Diaryheptanoid, a structurally related phytoestrogen inhibiting the growth of estrogen dependent breast cancer cells and its interaction with estrogen receptors

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Abstract

A decreased prevalence of breast cancer is associated with the excessive consumption of phytoestrogens which are biologically active plant-derived phenolic compounds that structurally mimic the mammalian estrogen, estradiol-17β. Consequently, hormone replacement therapy during the postmenopausal period is contraindicated in women with breast cancer. This study investigated the anti-estrogenic effects of DAH on the MCF-7 cell line, comparing its effects to the known anti-estrogen ICI182780. The anti-estrogenic effect was confirmed through an estrogen-inducible cell proliferation assay. The MTT assay demonstrated the anti-proliferative activity of DAH by confirming cell viability.

An estrogen receptor binding assay showed DAH's capacity to bind to estrogen receptors. Furthermore, mRNA expression levels of pS2, an early-stage estrogen-responsive gene in estrogen-dependent breast cancer cell lines, were downregulated, further confirming the anti-estrogenic effect. Additionally, docking analysis using Autodock demonstrated the binding ability of DAH to the estrogen receptor.

Keywords: Phytoestrogen, Diarylheptanoid (DAH), Breast cancer, Estrogen receptor, *In silico*.

Introduction

Breast cancer is one of the most common malignancies in women and is the leading cause of death worldwide for those aged 40 to 55 years. It is highly resistant to chemotherapy, with no effective cure for patients in advanced stages of the disease. In recent years, there has been considerable effort to search for naturally occurring phytocompounds to intervene in carcinogenesis. Many components from medicinal or dietary plants have been identified as possessing potential chemopreventive properties.[5] A lower incidence of breast cancer is associated with high consumption of phytoestrogens which are biologically active plant-derived phenolic compounds that structurally mimic the mammalian estrogen, 17-β estradiol.[8]

The sex steroid hormone estrogen is crucial for many physiological processes such as growth, differentiation and function of tissues in the reproductive system including the mammary glands, uterus and ovaries in females and the testis, epididymis and prostate in males. However, prolonged stimulation of breast ductal epithelium by estrogen contributes to the development and progression of breast cancer, making treatments designed to block estrogen's effects important in clinical settings.

The effect of estrogen is mediated through its binding to nuclear proteins called estrogen receptors (ERs) which regulate estrogen-responsive genes.[14] Phytoestrogens possess beneficial health effects by reducing the risk of cancer, osteoporosis and cardiovascular diseases and act as estrogen agonists/antagonists via the known ERα and ERβ subtypes.[11] While many compounds can bind to the estrogen receptor (ER), they can differ markedly in their stimulatory and/or inhibitory effects. Moreover, certain compounds, now referred to as selective estrogen receptor modulators (SERMs), have demonstrated agonistic/antagonistic effects depending on the tissue. SERMs alter receptor conformation and facilitate the binding of co-regulatory proteins that activate or repress the transcriptional activation of estrogen target genes like pS2 and the progesterone receptor.[19]

Phytoestrogens include isoflavonoids (genistein, daidzein), lignans (enterolactone, enterodiol), coumestans (coumestrol), flavonoids (kaempferol, quercetin) and stilbenes (resveratrol), all of which exhibit estrogenic and anti-estrogenic properties.[2] As such, phytoestrogens have been considered as an alternative to Hormone Replacement Therapy (HRT) due to their potential health benefits in alleviating menopausal symptoms and lowering the incidence of hormone-dependent diseases, including breast cancer. ERα promotes the proliferation of breast cancer cells, whereas ERβ acts as a tumor suppressor. The genes regulated by SERMs for ERα are distinct from those regulated for ERβ, indicating that drugs selectively targeting either ERα or ERβ could produce better clinical outcomes.[16]

A study shows that Ginkgo biloba extract (GBE) induces cell proliferation at low estrogen (E2) concentrations (10^-13 and 10^-12 M), which have little or no estrogenic activity, but block cell proliferation caused by higher E2 concentrations (from 10^-11 to 10^-9 M), which exhibit high estrogenic activity. Therefore, it was suggested that GBE has a biphasic response depending on the E2 concentration.[15] Most data support the notion that many known phytoestrogens compete more effectively with E2 for binding to ERβ than to ERα and demonstrate biphasic

activity, estrogenic action at low concentrations (<10^-6 M) and anti-estrogenic action at high concentrations (>10^-6 M) where they exert anti-estrogenic-cytotoxic activity.[18]

The preferential binding of known phytoestrogens to ERβ may be important in their anti-estrogenic effect in suppressing breast tumor growth, since ERα is mainly involved in promoting cell proliferation whereas ERβ has a counter-proliferative protective effect.[9] DAH, a naturally occurring phytochemical, is beneficial for nutraceutical health promotion. Pharmacologically, ginger contains a complex mixture of compounds such as gingerols, β-carotene, capsaicin, caffeic acid and curcumin.

Various formulations of ginger have been shown to act as dual inhibitors of many molecular target genes. *Alpinia officinarum*, traditionally used in China for relieving stomach ache, treating colds, invigorating the circulatory system and reducing swelling, contains DAH, has been reported to exhibit a variety of therapeutic actions *in vitro*, including inhibition of platelet aggregation, antioxidant,[21] anti-inflammatory, antimicrobial and anti-tumor promoting effects.[17] Therefore, we evaluated the anti-estrogenic effect of DAH isolated from the rhizome of *Alpinia officinarum* in human estrogen receptor-positive breast cancer cells. Docking analysis confirmed that the anti-estrogenic activity of DAH is similar to the ER antagonist Tamoxifen's interactions with the ERα domain.

Material and Methods

Cell lines and culture conditions: Breast adenocarcinoma cell lines MCF-7 (ER+ve) from ATCC were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, 2mM L-glutamine and 1% penicillin/streptomycin (Pen Strep) under a fully humidified atmosphere with 5% CO_2 at 37°C. For experimental conditions, MCF-7 cells were grown in phenol red-free DMEM medium containing 5% charcoal-stripped FCS (CCS, Sigma) and 0.2% Pen Strep (test medium). Untreated and vehicle-treated cells were included as controls in all experiments. For the experiments, cells were collected from sub-confluent monolayers using trypsin/EDTA. The studies were conducted using cells with low passage numbers and the cells were pre-incubated for 24 hours in the test medium to remove exogenous estrogens.

Extraction and bioactivity-guided isolation: *Alpinia officinarum* hexane extract (AOHE) (5 g) was prepared and subjected to solvent-solvent fractionation by dissolving it in 60% methanol. The soluble portion of AOHE in 60% methanol was partitioned with hexane, chloroform and butanol. Based on bioactivity, the hexane-soluble fraction was selected and further purified using column chromatography on Sephadex LH-20 with a hexane-ethyl acetate gradient mobile phase system (from 10:90 to 50:50), yielding five sub-fractions. Sub-fractions 3 and 4 yielded a yellow-colored oily substance with significant anti-proliferative activity. The pure molecule was subjected to NMR and mass spectroscopic studies for structure elucidation.

Cell viability (Anti-proliferative effect) determined by MTT assay: Cell growth inhibition by DAH was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with slight modification. Briefly, MCF-7 cells were seeded in 96-well plates at a density of 0.2 million cells per well. After treatment with various concentrations of DAH for 24 h, 10 µL of MTT (5 mg/mL) was added. After 4 h of incubation, 100 µL of DMSO was added to each well to dissolve the resulting formazan crystals. Absorbance was read at 492 nm using an enzyme-linked immunosorbent assay reader (Spectra Max; Molecular Devices, Sunnyvale, CA). Data were collected from three separate experiments and the percentage of DAH-induced cell growth inhibition was determined.

Estrogen-induced cell proliferation assay: Various concentrations of 17-β estradiol (E2), ranging from 2 nM to 50 nM, were used to assess cell growth. We found that 10 nM concentration induced maximum cell proliferation compared to control, so further assays were carried out using 10 nM of 17-β estradiol (E2) for induction. MCF-7 cells were seeded in 96-well plates at a density of 5 x 10^3 cells per well and treated with DAH (500 nM, 1 µM, 5 µM, 10 µM and 50 µM/mL) or 10 nM 17-β estradiol (E2) or 10 nM ICI 182-780. After incubating for 48 h, the medium was aspirated, 100 µL of MTT reagent (5 mg/mL) was added and the cells were incubated for 3 h at 37°C.

The viable cell number was directly proportional to the production of formazan following solubilization with MTT lysis buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide) which was measured spectrophotometrically at 570 nm. The cell proliferation data (RPE, relative proliferation effect) were calculated as RPE = [(S-1)/(E-1)] x 100 where S = proliferation of samples and E = proliferation of positive control (10 nM E2).[10]

Competitive ER binding assay: Hydroxyapatite (HAP) assay was carried out to check the competitive binding of DAH to ER. MCF-7 cytosolic extract was used for competition-binding studies. Cytosol was prepared from cells grown for 4 days in estrogen-depleted medium. The protein content was measured spectrophotometrically at 570 nm using Bradford reagent. About 40 µg of the total protein (ER preparations) was incubated overnight at 4°C with varying concentrations of DAH (500 nM, 1 µM, 5 µM, 10 µM and 50 µM/mL), 20 nM [^{3}H]-estradiol±100-fold molar excess of estradiol (E_2) in a final volume of 250 µL. 250 µL of a 60% HAP suspension in TEM buffer was added and the mixture was incubated at 4°C for 15 min.

The HAP-bound receptor-[^{3}H]-E_2 complex was separated by centrifugation at 200×g for 15 min. After washing twice with Tris buffer (10 mM), the HAP pellet was extracted with 1

mL of absolute ethanol.[20] These extracts were added to 4 mL of scintillation cocktail and the radioactivity was measured in a Wallac 1409 liquid scintillation counter. Data were expressed as the ratio of bound [^{3}H]-E_2 in the presence of a competitor to the bound [^{3}H]-E_2 in control × 100.

mRNA level expression of pS2 by RT-PCR: MCF-7 cells were seeded into 6-well plates at a density of 5×10^5 cells/mL per well. After 24 h of incubation, the seeding medium was changed to the experimental medium (phenol red-free 5% CDFBS-DMEM). The anti-estrogenic activity of DAH was determined by exposing the cells to E2 (10^-11 M) as control and ICI 182-780 as positive control. The medium was then removed and the cells were scraped from the dishes to determine the amount of pS2 mRNA. The total RNA was purified using TRIzol reagent (GIBCO) according to the manufacturer's protocol. The RNA was stored at −80°C. The cDNA sequences of human pS2 are available in the GenBank. The primers used are as follows: pS2-up: 5'-CATGGAGAACAAGGTGATCTG-3'; pS2-down: 5'-CAGAAGCGTGTCTGAGGTGTC-3'. The mRNA products were amplified by RT-PCR and were run on 1.5% agarose gel, then the product was detected and analyzed using the Gel documentation and Analysis system (UVP).[1]

Docking analysis: The 3D structure coordinates of ERα (3ERT) were taken from the RCSB protein data bank as PDB coordinates and protein preparation was performed by ADT (MGL Tools 1.5.6). The 4-OHT (4-hydroxytamoxifen) molecule from the 3ERT complex was separated and used as the reference ligand for ERα antagonism. The ligand molecule 17-β-estradiol from the 1A52 complex was separated and used as the reference ligand for ER agonist property. The DAH was drawn using J mol and obtained as PDB structures from the PRODRG 2.0 server. The 3D PDB structures were energy minimized for 1000 steps with Gasteiger charges using CHIMERA.

All the ligands were rigidly docked individually to the active site of ERα (3ERT) in a grid box of 66 x 66 x 66 with a spacing of 0.375 Å using the Lamarckian Genetic Algorithm, allowing all active torsions of the ligand to select the best 20 conformations from a population of 150 individuals using a cluster tolerance of 2 Å. Using AutoDock 4.2, the docking calculations were performed and the resulting interactions were analyzed by ADT 1.5.6.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 4.03 (San Diego). One-way Analysis of Variance (ANOVA) followed by Dunnett's post hoc test was used for other parameters. Data are expressed as mean ± S.E.M and $p < 0.05$ was considered to be statistically significant.

Results and Discussion

Phytochemicals have been reported to offer protection against the development of various cancers including breast cancer.[13] Exogenous estrogens, such as those used in hormone replacement therapy (HRT), are commonly prescribed to postmenopausal women to reduce the risk of osteoporosis and cardiovascular disease. However, many women are hesitant to use HRT due to their undesirable side effects and safety concerns.[12] Therefore, in this study, we aimed to investigate and to report the anti-estrogenic activity of DAH from *Alpinia officinarum* in MCF-7 (ER+ve) breast cancer cells.

The hexane extract of *Alpinia officinarum* (AOHE) demonstrated a significant anti-proliferative effect compared to the other two extracts (data not shown) in MCF-7 cells as determined by the MTT assay. Following solvent-solvent fractionation and based on its anti-proliferative activity, the hexane-soluble fraction was further purified using column chromatography which led to the isolation of a pure compound. Structural characterization through NMR and mass spectral analysis identified this compound as 1-(4-Hydroxyphenyl)-7-phenyl-hept-4-en-3-one, with the molecular formula $C_{19}H_{20}O_2$ and a molecular weight of 280.36.

The compound exhibited a characteristic yellow color and had an oily consistency. Chromatographic analysis of the *Alpinia officinarum* hexane extract (AOHE) and the isolated pure compound DAH were conducted at 546 nm and documented with photographs. The purity of DAH was verified by comparing the UV absorption spectra at the start, middle and end positions of the chromatographic bands.

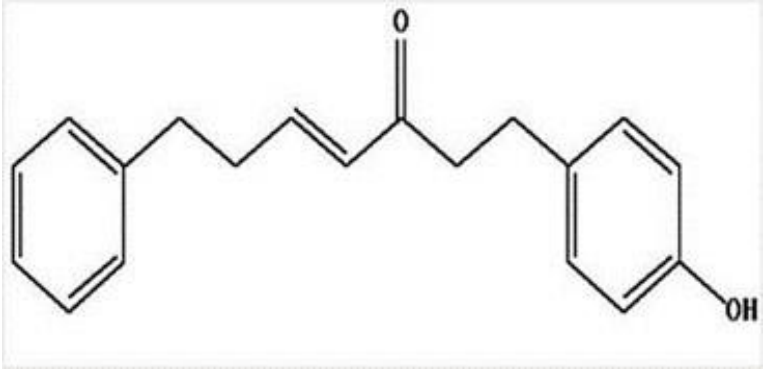

Figure 1: Structure of the bioactive compound chemically characterized as 1-(4 Hydroxyphenyl)-7-phenyl-hept-4-en-3-one with molecular formula $C_{19}H_{20}O_2$ (Molecular weight: 280.36) and identified as Diarylheptanoid (DAH).

The amount of DAH present in AOHE was determined to be 0.81% w/w using the HPTLC method. The MTT assay confirmed that DAH exhibited maximum anti-proliferative activity in a concentration-dependent manner, with an IC_{50} of 35.7 µM in MCF-7 cells and showed no cytotoxic effects. Consequently, further studies were conducted to elucidate the molecular mechanism of action for DAH at its IC_{50} value. Estrogens are known to stimulate the growth of breast cancer cells while antiestrogens inhibit cell growth.[4] To assess the anti-proliferative effect of DAH, an MTT assay was performed, which relies on the reduction of tetrazolium salt by the mitochondria of living cells to form a blue formazan product.[6]

The assay, which reflects the number of viable cells and their mitochondrial activity, showed a dose-dependent decrease in the viability of MCF-7 cells treated with DAH compared to the untreated control. Upon stimulation of MCF-7 cells with 17-β estradiol (10 nM), the proliferative effect was assessed by measuring the percentage of cell viability. MCF-7 cells were treated with different concentrations of DAH (500 nM, 1 µM, 5 µM, 10 µM and 50 µM) for 48 hours. A significant decrease in cell viability at 50 µM of DAH was observed along with inhibition of proliferation, compared to the ER antagonist ICI 182780 (10 nM) which was used as a positive control, suggesting an anti-estrogenic property of DAH. Co-incubation of DAH (50 µM) with ICI 182,780 exhibited a significant inhibition of cell proliferation.

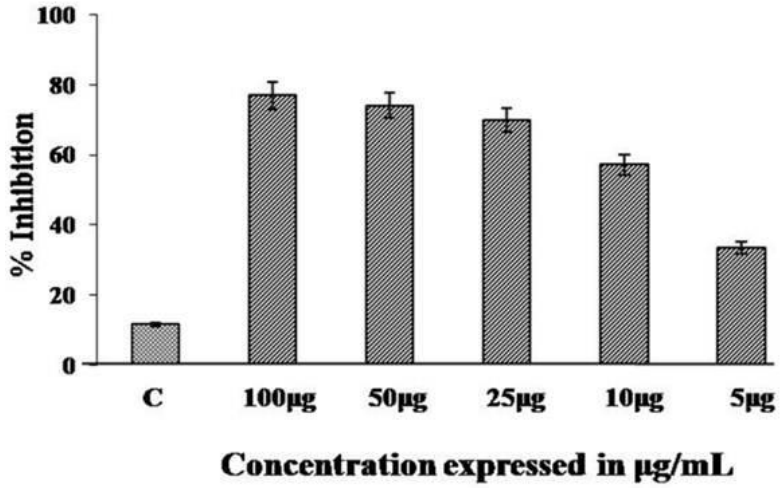

Figure 2: DAH inhibited the growth of MCF-7 cells in a dose-dependent manner. Cells were treated with different concentrations of DAH. After 24 h incubation, the cells were harvested and the proliferative effect was determined by MTT assay. The optimum concentration of DAH to inhibit growth of the cells was found to be 10 µg/mL (35.7 µM) respectively. Values shown as the mean±SEM were obtained from three independent experiments.

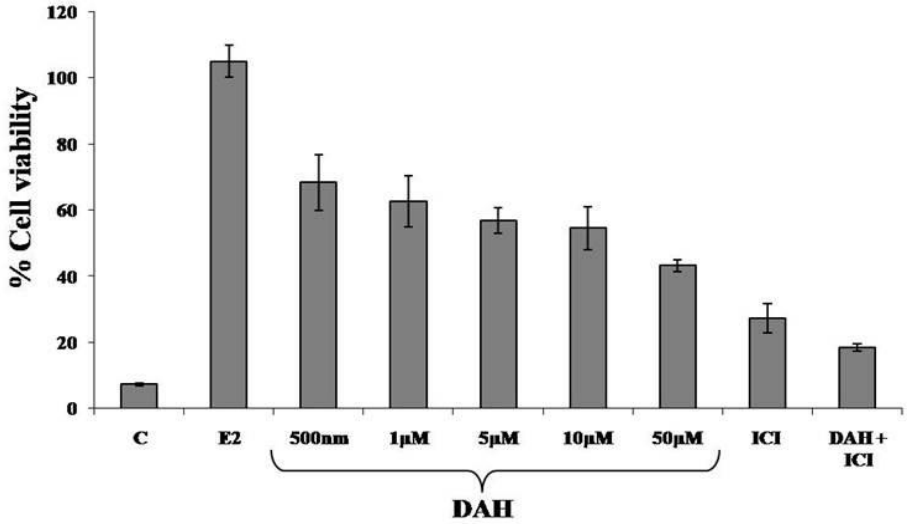

Figure 3: Effect of DAH on inhibition of MCF-7 cell proliferation induced by 17-β estradiol (10 nM) in comparison and co-treatment with positive control ICI182780 (10 nM). Maximum inhibition of MCF-7 cells at 50 µM of DAH was observed. Data represent the mean±SEM of triplicates of two independent experiments as compared with untreated control.

Generally, anti-estrogenic compounds exert their effects by competing directly with E_2 for binding to the estrogen receptor (ER), forming a functionally inactive ligand-bound complex. Alternatively, they may induce the metabolism of E_2 or inhibit its biosynthesis, indirectly depleting endogenous E_2 available for binding to ER. Inhibiting estrogen activity is a common therapeutic approach for estrogen-dependent breast tumors in postmenopausal women and for tumor prevention in premenopausal women. In this study, we analyzed the anti-estrogenic effect of DAH through direct binding to ER in ER-positive human mammary carcinoma cell line (MCF-7).

Cytosolic extracts from MCF-7 cells were treated with various concentrations (500 nM, 1 µM, 5 µM, 10 µM and 50 µM) of DAH and their ability to compete with [^{3}H]-E_2 binding was assessed to investigate the binding of DAH to the ER. DAH displaced [^{3}H]-E_2 specifically bound to ER in a concentration-dependent manner, with maximum binding observed at 50 µM DAH. Confirmation studies using competitive binding assays revealed that DAH binds to ER in a dose-dependent manner, exhibiting an IC_{50} of 50 µM. These findings underscore the role of ER-regulated genes in controlling cell proliferation.

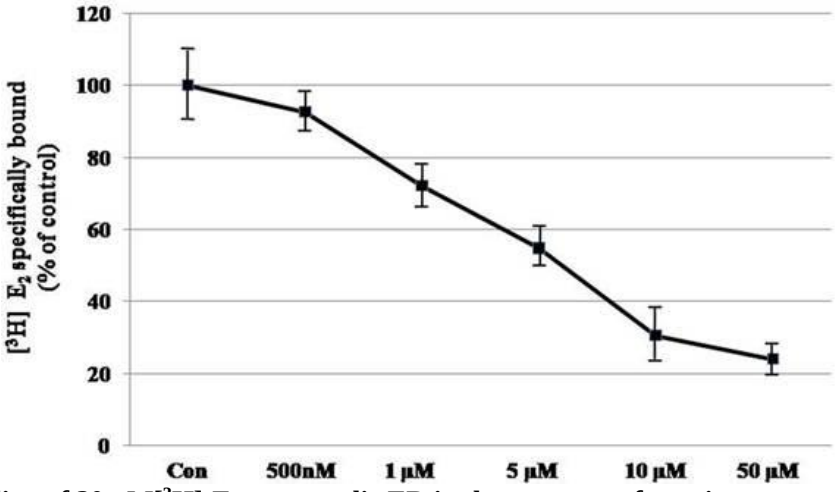

Figure 4: Binding of 20 nM[^{3}H]-E_2 to cytosolic ER in the presence of varying concentration of DAH. Specific bound radio ligand was calculated by subtracting non-specific bound counts from total bound counts. Data were presented as mean±SEM from three separate experiments for each data point.

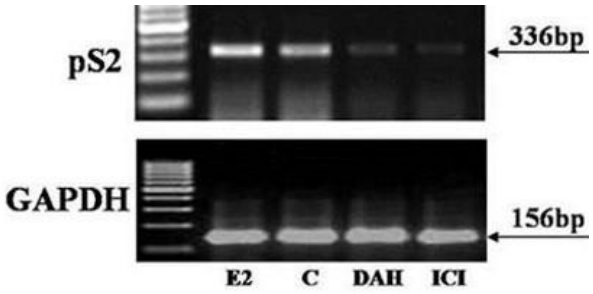

Fig.5.A

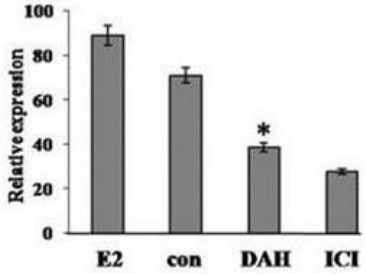

Fig.5.B

Figure 5: Effect of DAH on pS2 gene expression in MCF-7 cells for 24 h (Fig.5A) Lane 1- DNA ladder (100bp), Lane 2- 17-β estradiol (10 nM), Lane 3- Control, Lane 4- DAH (50 uM), Lane 5- ICI 182780 (10 nM). The graph (Fig.5B) shows the ratio of density of pS2 expression to that of endogenous control GAPDH and represents mean±SEM of three replicates when compared to untreated control.

However, ligands binding to ERα and ERβ may display varying degrees of agonism or antagonism depending on the estrogen-responsive tissue and ligand concentration, modulating estrogen-responsive genes through direct interaction with DNA at estrogen response elements (ERE) in the promoter region. The pS2 gene, an early estrogen-responsive gene in estrogen-dependent breast cancer cells, is directly regulated by estradiol.[7]

Hence, we investigated the effect of DAH on pS2 gene expression as a model for endogenous estrogen-responsive genes expressed in MCF-7 cells. Treatment with DAH (50 µM) resulted in downregulation of pS2 gene expression compared to untreated (control) and E2-treated cells (Fig. 5A).

The relative expression of pS2 was normalized with GAPDH compared to the control **(**Fig.5B**)**. The pS2 gene expression level was found to be downregulated in comparison to 17-β estradiol (10 nM). ER antagonist ICI182780 was used as agonistic and antagonistic control respectively and a significant down-regulation in comparison to ICI182780 was observed confirming the ER antagonistic property. The binding interactions of small molecules with receptor proteins for deriving thermodynamic data are determined by the three-dimensional orientation, relative binding affinity and many other parameters. The estrogen receptor (ER) is a member of the nuclear receptor (NR) super family[3].

These proteins are ligand activated transcription factors involved in a number of biological processes such as homeostasis, lipid metabolism, embryonic development and cell death.

Upon the dysfunction of NRs, diseases and malfunctions such as obesity, diabetes, infertility and cancer may develop. The NR family consists of 48 different proteins, each consisting of three functional domains. These are the N-terminal transactivation domain, the central DNA binding domain and the C terminal ligand binding domain (LBD), where the activation function-2 (AF-2) is positioned. The overall architecture of the LBD is conserved among all NRs; however, selective ligand interactions are entirely due to this domain.

Various isoforms of NR LBDs, all with their own particular ligand specificity, may be found in different tissues and thus provide opportunities for specific medicinal targeting of these domains. Previous *in silico* analysis of binding with ER alpha has suggested, tamoxifen to act as an effective known ERα antagonist through its hydrogen bonding interaction with Glu 353 and Arg 394 at the active pocket and another with a water molecule. To understand the interaction of DAH with estrogen receptor, *in silico* docking studies were initially performed with estrogen, tamoxifen (ER antagonist) and DAH towards Erα. To understand the interaction of DAH with ER, *in silico* docking studies were initially performed with estrogen, tamoxifen (ER antagonist) and DAH towards ERα.

Results of docking analysis of ERα agonist estradiol showed the best possible conformation with the least binding energy of – 9.83 Kcal/mol, forming 4 hydrogen bonds with Gly 521, Glu 353, Arg 394 and His 524 **(**Fig.6A**)**. Docking analysis of Tamoxifen (4-OHT) showed the best possible conformation with the least binding energy of -12.05 Kcal/mol and formation of 3 possible hydrogen bonds with Glu 353, Asp 351 and Arg 394 **(**Fig.6B**)**. Interestingly, docking analysis of DAH, showed the first best conformation with a least binding energy of -8.7 Kcal/mol forming hydrogen bonds with His 524 and Gly 521 and the second-best conformation with the least binding energy of -7.82 Kcal/mol forming hydrogen bonds with Arg 394 and Glu 353 (Fig.6C).

Thus, the results clearly show that DAH exhibits similar interactions as tamoxifen which could be suggestive of its ER antagonistic activity.

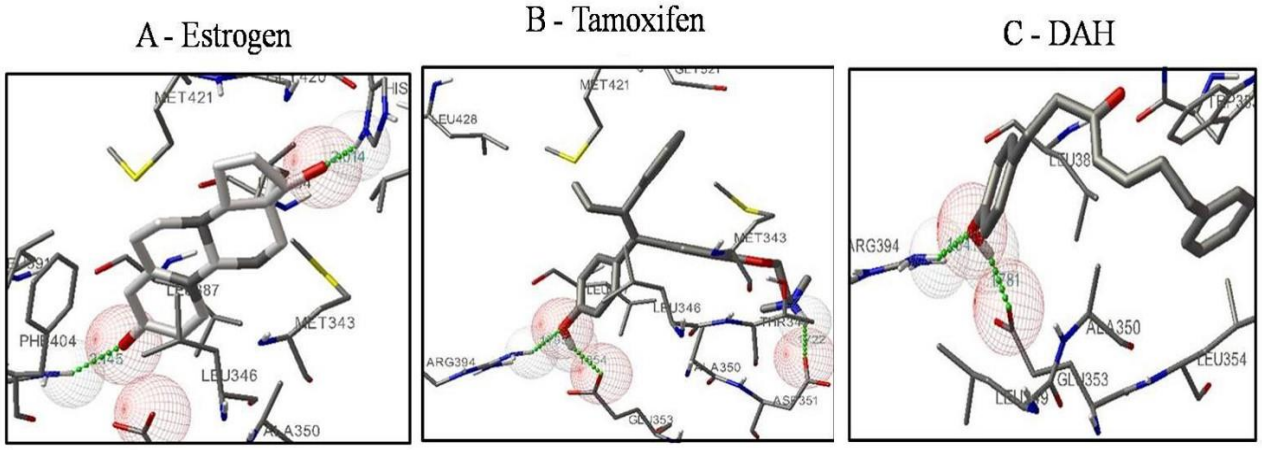

Figure 6: Effect of Estrogen (A), Tamoxifen (B) and DAH (C) interactions with ER-alpha domain. Estrogen, Tamoxifen and DAH are represented as thick stick model with surrounding residues as thin stick model (colored by atom type). The atoms forming hydrogen bond (Arg-394, His-524) are shown as meshed sphere. Hydrogen bonds are shown as green dotted line.

Conclusion

The anti-estrogenic activity of DAH isolated from *Alpinia officinarum* was confirmed through various assays. Furthermore, docking analysis affirmed that DAH interacts similarly to the ER antagonist Tamoxifen with the ERα domain. These findings provide evidence and rationale for the potential therapeutic effects of DAH, paving the way for further development in the prevention and treatment of hormone-dependent breast cancer.

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