Synthesis and antitumor activity of a 2-hydroxy substituted chalcone (C6)

Muhammad, Zahatu[1*], Ya'u, Jamilu[1], Zezi, Abdulqadir Umar[1], Magaji, Muhammad Garba[1], Hamza, Asmau Nasiru[2]

[1]Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria.

[2]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences Ahmadu Bello University Zaria, Nigeria.

Submitted: 15th April., 2023; Accepted: 19th July., 2023; Published online: 31st Aug., 2023
DOI: https://doi.org/10.54117/jcbr.v3i4.8
**Corresponding Author:* Muhammad, Zahatu; zahatumuhammad@abu.edu.ng, zahatu2016@gmail.com, +2348131302991

Abstract

Cancers account for approximately 13% of all deaths each year with breast cancer being one of the most common. Chemotherapeutic agents used in cancer usually have nonspecific toxicity which kills both tumor and normal cells and cause serious side effects that often limit their efficacy. This study aimed to synthesize chalcone and evaluate its antitumor activity.

Chalcone was synthesized using Claisen-Schmidt condensation and characterized using spectroscopic techniques. The LD_{50} of the synthesized compound was estimated using OECD-425 guidelines in rats. A mammary tumor was induced with a single subcutaneous administration of 65 mg/kg of 1-methyl nitrosourea (MNU). The rats were palpated weekly to determine the size of the tumor. Eight weeks post MNU administration, the rats were divided into five groups of six rats each and treated with graded doses (12.5, 25, and 50 mg/kg) of the compound and paclitaxel (10 mg/kg) for six weeks. Before treatment, three rats were randomly selected and sacrificed, and mammary gland samples were subjected to histological assessment to confirm the induction of the tumor. At the end of the treatment, the rats were euthanized, and mammary glands were collected and subjected to histological evaluation. The possible mechanism of action of the synthesized compound was elucidated *in silico* using molecular docking.

The compound was synthesized and named C6. C6 was found to be relatively safe with LD_{50} above 2000 mg/kg and exhibited remarkable antitumor activity. MNU-induced mammary tumor rats treated with C6 produced a significant decrease in tumor diameter when compared with the untreated group, histology slides displayed fewer signs of hyperplasia and small numbers of connective tissue with larger lobules when compared with the untreated group. *In silico* tubulin-binding interactions revealed that C6 binding to tubulin was like that of colchicine, and also has higher affinity to tubulin than colchicine.

The synthesized chalcone demonstrated significant antitumor activities in MNU-induced mammary tumors in rats postulated via inhibition of tubulin polymerization.

Keywords: Mammary tumor; Chalcones; Tubulin; Colchicine; Paclitaxel

Introduction

Cancer takes a heavy toll around the world through death, disability, and suffering (from those diagnosed with the disease and those whose lives are otherwise touched by it, including families, caregivers and medical workers) (Ferlay *et al*., 2021). Cancer is one of the major health problems that affect millions of people worldwide (Ali *et al.*, 2007). The Cancer Statistics Worldwide (WHO, 2021) documented that; more than one million new cases of female breast cancer are diagnosed each year. Breast cancer accounts for over one-third of the estimated annual 4.7 million cancer diagnoses in females and is the second most common tumor after lung cancer in both sexes. It is also the most common female cancer in both developed and developing countries with 55% of it occurring in developing countries (Ferlay, *et al.,* 2021).

Despite the nonstop discovery of new anticancer drugs, the treatment process is still inadequate and unsatisfactory due to several adverse effects on the normal cells combined with cell resistance to anticancer drugs (Yang *et al*., 2018 Jagsi *et al.,* 2019). As a result, there is a vital need for the discovery and development of new anticancer drugs with less toxicity and more ability to fight the development of drug resistance (Chan *et al.,*2021).

Proteins of different nature and dimensions are very important target in drug discovery and development. Structural proteins have also played a significant role in the discovery of vital drugs. Tubulin is a generic name for a family of globular protein which exist in solution as heterodimers α and β types of subunits. It is one of the most abundant cytoplasmic proteins that polymerizes to form microtubules (Banerjee *et al.*, 2018). Microtubules play a critical role in cell division by involving in the movement and attachment of the chromosomes during various stages of mitosis. Therefore, microtubule dynamics are important target for developing anti-cancer drugs (Banerjee *et al.*, 2018).

Colchicine is a natural product that was extracted from *Colchicum autumnale*. It has been used to treat acute gout. Although colchicine is a powerful antimitotic agent, the use of colchicine in cancers chemotherapy is hampered by its toxicity and the development of multidrug resistance (Lu *et al*., 2012). However, the observable biological activity of colchicine in microtubule destabilization has stimulated the exploration of new colchicine analogues with improved anticancer activity and low systemic toxicity (Dong *et al.*, 2016). Chalcones are a class of flavonoids that have been studied for their potential medicinal properties, including anti-inflammatory, antioxidant, and anticancer effects. Some studies have found that, chalcones relatively safe with no significant toxicity (Dong *et al*., 2016; Yang *et al*., 2018). Chalcones represents an attractive scaffold for the design of novel colchicine site ligands that inhibit tubulin assembly (Mengqi *et al.,* 2016). Several chalcones where synthesize and reported to exhibit an IC_{50} of less than 1 μM against K562, MCF7, Hep G2 cell lines (Prota *et al.,* 2014 and Mengqi *et al.,* 2016). Due to

relative ease in the synthesis of chalcones and the presence of potential skeleton of antiproliferative agent in their structures, they have been subjected to modification to enhance their antitumor potential (Lu *et al*., 2012). Research has found that chalcones have the potential to be used as anti-cancer agents against various types of cancer such as breast, prostate, lung, ovarian, and gastric cancer. However, most of the studies were conducted in the laboratory using cell lines, more research is needed to establish the safety and efficacy of chalcones as an anti-cancer treatment in animals and humans. Clinical trials are needed to confirm the safety and efficacy of chalcones as an anti-cancer agent. It's important to note that research on chalcones as anti-cancer agents is still ongoing and more studies are needed to fully understand their potential as a treatment for cancer (Yang *et al*., 2021). The aim of this work was to synthesize and evaluate the antitumor activity of a compound bearing chalcone moiety.

Materials and Methods

Equipment and glassware

Most of the equipment were sourced from the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University (ABU), Zaria. Nuclear Magnetic Resonance (NMR) experiments were performed in the department of chemistry, Kwa-Zulu Natal University, Durban South-Africa. Some of the equipment used include melting point apparatus, microscope, Agilent FTIR spectrometer (IndiaMART), 400MHz Agilent and 500MHz Bruker NMR spectrometer (IndiaMART).

Reagents, solvents, and standard drugs

All the starting reagents and solvents used for the experiments were of analytical grade and were used without further purification, these include acetyldehyde, acetophenone, Sodium hydroxide (50%), Acetophenone, iodine crystals, Chloroform, Ethylacetate, N-hexane, Ethanol, Hydrochloric acid, 10% Giemsa stain, Acacia, 1-methyl nitrosorea (Sigma Aldrich, Germany).
, Paclitaxel (Sigma Aldrich, Germany).

Synthesis of 2-Hydroxy Substituted Chalcone (Claisen –Schmidt Condensation)

The chalcone derivative synthesized via base catalyzed condensation of 0.1 molar of acetophenone and acetyldehyde were mixed with 20 ml ethanol in a round bottom flask. To this, 10 ml of 40%, sodium hydroxide solution was added drop wise with continuous stirring for 30 minutes while keeping the mixture cold. The mixing was then continued for another 2 hours at room temperature using magnetic stirrer and kept in a refrigerator overnight until it formed a colored solid mass. Drops of conc. 10% HCl were added to neutralize the reaction. The mixture was diluted using 40 ml ice-cold distilled water and then filtered; the residue was washed well with more ice-cold distilled water and dried in air. The product was recrystallized with ethanol, dried, final weight taken, and the percentage yield was calculated (Kumar *et al.,* 2010).

Characterization of the 2-hydroxy substituted chalcone

Characterization of C6 was done using the method prescribed by Kumar *et al,* (2010). Melting point was recorded in open capillaries with gallenklamp melting point apparatus. Detailed structural analysis of the synthesized compound was performed using Nuclear Magnetic Resonance (NMR) of; proton (^{1}H) NMR and carbon-13 (^{13}C) NMR

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Data for ^{1}H NMR were reported as follows: chemical shift (δ ppm), multiplicity (s =

singlet, d = doublet, dd = doublet of a doublet, m = multiplet), integration (J in Hz). Data for ^{13}C NMR reported in terms of chemical shift (δ ppm).

Animal management

Ethical approval was sought from the ABU Committee on Animal Use and Care (ABUCAUC) with an approval number of ABUCAUC/2022/003. Sixty Wistar rats weighing between 60-80 g of not more than 50 days of age were obtained and housed in the Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria. The animals were fed with standard feed and given access to water *ad libitum.*

Acute toxicity studies in rats

The oral median lethal dose (LD_{50}) was determined using OECD 425 guidelines in rats. Three rats were fasted prior to treatment for 3 hours, fasted body weight was determined for each animal and dose was calculated according to the body weight. Food was further withheld for two hours after the administration of the new compound. A rat was dosed 2000 mg/kg (limit test) and was observed for 48 hours. The first rat survived; thus, additional two rats were dosed 2000 mg/kg. The rats were observed for signs and symptoms of toxicity at least once during the first 30 minutes, periodically during the first 24 hours and then daily for 14 days after dosing.

Induction of mammary tumor

Female Wistar rats 45-50 days old were used for the study. Mammary tumor was induced according to the method of Thompson *et al.* **(1992)** with modification. Methyl nitrosourea was dissolved in normal saline and 65 mg/kg was administered by subcutaneous injection beneath the mammary gland of each rat. The rats were observed and palpated weekly to determine the development, localization, and size of neoplasia on the mammary gland. Eight weeks post-tumor induction, the rats were divided into five groups of six rats each. Rats in group I served as negative control and received normal saline (1 ml/kg), group II served as positive control and received Paclitaxel (10 mg/kg, *i.p*) in alternate days. Group III, IV and V rats were treated with graded doses of the synthesized compound (12.5, 25 and 50 mg/kg respectively via oral route) daily. Tumor diameter of each rat was measured before commencement of treatment and weekly during treatment with the synthesized compound. All the animals were treated for six weeks.

Samples collection

At the end of the study, rats were euthanized, and mammary gland samples were collected, preserved in 10%$^{v}/_{v}$ formalin in normal saline for histological assessment.

Molecular docking

The protein of interest is tubulin, and it was obtained from protein data bank with PDB ID 4O2B. Best resolved monomers were chosen for the study. All non-standard residues were removed from the 3D structures of the protein using UCSF Chimera. Isolated receptors and co-crystallized ligands were prepared on Chimera and saved as rec.pdb and Lig.mol2, respectively. Rec.pdb and Lig.mol2 were edited on ADT by adding polar hydrogen and Gastier charges then saved as pdbqt files.

The 2D structure of the ligand studied (2-Hydroxy chalcone) was generated using Chem Draw while Spartan was used to convert the 2D structures to 3D. Geometrical optimization using the AMI semi-empirical method was performed on the compound using the Spartan software and saved as mol2 file. Hydrogen and Gasteir charges were

added on ADT and mlo2 file converted to pdbqt.

Docking procedure for the protein was validated prior to docking the test compounds by separating the co-crystallized ligand from the protein crystal structure and re-docking it using the set-up parameters. Procedure that gives conformation superimposable with geometrical conformation of the co-crystallized ligand in the active site was chosen (De Oliveira *et al.,* 2013).

Molecular docking was performed considering a flexible ligand and rigid receptor (Oliveira *et al.,* 2013; Syahri *et al.,* 2017). Docking was carried out using virtual screening software AutoDock vina (Trott and Olson, 2010). The synthesized chalcone was docked on the active site of the protein. The grid box parameter for the protein is as shown on the (Table 1). The gridbox parameter was used to write configuration file (config.txt). AutoDock Vina generated results in the pdbqt pdbqt format. Compound having the best binding energy and optimal geometric conformation of the protein studied was selected from the ViewDock feature of Chimera and saved in complex with the reference protein. The following parameters were accessed using ViewDock; The binding interactions between the compound and the amino acids of the protein and the binding poses of the compound with respect to the protein (active sites).

Table 1: Grid Box Parameters for the Protein (4O2B)

	X	**Y**	**Z**
Grid box Center	17.24	63.99	37.12
Grid box Size	9.10	13.91	17.37

Statistical Analysis

Statistical analysis was carried out using SPSS (Version 20) and data obtained were expressed as mean ± SEM. Difference between mean were analyzed using one-way or repeated measures analysis of variance (ANOVA) where applicable, then, followed by Bonferoni post hoc test, for multiple comparison, values with $p \leq 0.05$ were considered significant.

Results

Thin layer chromatography of C6

The thin layer chromatography of C6 revealed the appearance of a single new spot and disappearance of the reactants spot (Figure 1).

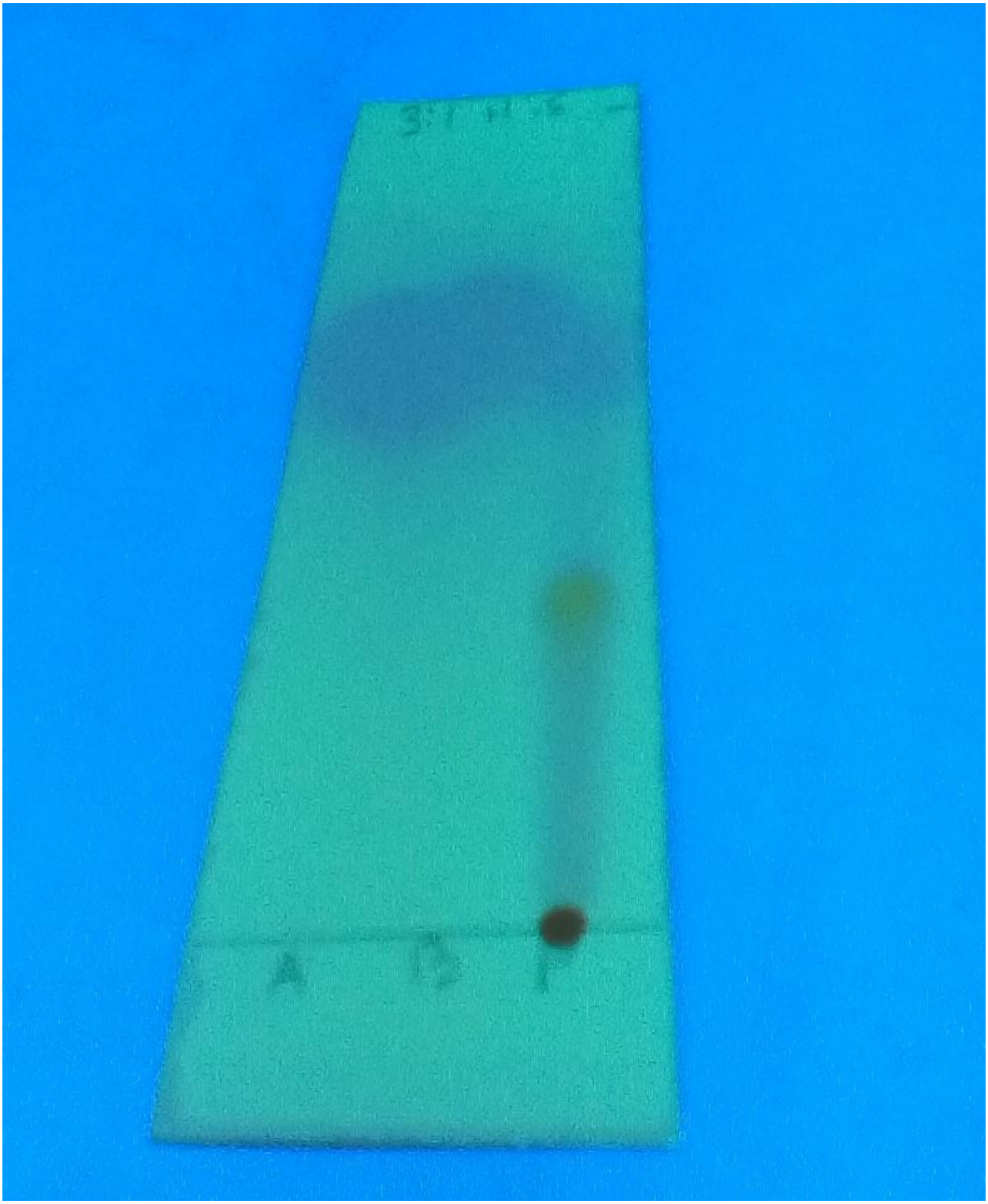
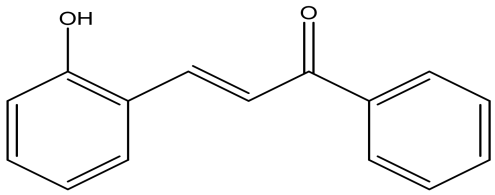


Figure 1 Thin layer chromatography (TLC) plate of C6 with N-hexane ethylacetate (3:7) as development solvent. P = C6

Yield and Some Physical Properties of the Substituted Synthesized Chalcones

The synthesized compound is a 2-hydroxy substituted chalcone with a proposed molecular formula of $C_{15}H_{12}O_2$, and molecular mass of 224.26 was named C6 (Figure 2). It is a pale-yellow powder with a retention factor of 0.40, percentage yield of 50.6% and melting point range of 109-111°C.



(*E*)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one

Figure 2: Structure and IUPAC name of C6

^{1}Hand ^{13}C NMR of the Synthesized Chalcone

The ^{1}H and ^{13}C NMR data of C6 are shown in supplementary information.

C6: 1H-NMR (DMSO) 6.38 (1H, s), 6.71 (1H, d, J=8.04 Hz), 7.04 (1H, t, J=7.26 Hz), 7.54 (1H, m, J=8.61 Hz), 8.05 (1H, q, J=15.10 Hz).

Acute Toxicity Study of the Synthesized Chalcone

The oral median lethal dose (LD_{50}) of C6 was found to be greater than 2000 mg/kg body weight in rats. The preliminary assessment during the first forty-eight (48) hours of the study revealed that, treatment with C6 did not show any critical effects that could lead to death of the rats. Assessment of the rats for fourteen days (14) revealed that, there was no alteration in functional and behavioral observations following the single administration of C6.

Effect of the Synthesized Chalcones on Mean Tumor Diameter in MNU-Induced Mammary Tumor Rat

There was a significant decrease in the mean tumor diameter of the group of rats treated with C6 at all the tested doses when compared with the tumor diameter of the rats before treatment. There was an increase in the mean tumor diameter of the group of rats treated with normal saline (Figure 3)

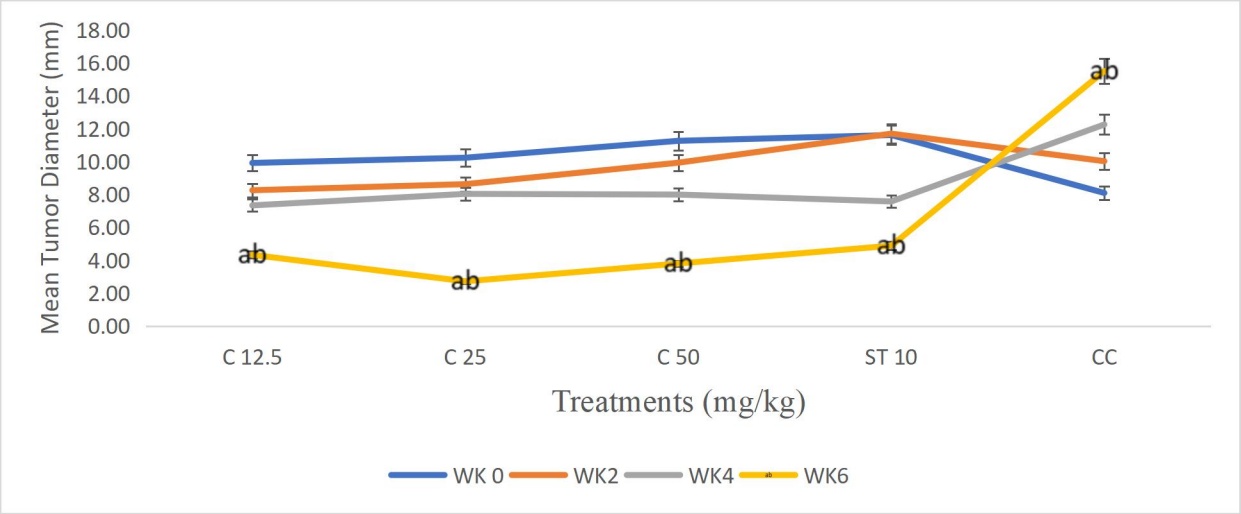
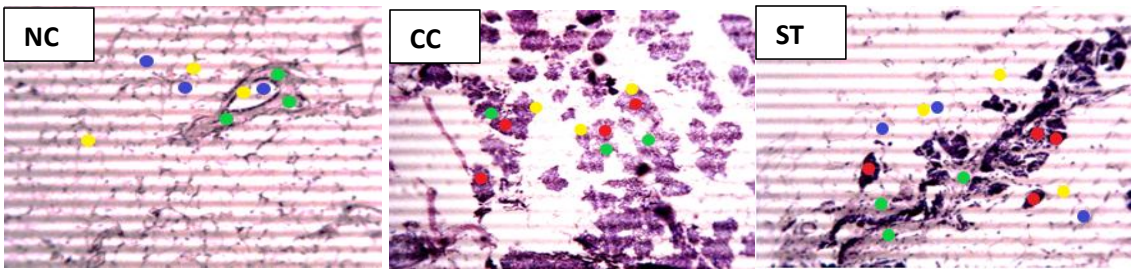


Figure 3: Effects of compound 6 administration on average tumor diameter in MNU induced mammary tumor rats.

Values are expressed as Mean ± S.E.M. Data was analyzed using repeated measures ANOVA followed by Bonferoni post hoc test. n=6, C = C6, ST = Paclitaxel CC =cancer control, 12.5, 25, 50 and 10 = doses in mg/kg, a = difference within, b = difference between the groups

Effect of the Synthesized Chalcones on Histological Examination in MNU-Induced Mammary Tumor Rat

Mammary tissues of control rats were shown to have normal fibrous connective tissue, well differentiated ducts, and tiny lobules. The induction of mammary gland tumor, characterized by dilated ducts filled with tumor cells and extreme hyperplasia of mammary lobules and decreased connective tissue, was shown by MNU-induced mammary tumor rats. Rats with MNU-induced mammary tumor treated with C6 displayed fewer signs of hyperplasia and small numbers of connective tissue with larger lobules when compared with the untreated group. The group of rats treated with (25 mg/kg) showed no signs of hyperplasia, ducts were well-differentiated, and tiny lobules that is almost the same with the control group were observed (Figure 4).



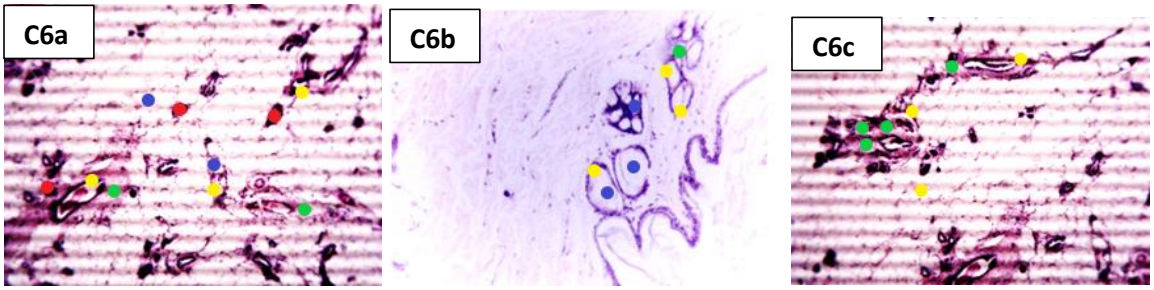


Figure 4: Photomicrograph of a Sections of the Mammary Gland of Female Wistar Rats Showing the Lactiferous Glands of the Rats Treated with C6

NC=normal control, CC=cancer control, ST=Paclitaxel (10mg/kg) C= C6, a=12.5 mg/kg, b=25 mg/kg and c=50 mg/kg, Red dots=cancer cells, blue dots=milk duct, yellow dots=lobules, green dots=connective tissues.

Molecular Docking

The docking procedure applied on the protein was validated. As shown on Figure 5, the co-crystallized ligand (ash) and re-docked ligand (colored by element) on its respective protein was well super imposed on its original PDB structures.

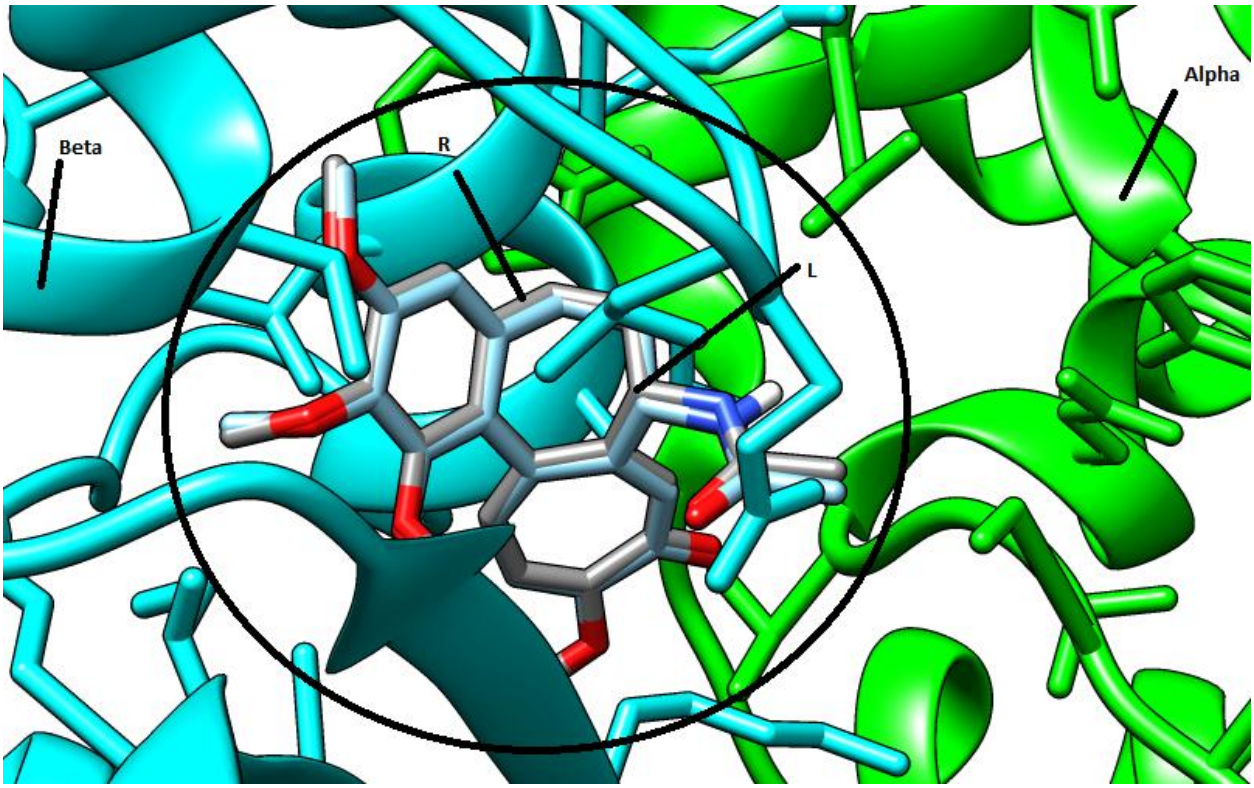


Figure 5. Molecular Docking Validation with the Co-Crystallized Ligand (Colchicine) of Tubulin Protein (PDB ID 4o2b)

cyan = alpha-tubulin, green = beta-tubulin, L= docked Colchicine (colored by elements), R = co-crystalized ligand (blue) binding in 4O2B protein

Docking results indicated that C6 exhibited better binding energies to 4O2B (-8.4 Kcal/mol) than the co-crystallized ligand (-7.5 Kcal/mol). The binding conformation and interaction with amino acid residues on the active site of the protein studied showed formation of bonds between the atoms of C6 and amino acids of 4O2B (Figure 6).

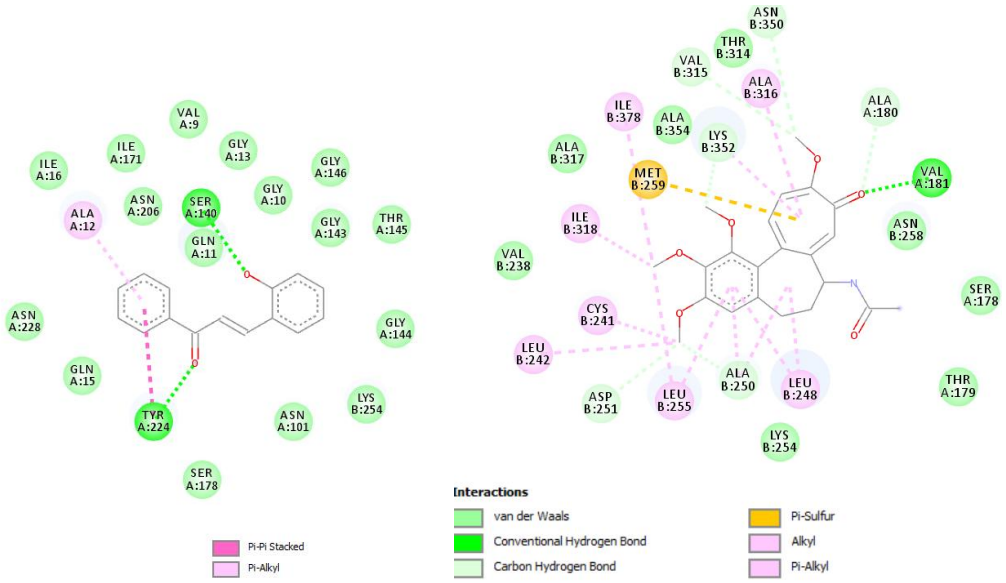


Figure 6: 2D intermolecular interactions between docked compounds (Colchicine ® and synthesized chalcones (C6)) and 4O2B protein

Discussion

The first step in confirming the synthesis of C6 is the appearance of new spot in the thin layer chromatography that is distinct to that of the reactants. This is line with study conducted by Kumar *et al* which states that 'appearance of a single new spot and disappearance of the reactants spot in thin layer chromatography confirmed the formation of the product' (Kumar *et al*, 2010), The synthesis of C6 furnished good yields, this agrees with the synthesis of chalcones and other chalcone derivatives reported in the literature (Li *et al.,* 1995). The sharp melting points observed with the compound suggests that the compound had high degree of purity (Vogel, 2006; Sinha *et al.,* 2013).

The ^{1}H NMR spectra was used to propose the structure of C6. In addition to the characteristics *α,β*-unsaturated ketone linker protons observed as doublets in the region of 7.42 – 8.33 ppm (H- α) and 7.89 ppm (H- β) with coupling constants of 15.9Hz , the rest of the protons appear in their expected regions with their usual coupling constants.

Additional support for the structures of C6 also came from the ^{13}C NMR spectra, where the carbonyl carbon (C=O) of the *α, β*-unsaturated ketone linker is observed in the region 186 ppm. Also, the α and β- carbon atoms with respect to the carbonyl group gave rise to signals between δ 100 and δ150 ppm (δ120.3 ppm and δ 139.5 ppm respectively)

which is characteristics of carbon atoms on either end of a non-aromatic double bond(Supplementary information).

The LD_{50} of a compound provides an insight on the potential toxicity of that compound and serves as a guide in dosage determination(Gbolo *et al.,* 2019). The synthesized compound (C6) was found to be relatively safe with LD_{50} above 2000 mg/kg per oral.

The size of a tumor is one of the indices of assessing the progress of cancer in patients and the mean tumor diameter is one of the tools that was used to assess the treatment in this study. The observed ability of C6 to significantly decrease the tumor size in the rat model, indicates that, C6 possesses antitumor activity in MNU-induced mammary tumor. This is because, increase in size of the tumor, signifies progression of the disease. Any substance that is capable of significantly decreasing the tumor diameter implies that, it possesses cytotoxic activity. Histological evaluation is a crucial step in the assessment of breast cancer and helps to determine the type, grade, and stage of progression which are important factors in guiding treatment decisions. Histological features of breast cancer (ductal carcinoma) include inflammation and dilation of duct that is filled with tumor cells, hyperplasia of the lobules and decrease number of connective tissues (Jagsi *et al.,* 2019). All the features were present in the group of rats that was treated with MNU, and thus, confirmed the induction of cancer. Induction of tumor was done by MNU, which is a chemical carcinogen that is known to destroy P53 gene. This gene is responsible for inhibition of cell proliferation and induction of apoptosis (Mantovani, 2018). Treatments with the C6 was found to have a remarkable antitumor activity at all the tested doses in MNU-induced mammary tumor. The ability of C6 to to reduce the tumor diameter was a result of its cytotoxicity against cancer cells and control hyperplasia as seen in the histology slides (Figure) that led to shrinking of the tumor. The best activity was seen in the group of rats treated with 25 mg/kg of C6, this implies that, C6 at this dose was able to almost completely kill the cancer cells and prevent further abnormal proliferation of the cells of the lactiferous gland. Chalcones have been shown to exert cytotoxic activity against many cancer cells lines through multiple mechanisms such as inhibition of angiogenesis, inhibition of cell proliferation and induction of apoptosis (Banerjee *et al.,* 2018). The ability of C6 to inhibit or kill the cancer cells induced by MNU could be suggestive of the activity of the compound through one or more of those mechanisms.

Following the observed *in vivo* anticancer activity, the possible mechanism of of action was evaluated using molecular docking. From the docking scores, it could be deduced that C6 had good affinity for 4O2B, which could be one of the reasons behind its antitumor activity seen in this study. Tubulin-binding interactions revealed that, C6 binding to tubulin was similar to that of colchicine.The ability of a compound to alter the biological function of a protein/enzyme depends on the affinity of that compound to the protein/enzyme. When colchicine binds to tubulin, it was reported to alter the function of tubulin by inhibiting its proliferation because of formation of chemical bonds between the atoms of colchicine and some of the amino acids of the tubulin (Pirhadi *et al.,* 2013). Since the binding interaction of C6 is similar to that of colchicine, with higher affinity to the tubulin than colchicine, C6 is postulated to act via the same mechanism with colchicine in addition to its 4O2B mediated action. It

was reported that, the microtubule-destabilizing activity of colchicine can be explained by the binding of colchicine to the tubulin preventing the curved to straight structural transition in tubulin, a process that is necessary for microtubule formation (Prota *et al.,*2013; Banerjee *et al.,* 2018). When C6 binds to tubulin, it is postulated to act like colchicine by inhibiting further polymerization of tubulin and thus inhibit its function. Microtubules function in many essential cellular processes, including mitosis. Tubulin-binding drugs kill cancerous cells by inhibiting microtubule dynamics, which are required for DNA segregation and cell division (Dumontet and Jordan, 2010). The antitumor activity of C6 seen in MNU-induced mammary tumor could be because of inhibition of the tubulin.

Conclusion

The synthesized chalcone analogues possesses significant antitumor activities in MNU-induced mammary tumor in rats possibly via inhibition of tubulin polymerization and was to be found relatively safe in Wistar rats.

Conflicts of interest

The authors declared no conflicts of interest.

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