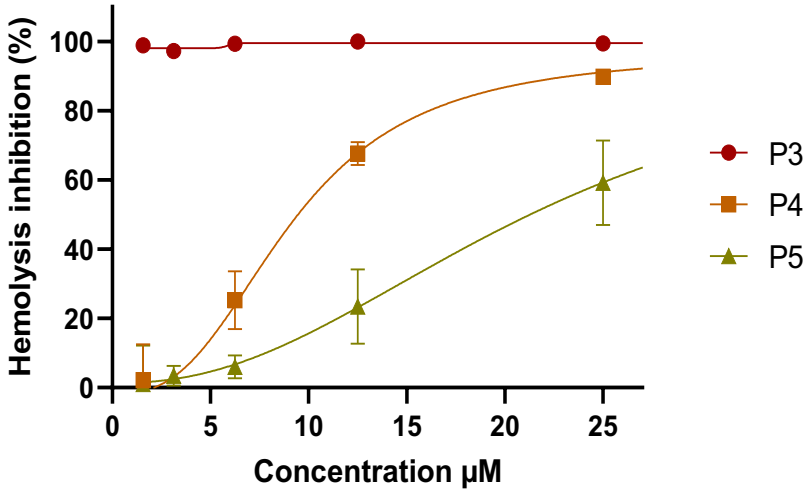

Figure 6. Protective effect of **EA** derivatives against H_2O_2- induced hemolysis of human RBCs *in vitro*. Data are expressed as % inhibition of H_2O_2 induced hemolysis. Values are mean of three independent experiments ± standard deviation.

Table 2. IC_{50} values for *in vitro* hemolysis and membrane protective effects of **EA** derivatives on human RBCs

	IC50 (µM)			
Compounds	P3	P4	P5	Paclitaxel
Hemolysis induction	>50	>50	>50	15.6 ± 1.37
Hemolysis inhibition	<1.5 [a]	9.1 ± 1.6 [b]	22 ± 5.3 [c]	-

Values are means of three independent experiments ± Standard deviation. Different lowercase letters indicate significant differences among treatments according to one-way ANOVA and Tukey's multiple comparison tests (*p*-Value <0.05).

Conclusion

In the present study, we investigated the *in vitro* antitumor and antioxidant potential of three promising **EA** derivatives bearing piperazine moiety. This is the first investigation based on the antiproliferative activity of **EA** derivatives against TNBC subtype in comparison with a Luminal cancer cell line. In brief, we demonstrated strong *in vitro* antitumor activity against the TNBC cell line (MDA-MB-468) as well as the MCF7 cell line. It was also noted that the thioredoxin and glutathione systems were disrupted simultaneously. *In vitro* safety screening tests on PBMCs and RBCs revealed a satisfactory safety and hemocompatibility profile, with high selectivity ratios and no hemolytic potential, even at the highest tested concentrations. In contrast to antitumor activity, structural differences were found to influence the antioxidant activity of the compounds. The presence of a hydroxyl group on the phenol ring resulted in derivative **P3** having the highest radical scavenging and lipid peroxidation inhibition activity, and was especially effective at protecting RBCs from oxidative stress in the presence of H_2O_2. Overall, the inclusion of pharmacologically active pharmacophores such as piperazine moieties significantly enhanced the poor intrinsic anticancer and antioxidant properties of **EA**. The pharmacological activities of the tested **EA** derivatives were extremely spectacular compared to their parent molecule (**EA**), and revealed some intriguing structure-activity relationships. Derivative **P3** has

the most promising potential for further preclinical investigations owing to its safety profile and significant antitumor and cytoprotective properties

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Compliance with Ethical Standards: Human blood samples from healthy volunteer donors were used after approval by the Biomedical Research Ethics Committee of Mohammed V-Souissi University of Rabat (IORG0006594 at Office for Human Research Protections). Deposit N° 592/2014

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