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In Vitro Antioxidant Evaluation and *In Silico* Prediction of Antiestrogenic and Pharmacokinetic Properties of Ketamine Derivatives

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ABSTRACT

Background: Breast cancer is the most prevalent cancer found among women; 12 out of 100 females are affected during their lifetime.
Objective: *In vitro* antioxidant and in silico methods were applied to determine the anti-breast cancer properties of the derivatives of ketamine.
Methods: Their antioxidant abilities were assessed by the following methods: ferric reducing antioxidant power (FRAP), ferrous chelating assay, 2.2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid(ABTS) ABTS radical scavenging assay, and antioxidant peroxidation assay. The compounds were docked against human estrogen alpha receptor (ERα) PDB: 1SJO and cyclin D-dependent kinase 4 (CDK4) PDB: 2W96, which were always overexpressed in breast cancers. The standard drug employed during the docking process was 5-fluorouracil. The docking, drug likeness, and ADMET analysis were carried out by Maestro Suite.
Results: All tested compounds (D11-D15) showed antioxidant effects. In addition, all tested compounds exhibited stronger binding affinity than the reference compound, 5-fluorouracil. Also, the results for the docking of D11–D15 are -7.26, -7.56, -7.96, -7.77, and -7.43 kcal/mol, respectively, against 1SJ0. It also revealed that all compounds were better than the reference drug 5-fluorouracil (-4.81 kcal/mol). In the docking study of cyclin-dependent kinase 4, both the standard drug and the derivatives of ketamine are all in very close range of binding energies. All the drugs can be excreted with high safety, they do not violate Lipinski's rule of five, and their molecular weights are within the normal range.
Conclusion: Therefore, these compounds can serve as potential lead compounds in the treatment and management of breast cancers.

Keywords: Ketamine derivatives, breast cancer, antioxidant assays, in silico, CDK 4 and ERα

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INTRODUCTION

Predictions from the Global Cancer Project (GLOBOCAN 2020) show that by the year 2040, the burden of breast cancer will be over 3 million new cases annually and a million deaths [1]. Breast cancer is the most prevalent cancer found among women; 12 out of 100 females are affected during their lifetime [2]. Breast cancer is due to abnormal molecular changes in normal cells of the breast leading to unstoppable growth and proliferation of cells [3]. Breast cancer affects the normal functions of the breast; it can be invasive, aggressive, and metastatic. Heredity, hormonal therapy, lifestyle, and obesity are major factors that can lead to breast cancer [4] based on molecular subtype classification: estrogen receptor 2 (HER-2), cell proliferation regulator Ki-67, and progesterone receptors (PR) [5]. HER-2 is the most prevalent, which accounts for 70% of all breast cancer cases [5]. ERα is majorly expressed by the breast and the womb. The estrogen receptor in females plays significant roles in inflammation, apoptosis, maturation, and proliferation of breast cancer cells [6]. Overproduction of the estrogen hormone leads to multiplication of ERα in the mammary gland, leading to maintenance and proliferation of breast cancer cells [7]. Homeostasis is due to cell death and division; these processes are governed by the cell cycle, which has control points or checkpoints [8]. These points are regulated by cyclins and CDK4 and CDK6. Activated CDK-4 or 6 by cyclin D phosphorylates retinoblastoma-associated protein (RB). This phosphorylation releases RB from E2F, making the cell transit from G1 to S [8]. Estrogen receptor-positive cancer overexpression of D cyclin is common; therefore, inhibiting CDK-4/6 is a very good therapeutic approach to restrict breast cancer cells from transiting from G1 phase to S phase.

The approved inhibitors of ERα by the FDA are tamoxifen, raloxifene, fulvestrant, and the CDK-4/6 inhibitor palbociclib; their efficacies are declining as years go by due to recorded resistances, poor pharmacokinetics, and toxicity [9]. Therefore, there is a great need to search for approved drugs as better alternatives for the treatment of breast cancer. Ketamine is a hydrophobic N-methyl-D-aspartic acid (NMDA) receptor antagonist. It has a high safety profile. Ketamine also targets other receptors, such as opioid, cholinergic, dopaminergic, and serotonergic receptors [10]. Ketamine is eliminated rapidly from blood after metabolism by cytochrome P-450 [11]. Ketamine is effective for obstetric anesthesia for cesarean section [12]. Ketamine binding to the NMDA receptor decreases Ca2+-mediated cellular signaling, thereby reducing channel opening and inhibiting pain [13]. Ketamine also regulates analgesia for painful fractures, burns, and traumatic amputations [14]. Ketamine also regulates inflammation by decreasing TNF-α, IL-6, IL-8, and IL-Iβ [15]. Ketamine as an adjuvant for the management of cancer-related pain. The NMDA receptor is seen expressed on many cancer cells, including breast cancer, liver, prostate, and gastric cancer cells [16]. The tumor-inhibiting ability of ketamine was displayed by [17]. They found that CD-69, a white blood cell and natural killer cell activator marker, was insignificantly expressed in lung cancer tissues, but ketamine upregulated CD69, resulting in a significant apoptosis of cancer cells.

Therefore, ketamine was modified chemically in the Pharmaceutical and Medicinal Chemistry laboratory, Niger Delta University. This study aims to evaluate the antioxidant activity of chemically modified ketamine as an anticancer drug in silico, drug likeness, and ADMET studies.

METHODOLOGY

Apparatus/Chemicals

UV-vis spectrophotometer (S23 A Gulfex Medical and Scientific), spatula, micropipette, electronic thermostat water bath (model HH W21), pH meter, analytical balance, test tubes, test tube rack, beakers, reagent bottles, and measuring cylinder were used in this study.

Ketamine derivatives (D11-D15) were obtained from the Department of Medicinal Chemistry, Niger Delta University, Bayelsa State; ethylenediaminetetraacetic acid (EDTA), Trolox, ferrous sulfate, ferrozine, Fe3-tetra(2-pyridyl)pyrazine (TPTZ), ferric chloride (FeCl3.6H2O), trichloroacetic acid (TCA), sodium acetate trihydrate (CH3COONa.3H2O), glacial acetic acid, HCl, potassium persulfate (K2S2O8), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), dimethylsulfoxide (DMSO), thiobarbituric acid (TBA), disodium hydrogen phosphate (Na2HPO4), sodium dihydrogen phosphate (NaH2PO4), and ferrous chloride—all chemicals are from Sigma Aldrich USA and SD-Fine Chemicals.

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Antioxidant Peroxide Assay

Inhibition of lipid peroxidation by derivatives of ketamine was assayed based on [18]. Mammary tissue of a goat (10 g) was homogenized using a Potter-Elvehjem homogenizer in cold phosphate buffer at pH 7.4. The homogenized tissues were centrifuged at 400 rpm at 4°C. Different doses of derivatives of ketamine (100-1000 µg/ml) were added to the homogenized mixture for reaction by adding 0.1ml $FeSO_4$ (15 mM) to 3 ml of the tissue homogenate mixture. After 30 minutes, 100 µl was taken in a centrifuge tube containing 1.5 ml of 10% TCA. All the tubes were centrifuged for 10 minutes at 4000 rpm, and the supernatant was mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated at 100°C for 30 minutes in order to develop the color. The pink color complex was measured at 535nm.

Cationic Radical Assay

Generation of ABTS+ was by reacting ABTS (7 mM) and potassium persulfate (2.45 mM) in a solution ratio of 1:1 (v/v) kept in the dark for 1 day at 25°C. Later, 500 µl of ketamine derivatives (100-1000 µg/ml) were mixed with 3 ml of diluted ABTS+ solution having an optical density of about 0.8 at 734 nm. The absorbance of the mixture and the standard antioxidant Trolox were measured at 734nm after 20 min of incubation [19].

FRAP Assay

Sodium acetate buffer 0.3M (pH 3.6) 100 ml, then 10 ml of 20 mM $FeCl_3$ and 10 ml of 10 mM solution of TPTZ in 40 mM HCl were prepared to form FRAP reagent (v/v/v). Thereafter, 1 ml of FRAP reagent was incubated at 37°C for 20 min and was mixed with different doses of ketamine derivatives and ascorbic acid (100-1000 µg/ml) and kept in the dark for 30 min. Later the absorbance was determined at 593 nm [20].

Fe2+ ion Chelating Ability

The ferrous ion sequestering ability of ketamine derivatives was determined according to [21]. Ketamine derivatives and EDTA as a reference drug (100-1000 µg/ml) were dissolved in dimethylsulfoxide and mixed with ferrous sulfate solution (1 mM) and distilled water. The mixture was incubated for 10 min at 25oC. Ferrozine 5 mM (40 µl) and further incubated for 10 min at 25oC. Absorbance was measured at 562nm.

In Silico Docking Of Derivatives of Ketamine

In these computational studies, the 3D structures of the proteins estrogen receptor alpha (ERα) PDB:1JSO and cyclin D department kinase 4 (CDK4) PDB:5W96 were retrieved from the protein data bank. The ligands D11-D15 and the reference anticancer drug (5-fluorouracil) were obtained from Maestro 3D sketcher of Schrodinger suite, but the proteins were retrieved from research collaborator for structures bioinformatics (RCSB) and downloaded in protein data bank format (www.rcsbpdb.org). The proteins were edited using the preparation wizard of the Schrodinger suite. Protein editing includes eliminating water molecules and adding H atoms. After protein editing, molecular docking and calculations were carried out by Maestro 2023[22] and the extra precision mode of the Glide module in the Schrodinger suite. The docking score with the lowest values or highest in terms of negative sign is considered better [23].

Determination of ADMET and Drug Likeness of D11-D15

The Qikpro module of Maestro was used to predict ADMET and drug likeness: absorption, distribution, metabolism, excretion, and toxicity and violation of Lipinski's rule of five [24].

Statistical Analysis

All results were calculated and presented as mean ± S.D., using graphical prism software, USA, n = 5. The level of significance was $p < 5\%$.

RESULTS

The chemical structures of ketamine derivatives (D11-D15) are shown in figure 1. The antilipid peroxidation results revealed antioxidant effects of all tested compounds (D11-D15), while the standard compound showed significant effect ($p < 0.05$) compared to tested compounds (Figure 2). In addition, the ABTS cation radical scavenging ability of tested compounds displayed effect and the effect of standard compound trolox was significant compared to D11-D15 compounds (Figure 3). Furthermore, the tested compounds showed Ferric reducing antioxidant power with significant effect of the standard compound ascorbic acid compared to D11-D15 (Figure 4). The ferrous ion chelating ability of tested compounds was clearly displayed as shown in figure 5.

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Compounds D11-D15 were docked into the active sites of estrogen receptor alpha (PDB: 1SJ0) and cyclin D-dependent kinase 4 (PDB: 2W96). The docking scores of D11 – D15 and the reference drug 5-fluorouracil against human estrogen receptor alpha were depicted in Table 1, Figures 6, 7, 8, and 9. All the derivatives of ketamine D11 – D15 showed better docking scores than the standard drug 5-fluorouracil. The ADMET and drug-likeness parameters of derivatives of ketamine show that all compounds can be excreted safely. It also shows that all compounds have a molecular weight of less than 400 g/mol and a polar surface area of 50 angstroms. The number of hydrogen bonds and hydrogen bond donors/acceptors are within the normal range (Table 2).

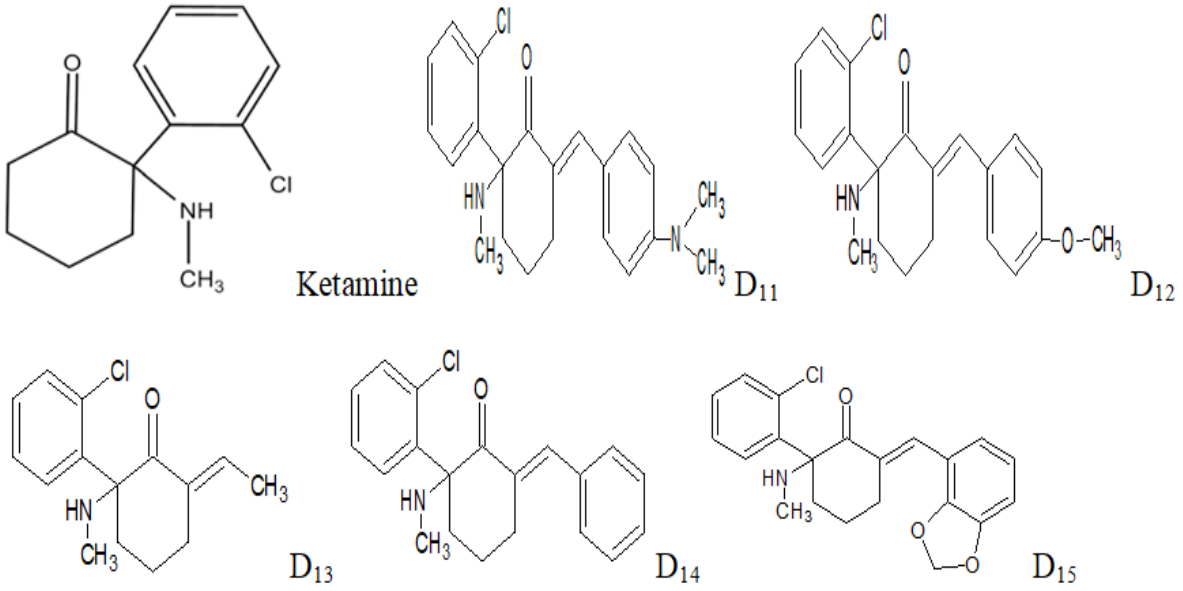

Figure 1: Different ketamine derivatives synthesized in Pharmaceutical and Medicinal chemistry Department Niger Delta University (D11-D15). D_{11}:(6*E*)-2-(2-chlorophenyl)-6-[(4-dimethylaminophenyl)methylidene]-2(methylamino)cyclohexan-1-one, D_{12}:(6*E*)-2-(2-chlorophenyl)-6-[(4methoxyphenyl)methylidene]-2-(methylamino)cyclohexan-1-one, D_{13}:(6*E*)-2-(2-chlorophenyl)-6-ethylidene-2-(methylamino)cyclohexan-1-one, D_{14}:(6*E*)-6-benzylidene-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one and D_{15}:(6*E*)-6-[(2*H*-1,3-benzodioxol-4-yl)methylidene]-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one.

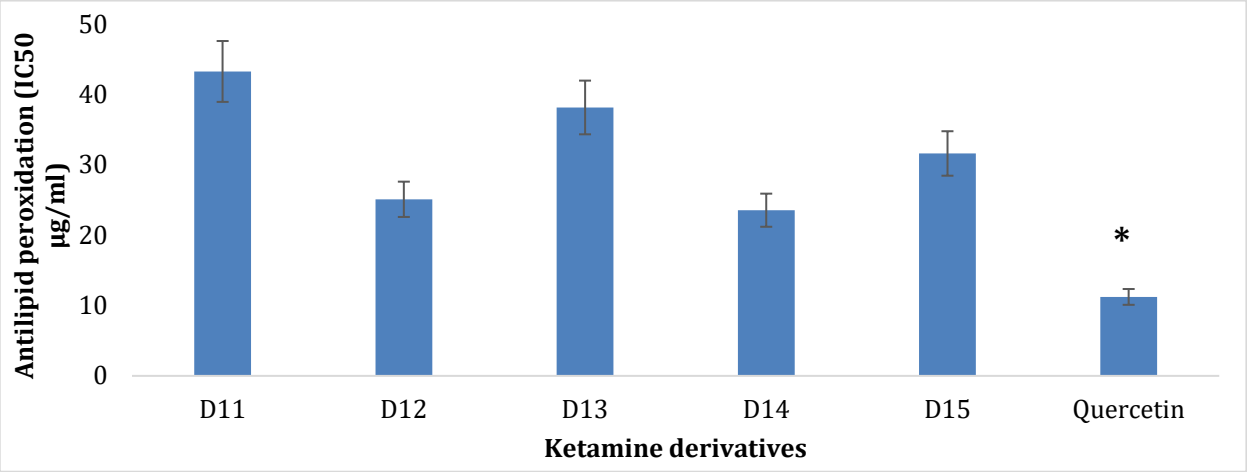

Figure 2: Antioxidant peroxide assay of derivatives of ketamine and quercetin as standard at concentrations of (100 – 1000 µg/ml), n = 5. * = p <0.05.

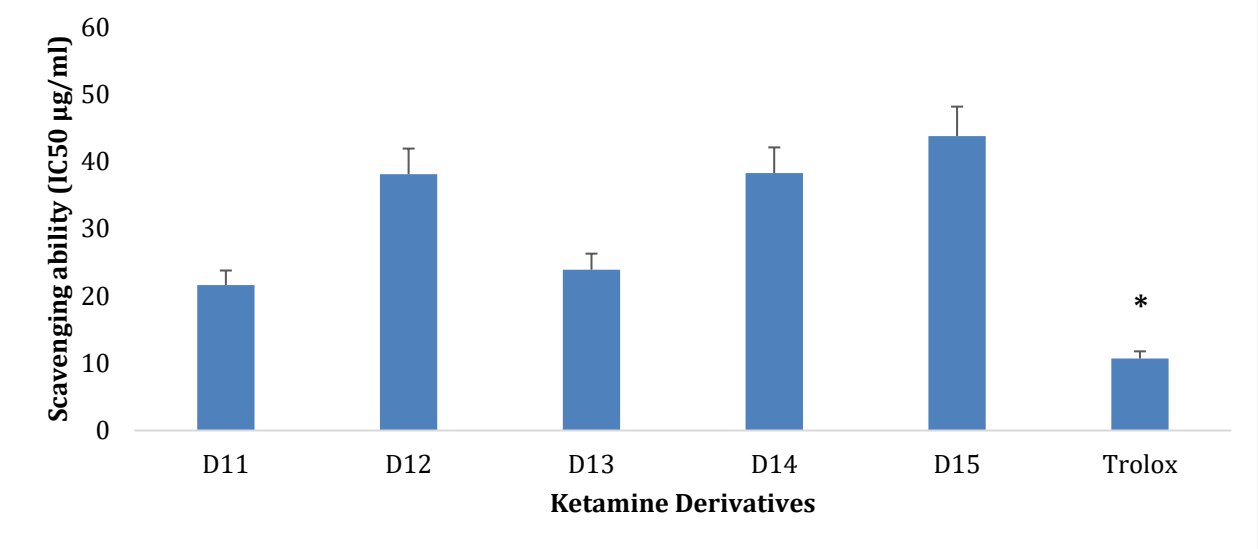

Figure 3: ABTS cation radical scavenging ability of derivatives of ketamine and trolox as standard at concentrations of (100 – 1000 µg/ml), n = 5. * = p <0.05.

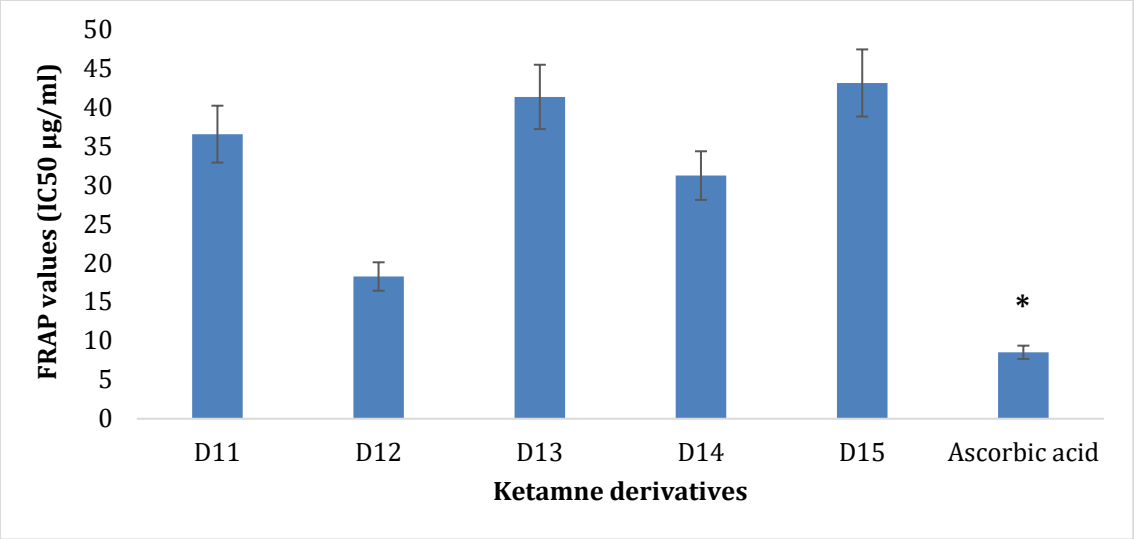

Figure 4: Ferric reducing antioxidant power of derivatives of ketamine and ascorbic acid as standard at concentrations of (100 – 1000 µg/ml), n = 5. * = p <0.05.

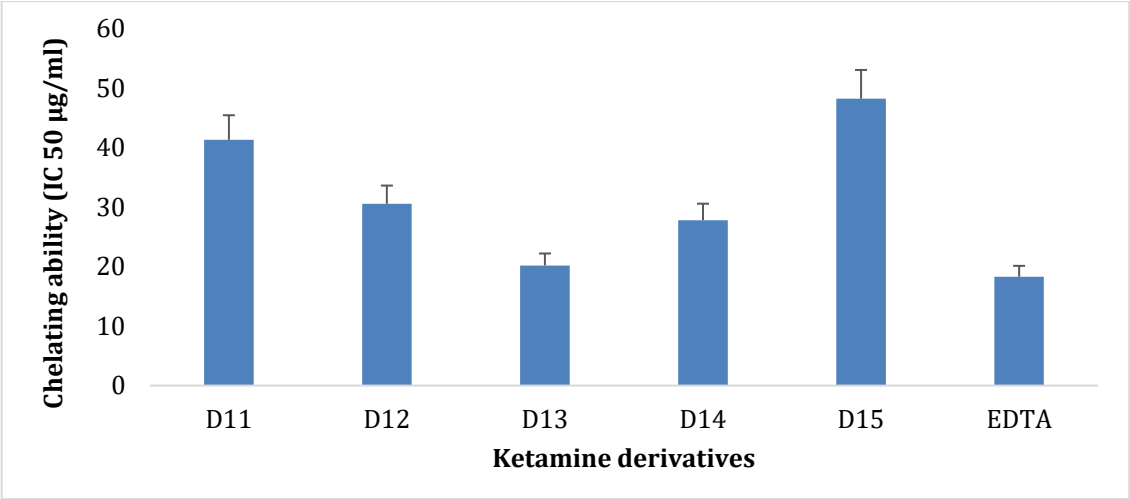

Fig. 5. Ferrous ion chelating ability of derivatives of ketamine and EDTA as standard at concentrations of 100–1000 µg/ml, (n = 5).

Table 1: Docking Result of Derivatives of Ketamine Against Estrogen Receptor Alpha (PDB:1SJ0) and Cyclin D Dependent Kinase 4 (PDB:2W96)

Sample ID	PDB: 1SJ0		PDB: 2W96	
	Docking Score	Glide model	Docking Score	Glide model
D11	-7.26	-63.47	-4.41	-39.30
D12	-7.56	-49.92	-4.78	-45.84
D13	-7.96	-51.43	-4.17	-31.08
D14	-7.77	-51.74	-3.81	-33.32
D15	-7.43	-51.19	-4.43	-43.12
5-fluorouracil	-4.81	-25.57	-4.90	-29.59

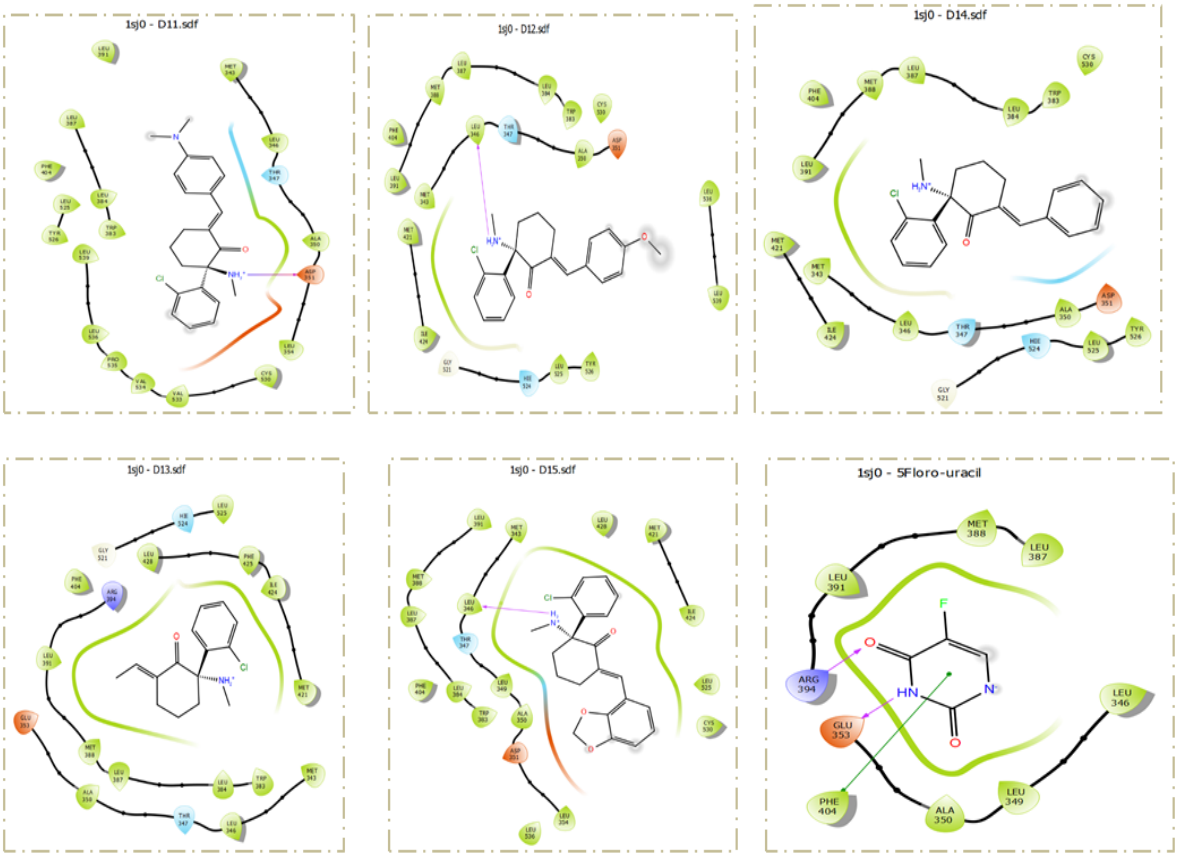

Figure 6: 2D interactions of ligands with 1SJ0

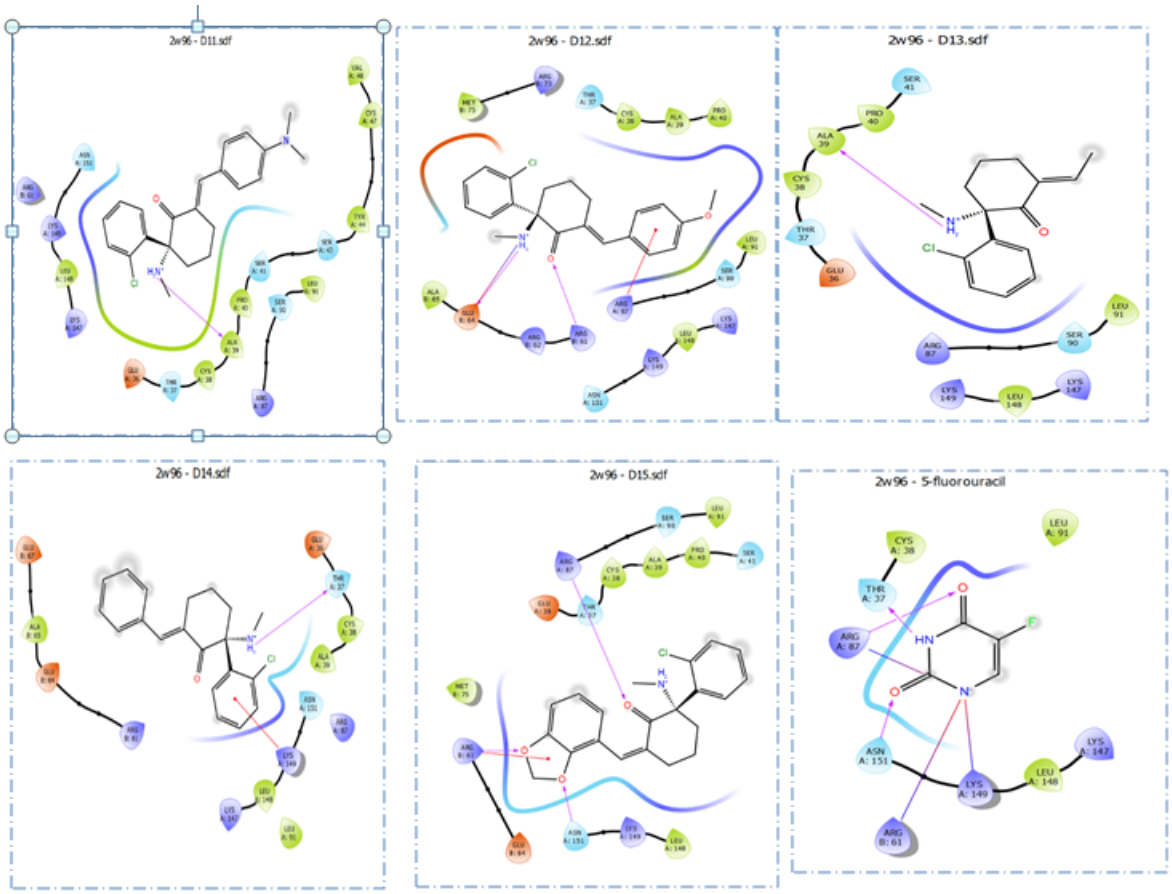

Figure 7:2D interactions of ligands with 2W96

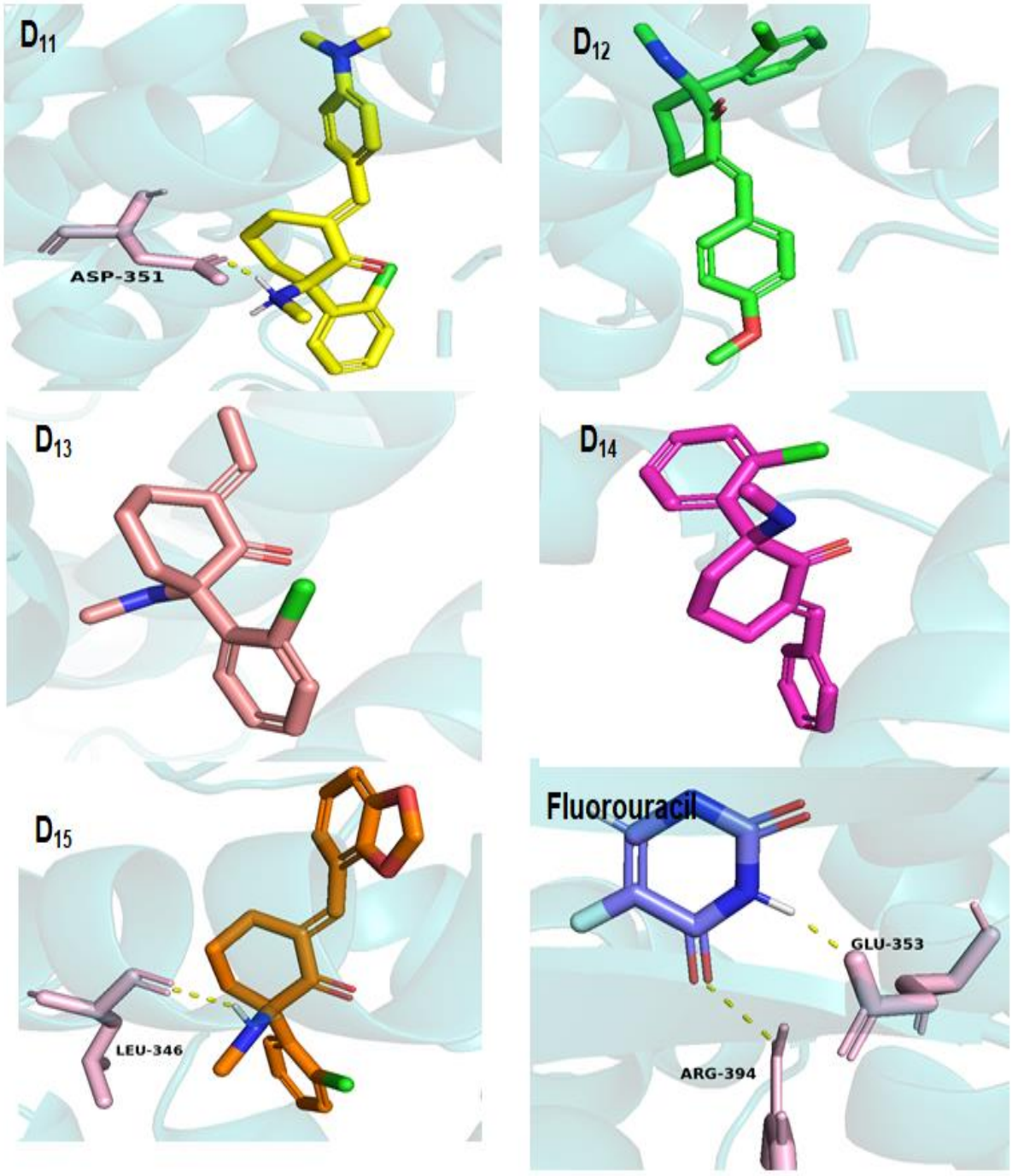

Figure 8: 3D interactions of ligands with 1SJ0

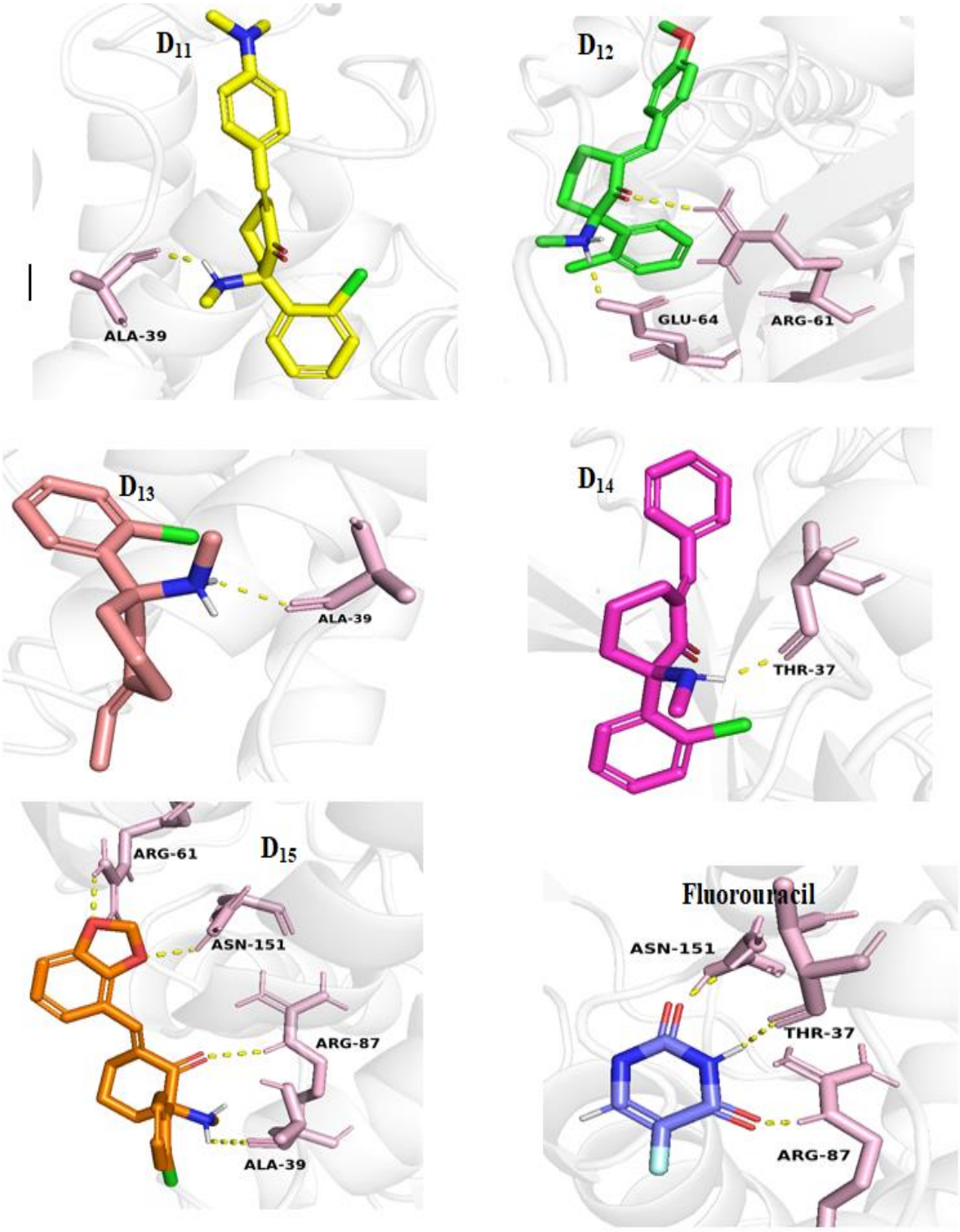

Figure 9: 3D interactions of ligands with 2W9

Table 2: ADMET parameters prediction of D11-D15 showing that the compounds are safe based of different tested parameters

SAMPLE ID	D11	D12	D13	D14	D15	Standard Rage
Metabolism likely	Amine dealkylation	Ether dealkylation	Allylic H -> alcohol	Amine dealkylation	Amine dealkylation	Range 95% of Drugs
Molecular Weight	368.91	355.86	263.77	325.84	369.85	130.0/725.0
Weakly Polar SASA	42.604	41.976	45.941	42.032	283.989	0.0/175.0
vdW Polar SA (PSA)	32.782	39.284	30.92	31.106	47.895	7.0/200.0
No. of Rotatable Bonds	4.0	4.0	2.0	3.0	3.0	0.0/15.0
Hydrogen Bonds Donor	1.0	1.0	1.0	1.0	1.0	0.0/6.0
Hydrogen Bonds Acceptor	4.0	3.75	3.0	3.0	4.5	2.0/20
Globularity (Sphere = 1)	0.818	0.836	0.878	0.850	0.851	0.75/0.95
QP Polarizability (Angstroms^3)	41.921M	38.970M	28.803M	37.041M	37.862M	13.0/70.0
log P for hexadecane/gas	11.983M	11.418M	8.268M	11.098M	11.015M	4.0/18.0
log P for octanol/gas	16.942M	15.981M	12.105M	15.125M	16.059M	8.0/35.0
log P for water/gas	7.853M	7.674M	6.241M	7.435M	8.551M	4.0/45.0
log P for octanol/water	4.874	4.500	3.044	4.385	3.937	-2.0/6.5
log S for aqueous solubility	-5.302	-4.612	-2.990	-4.347	-4.019	6.5/0.5
log S - conformation independent	-4.884	-4.715	-2.768	-4.393	-4.819	6.5 /0.5
log K hsa serum protein binding	0.945	0.783	0.325	0.764	0.564	1.5 /1.5
log BB for brain/blood	0.529	0.502	0.640	0.572	0.600	-3.0/1.2
No. of Primary Metabolites	4.0	4.0	4.0	3.0	3.0	1.0 /8.0
Predicted CNS Activity	++	++	++	++	++	-- to ++
HERG K+ channel blockage: log IC50	-6.451	-6.262	-4.978	-6.300	-6.016	concern < -5
Apparent Caco-2 Permeability (nm/sec)	1289	1182	1117	1180	1256	<25 poor, >500 great
Apparent MDCK Permeability (nm/sec)	1232	1114	1101	1112	1193	<25 poor, >500 great
log Kp for skin permeability	-2.822	-2.820	-3.503	-2.726	0.048	Kp in cm/hr
Jm, max transdermal transport rate	0.003	0.013	0.085	0.028		mcg/cm^2 -hr)
Lipinski Rule of 5 Violations	0	0	0	0	0	max is 4
Jorgensen Rule of 3 Violations	0	0	0	0	0	max is 3
% Human Oral Absorption in GI (+-20%)	100	100	100	100	100	<25% is poor
Qual. model for human oral absorption	High	High	High	High	High	>80% is high)

DISCUSSION

The derivatives of ketamine showed antioxidant capacity, ferrous ion chelation, FRAP, ABTS, and antilipid peroxidation activity. Molecular docking studies against CDK4 and estrogen receptor alpha show that the derivatives of ketamine can act as better inhibitors in the management of breast cancer burden.

Free radicals like hydroxyl radical and superoxide are capable of damaging deoxyribonucleic acid by breaking strands, modifying bases, and increasing methylation [24]. Breast cancer cells produce numerous free radicals by the enzyme lactoperoxidase that catalyzes the removal of an electron from 17-β-estradiol to form a phenoxyl free radical [25]. Another enzyme overexpressed in breast cancer is thymidine phosphorylase, which converts thymidine to a glycating and free radical-producing agent known as 2-deoxy-D-ribose-1-phosphate [26]. The results of the ferric reducing antioxidant power assay and ABTS radical scavenging assay show that ketamine derivatives act as antioxidants by reducing both Fe3+ and ABTS+. Although values were reported as IC50 µg/ml for FRAP: Trolox > D12 > D14 > D11 > D13 > D15, as depicted in figure 4, for ABTS, Trolox as reference was 10.7 ± 0.38 µg/ml, and D11 was 21.63 ± 1.14 µg/ml. These results show that the derivatives of ketamine act as reducing agents, and the findings of [27].

One of the most notorious free radicals is the hydroxyl radical that can react with DNA, carbohydrates, and lipids. This radical is produced through Fenton chemistry. Therefore, chelating copper ions or ferrous ions will avert production of hydroxyl radicals [28]. The ferrous chelating potential of ketamine derivatives was assayed according to the method outlined by [21] utilizing ferrozine. The results showed D13<D11<D12<D14<D13<EDTA as depicted in figure 5. This shows that ketamine derivatives can chelate ferrous ions and spare macromolecules from the deleterious effects of hydroxyl radicals. Our reports are similar to the work of [29].

The end products of lipid peroxidation, malondialdehyde and 4-hydroxylnonenal, are very toxic to nucleic acids and protein [30]. Also, lipid peroxidation leads to loss of membrane fluidity, inhibition of enzymes and receptors, and loss of functionality of the membrane [31]. The results of our present study on the derivatives of ketamine showed their potential as drugs inhibiting the process of lipid peroxidation as compared to the standard quercetin, which had an antilipid peroxidation of 11.23 ± 0.17 mg/ml and was closely followed by D14 with an IC50 value of 23.56 ± 1.21. These reports are closely related to [29].

Despite tremendous advancement in the traditional way of discovering drugs, the process is slow, time-consuming, and expensive, with fewer success rates [32]. Therefore, it is important to discover drugs utilizing molecular docking. In the present study, five chemically modified drugs, derivatives of ketamine, were docked into the active sites of estrogen receptor alpha (PDB: 1SJ0) and cyclin D-dependent kinase 4 (PDB: 2W96). The docking scores of D11 – D15 and the reference drug 5-fluorouracil against human estrogen receptor alpha were depicted in Table 1, Figures 6, 7, 8, and 9. All the derivatives of ketamine D11 – D15 showed better docking scores than the standard drug 5-fluorouracil, as depicted in Table 1. The derivative with the highest docking score was D13 with a score of -7.96 kcal/mol and 5-fluorouracil -4.81 kcal/mol. D13 shows a lot of hydrophobic interactions between PHE 425, ILE 424, LEU 428, and LEU 398 and the estrogen receptor, which afforded a better bonding affinity of -7.96 kcal/mol. The findings are similar to the work of [33].

Although the docking scores of the derivatives of ketamine, D11 –D15, against cyclin-dependent kinase 4 were lower than that of 5-fluorouracil, they were in a very close range, as depicted in Table 1 above. D12 made an ionic interaction between GLU 64 and the amino group on the compound. Also, there is a hydrogen bond between the polar ARG 61 and oxygen on D13; pi-pi stacking also occurs between ARG 87 and the benzene ring of the compound. These interactions afforded D13 a higher docking score of -4.78 kcal/mol. This is very close to the reference drug 5-fluorouracil of -4.90 kcal/mol. The study runs parallel to the works of [34].

The ADMET and drug-likeness parameters of derivatives of ketamine show that all compounds can

be excreted safely. It also shows that all compounds have a molecular weight of less than 400 g/mol and a polar surface area of 50 angstroms. The number of hydrogen bonds and hydrogen bond donors/acceptors are within the normal range. There is no violation of any of the compounds based on Lipinski's and Jorgensen's rules of 5 or 3, respectively. The report is in accord with the reports of [34]. Although the study lacks *in vivo* and cell line cytotoxicity, these are recommended for further studies.

CONCLUSION

The reports of the present study reveal that the derivatives of ketamine showed strong antioxidant capacity, ferrous ion chelation, and peroxide antioxidant activity. Also, the molecular docking studies against CDK4 and estrogen receptor alpha show that the drugs can act as better inhibitors in the management of breast cancer burden. Further studies are required in the area of *in vivo* cancer cell line studies.

Conflict of interest

The authors declare that no conflict of interest.

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