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RESEARCH ARTICLE

Dehydrocostus lactone from the root of *Ajuga integrifolia* Buch.-Ham.: Quantitative determination and *in silico* study for anti-breast cancer activity

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Abstract

Many biological activities were reported for the *Ajuga* species, specifically for *Ajuga integrifolia* and its synonyms. These include anti-oxidant, anti-inflammatory, anti-diabetic, anti-bacterial, blood purifier effects, and anti-cancer activity. This study quantitatively determines dehydrocostus lactone (DHCL) from the root of *A. integrifolia* and its *in silico* study for anti-breast cancer activity. Camag HPTLC was used for TLC-densitometric estimation of dehydrocostus lactone. Estrogen receptor alpha (ERα) protein (PDB ID: 3ERT) was selected for its involvement in cell proliferation within the breast cancer cell. Tamoxifen is a reference drug commonly used in hormonal therapy, and DHCL was used as a ligand. Molecular docking was performed using AutoDock Vina in PyRx v.0.8 to get the best conformational pose for forming the expected receptor-ligand complex. The docking result visualization was performed using LigPlot v.1.4.5 software for 2D, and the interactive visualization in 3D was done using Biovia Discovery Studio software. The presence of DHCL in the root of *A. integrifolia* was not reported so far. DHCL content in the root of *A. integrifolia* was estimated to be 16.5 ± 0.25 mg/g of crude extract using the TLC-densitometric method. From the molecular docking study, DHCL was found to be a promising inhibitor for estrogen receptor interaction in the breast cell and can be selected for further *in vivo* research to develop an anti-breast cancer drug.

Keywords

Ajuga integrifolia; breast cancer; dehydrocostus lactone; molecular docking; thin layer chromatography

Introduction

More than 5000 indigenous medicinal plants are known in Ethiopia. *Ajuga integrifolia* (Syn: *A. remota, A. bracteosa*) is one of these medicinal plants (1). As part of the traditional medicine in many parts of Ethiopia locally known by different names such as Armagussa, Etse Libawit, and Medhanit (2). *A. integrifolia* is a member of the Lamiaceae family and pharmacologically active perennial herb (2). Many biological activities were reported for the *Ajuga* species, specifically for *A. integrifolia* and its synonyms. These include anti-oxidant and anti-inflammatory (3), anti-diabetic (4), anti-bacterial, diuretic, stimulant, astringent, rheumatism, febrifuge, blood purifier effects (5), and anti-cancer activity (6).

Dehydrocostus lactone (DHCL) is a natural sesquiterpene lactone reported as a principal and biologically active component of the roots of *Saussurea lappa,* Chinese traditional herbal medicine (7)*,* and *Laurus nobilis* (8). DHCL as a guianolide sesquiterpene lactone, also reported its presence in abundance in *S. costus*, a plant popularly known as 'costus' and renowned worldwide for its medicinal properties (9). DHCL, is among the two known lactone potential anti-breast cancer compounds identified using a bioactivity-oriented screening strategy (7). Reports showed that DHCL has an anti-cancer effect for some carcinomas, including anti-breast cancer (8, 10) and anti-tumor activities (11). Peng *et al* (10) and Kuo *et al* (12) suggest mechanisms of action as inducing cell cycle arrest and apoptosis.

Gas chromatography is one of the possible ways of quantifying volatile components of extracts and essential oils (13, 14). As an alternative, a rapid densitometric TLC method without derivatization was used to analyze DHCL in *S. costus* root (9). TLC-densitometric method has been used as an efficient tool for the phytochemical evaluation of herbal drugs.

Cancer is a group of more than 100 different diseases. It is among the most common public health problems. It can develop almost anywhere in the body. Breast cancer is one of the most common carcinomas that happens when cells in the breast grow and divide uncontrollably, creating a mass of tissue called a tumor. In Ethiopia, the incidence of breast cancer accounts for 22.6 % of all cancer cases and 17 % of cancer mortality annually (15). Breast cancer treatment includes chemotherapy and hormonal therapy targeting apoptotic proteins and hormones binding to cancer cells (15, 16). Estrogens are important for mammary gland development (17). Estrogen receptor interactions activate co-activator proteins like SRC-3, which triggers the replication of cancer cells (18). ERα inhibitors have been proven effective in inhibiting cell proliferation in hormonal-sensitive breast cancer (19).The presence of DHCL was not reported before in *A. integrifolia* and the genus *Ajuga*. Ethnobotanical and pharmacological studies reported the anti-cancer effect of the *Ajuga* genus's root and aerial parts (20–22). Apart from iridoid glycosides (23, 24), sesquiterpene lactones were not reported for anti-cancer activity from the *Ajuga* genus. Sesquiterpene lactones are known mainly in the root parts of medicinal plants and are responsible for anti-breast cancer activity (23, 25, 26). The literature search revealed that despite its many uses in different varieties of traditional medicine formulations and different parts of the world, no attempt had been made to quantify sesquiterpene lactones as an active constituent of *A. integrifolia.* Planar TLC detection of the lactone initiates the quantitative determination in the root sample of *A. integrifoila*.

In silico study is recommended (27) as a preliminary test before working on other pharmacological tests. In this study, we used an *in silico* analysis to estimate the minimal inhibitory concentration of one of the sesquiterpene lactones responsible for the anti-breast cancer action of the root of *A. integrifolia.* In this current study, we present a TLC-densitometric estimation of DHCL in an *A. integrifolia* root sample and assess its potential for use in the development of anti-breast cancer treatments using *in silico* studies.

Methods

Chemicals and Reagents

All chemicals and reagents were HPLC grade (Merck India, Mumbai, and S.D. Fine-Chem, Mumbai, India). Pre-coated silica gel F254 plates (HPTLC Silica gel 60 F254, glass plate, Merck KgaA, 64271 Darmstadt, Germany, HX229037, #1.05642.0001) of appropriate sizes were used for analysis. Water was distilled and purified by a MQ (18.2) at a 20.6 °C water purification system (Purelab flex 4 Elga) for washing plant materials. Dehydrocostus lactone (purity ≥ 98 %) was purchased from Shanghai Yuanye Bio-Technology Co. Ltd (Shanghai, China).

Plant Material Collection and Identification

The whole plant material of *Ajuga integrifolia* was collected from around the Addis Ababa Science and Technology University (AASTU) campus and Koye area (VRP5+3W6, 88852 °N, and 38.8098 °E, Elevation: 2,840 m) and identified by Ato Melaku Wondafrash at National herbarium, Department of Biology, Natural and Computational Science College, Addis Ababa University, Ethiopia. The sample specimen was deposited at the national herbarium. Identification made by a known botanist based on morphological information. Habitat: Shaded grassland, roadsides, ditches, humid or wetter places, 1500–3400 m. Collection season: Collected at the end of November to January following its flowering time. After collection, the plant material is sorted into aerial and root parts, washed with double distilled water, and dried in air/shade. The air-dried material was crushed with a coffee grinder, packed in a polyethylene bag, and kept in a cool area until the next activity.

Extraction of Terpenoids (Sesquiterpenes)

The fresh aerial part was extracted with acetone by dipping it for 15 s, following a method used to extract terpenoids and flavonoids (28). The dried aerial part was extracted with methanol using an ultrasonic sonicator for comparison. The air-dried and powdered root part (2.5 g) was extracted using maceration in n-Hexane (25 mL). Then sonication for 1 hr. at room temperature with hexane as in the proposed method for extracting sesquiterpenes for anti-breast cancer activity (7). The filtrate was collected, and residues were then extracted similarly twice. The three filtrates were combined and evaporated to dryness on a rotary evaporator. All the acetone, methanol, and hexane extract were dried under reduced pressure, labeled as AIA-Act, AIA-MeOH, and AIR-Hex, and kept in a cool place (refrigerator). On a larger scale, hexane extract was fractionated on a column and purified via recrystallization in an appropriate solvent system from 500 g of the root sample for Carbon 13-NMR (13CNMR) (Bruker ACQ 400 AVANCE spectrometer) in comparison with literature data. TLC comparison of the isolated compound with reference

standard was done. As DHCL is a well-known lactone, NMR data is sufficient to confirm.

Preparation of Standard Solutions of Dehydrocostus Lactone and Calibration Plot

Stock solutions of dehydrocostus lactone were prepared by dissolving 100 mg, accurately weighed in methanol and diluting to 100 mL with the same solvent to get 1000 ppm (1 g/L). Serial dilution to make up 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 ppm. The standard solutions of DHCL were applied in 20 µL volumes (Application position Y 8.0 mm, First track position X 20.0 mm, Application length 8.0 mm, application width 1.0 mm) in triplicate by use of a Camag Linomat IV automatic sample spotter on pre-coated silica gel glass HPTLC plates 60 F_{254} (20 cm × 10 cm; Merck, Germany). A constant application rate was applied, and the space between tracks was 11.4 mm.

The plates were developed (linear ascending) with a solvent system petroleum ether-toluene- methanol (4:5:1, v/v, 10.0 mL) as a mobile phase in a Camag glass twin through saturated chamber (20 cm × 10 cm), to a distance of 85 mm, under controlled conditions (temperature 21.1^{0}C, relative humidity 44.1 %). Before and after development, the plates were dried in hot air and densitometric scanning was performed with Camag TLC scanner III in the reflectance–absorbance mode at 220 nm and 600/620 nm (for the case of derivatization) and operated with Vision cat software (Version 2.3.16286.1). The specific wavelengths were determined after multiple wavelength scanning. The slit dimension was kept at 5.00 × 0.20 mm, macro, and the scanning speed was set at 20 mm/s. The purity of each peak was confirmed by recording the absorption spectra at the start, middle, and end of the band. The peak areas were recorded, and calibration plots for dehydrocostus lactone were constructed by plotting the peak area under the curve against the amount applied. The chromatographed compounds' concentration was determined by the intensity of diffusely reflected light. The evaluation was carried out by comparing peak areas with linear regression. The same procedure was followed after derivatization using vanillin ethanol solution as a band staining agent (1 g of vanillin, 100 mL of 95 % ethanol, and 5 % sulphuric acid) (29). The plate was heated to 100^0 C until the color spots became visible.

Preparation of Sample Solutions for Quantitative Estimation

6 g of dried instant dip acetone extract of the fresh aerial part of *A. integrifolia* dissolved in 1 mL of methanol and 90 mg of air-dried aerial part of the extract of the same plant dissolved in 1 mL methanol. Further, 4 mg of hexane extract from the root sample was dissolved in 1 mL of methanol.

Quantitative Estimation of Dehydrocostus Lactone

A densitometric TLC method has been established to quantify important bioactive components (9). All three extracts were used for the quantitative determination of the known sesquiterpene DHCL following a modified method indicated by Vijayakannan *et al.* (9). Reference DHCL was used for quantitative analysis. Each test solution (20 µL) was applied in triplicate to a TLC plate using a Camag Linomat IV automatic sample spotter (Muttenz, Switzerland) CAMAG HPTLC system. The plate was developed, derivatized, and scanned as described above. The peak areas and absorption spectra corresponding to dehydrocostus lactone were recorded. The amounts in the samples were estimated using the respective calibration plots for the target dehydrocostus lactone. Two solvent systems namely (i). toluene-ethyl acetate (9:1, v/v; 10.0 mL) and (ii). petroleum ether-toluene- methanol (4:5:1, v/v, 10.0 mL) were used as mobile phase. Comparing the two solvent systems, better separation was observed for the second solvent system.

Validation of TLC-densitometric method: the method was validated using parameters used by Patel *et al* (30) with slight modification. Linearity was studied by using the calibration curve developed by plotting absorbance units versus concentrations. The areas of peaks were treated by least square linear regression analysis. The limit of detection (LOD) and limit of quantification (LOQ) were determined using the following equation Eqn. 1 and 2:

$$LOD = \frac{3.3 \times \text{standard deviaation of the y-intercept}}{\text{Slope of the calibration curve}} \quad \text{Eqn. 1}$$

$$LOQ = \frac{10 \times \text{standard deviaation of the y-intercept}}{\text{Slope of the calibration curve}} \quad \text{Eqn. 2}$$

Sensitivity was also computed from the relation:

Sensitivity = 3 × SD/LOD.

The specificity of the method was ascertained by the peak purity of the component overlaying the spectra of the standards at the start, middle, and end positions. The statistical analysis was performed using Microsoft Excel 2007.

In silico Study on Anti-breast Cancer Activity

Selection and Preparation of Protein and Ligands

Estrogen receptor alpha (ERα) protein was selected for its involvement in cell proliferation in the breast cancer cell. Tamoxifen was used as a reference drug commonly used in hormonal therapy (27). Reptoside is a representative of iridoid glycosides included in the interaction study following the report by Goren *et al*. on their anti-cancer activity (31). The 3D structure of ERα was downloaded from Protein Data Bank (RSC) PDB ID: 3ERT (http://www.rscb.org). DHCL, Reptoside, and reference drug (Tamoxifen) structures were retrieved in 3D format from PubChem (http://pubchem.ncbi.nlm.nih.gov). Protein preparation is done with UCSF Chimera Version 1.15. The preparation includes removing water and hetero atoms and adding polar hydrogens.

Molecular Docking Analysis and Visualization

Molecular docking was performed using AutoDock Vina in PyRx v.0.8 (32) to get the best conformational pose for

forming the expected receptor-ligand complex. Using the prepared receptor protein and minimized ligands, interaction docking started by setting the grid box on the receptor's active site, which was previously identified from the RSC information. The docking result was saved in PDB format, and the values of the binding affinities with root mean square deviation (RMSD) values were saved in comma-separated values (CSV) (Microsoft Excel format). From the binding energy in Kcal/mol, Ki is computed using the relation Ki= exp(ΔG × 1000)/(Rcal × TK), where ΔG is the docking energy, Rcal is 1.98719, and TK is 298 (33). The docking result visualization was performed using LigPlot+ v.2.2 software for 2D, and the interactive visualization in 3D was done using Biovia Discovery Studio software.

Pharmacokinetics Analysis

From preADMET web-based application (https://preadmet.webservice.bmdrc.org/), absorption, distribution, metabolism, and excretion (ADME), toxicity, and drug -likeness data can be retrieved. Human intestine absorption (HIA) test result is found in the ADME data. HIA is used to determine the absorption of active compounds into the human digestive system (27). Drug-likeness test was performed on the SCFBIO server (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp) (34) using the mol file of the ligands as an input.

Prediction of Activity Spectra for Substances (PASS) Test

The PASS test was performed using the PASS online software (http://www.pharmaexpert.ru/ pass online). Sdf/mol files retrieved from PubChem (http://pubchem.ncbi.nlm.nih.gov) were used as input for activity prediction for the ligand compounds. The biological activity test was essential to confirm before lab tests were conducted (27). The results would be given as the probability activity (Pa) score and inactivity score (Pi), which predict success if tests were performed in the lab.

Results

Crude Extraction and Extraction of DHCL

The yield of hexane extract from the root of *A. integrifolia* was 8 mg (0.32 %) from 2.5 g of air-dried root sample. The methanol extract yielded 90 mg from 5 g of the air-dried aerial part. Similarly, the acetone extract yield was 240 mg from 280 g of the fresh aerial part. For NMR comparison from 500 g of root sample, 2 g of extract, and after further separation and purification, 20 mg of white crystal (compound 1) isolated from hexane extract and sent to NMR characterization, and the ^{13}CNMR result is compared with literature data (6).

^{13}CNMR is known to be significantly powerful for characterization (35), and DHCL is a well-known lactone, so we used ^{13}CNMR for comparison with literature data for confirmation. The spectra were compared with literature data (6) for dehydrocoustus lactone, as shown in Table 1. NMR results show close agreement with the reported data. Both ^{13}CNMR and DEPT 135 spectrum for compound 1 are provided (Fig. 1S and 2S, Supplementary data).

Table 1. Comparison of 13CNMR data of compound 1 with literature values of DHCL.

C atom No	δ 13C-NMR Compound 1	δ 13C-NMR DHCL (6)
1	47.53	47.61
2	30.29	30.31
3	32.61	32.61
4	151.34	151.24
5	45.08	45.12
6	85.32	85.29
7	51.99	52.02
8	30.94	30.94
9	36.34	36.28
10	149.24	149.23
11	139.70	139.74
12	170.32	170.33
13	120.27	120.33
14	112.59	112.62
15	109.52	109.61

HPTLC Quantitative Determination

The linear regression equations for HPTLC quantitative estimation of DHCL was y = 1.029 × 10^{-3}x + 1.533 × 10^{-3} (R^2=0.9875) as shown in Fig. 1 with a relative standard deviation of nearly 2 %, expressed as % CV, indicating that the method was reasonably precise and reproducible (9). The

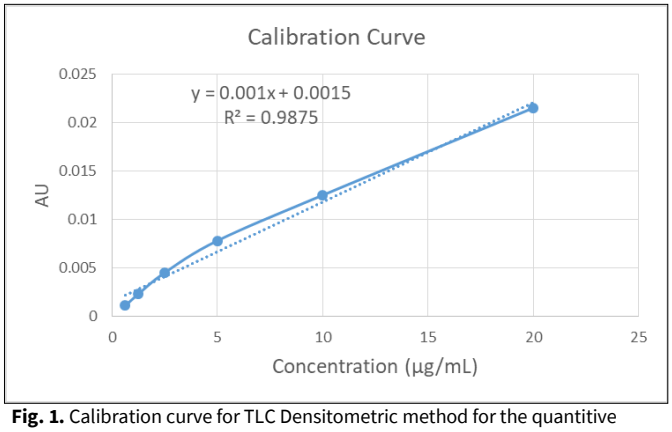

Fig. 1. Calibration curve for TLC Densitometric method for the quantitive estimaton of DHCL.

R^2 value shows reasonable linearity of the data obtained towards the quantitative estimation of DHCL. DHCL content of the three extracts investigated and estimated using the above-mentioned method is shown in Table 2. The estimation was done in duplicate. DHCL detected and quantitatively estimated its presence in the hexane extract of the root of *A. integrifolia*. It was not detected in the acetone and methanol extract of the aerial part. The acetone extract was included in the study as it is expected to contain terpenes. The methanol extract was included for com-

Table 2. Estimated DHCL contents of sample extracts of *A. integrifolia* in hexane, acetone, and methnaol.

S. No.	Sample Description	DHCL Content
1	AIR Hex	16.5 ± 0.25 mg/g of crude extract.
2	AIA Acetone	nil
3	AIA MeOH	nil

parison as it gives the complete profile of the aerial part.

The extraction method is optimized and rapid as used by Guccione *et al.* (36). The method used for quantitative estimation was validated for repeatability, LOD, LOQ, specificity and sensitivity. The repeatability of the method was found to be (% CV) = 2.67 %. LOD = 30.75 ng, LOQ = 93.2 ng and sensitivity = 0.935 ng. The range of linearity for DHCL was found to be 312.5 ng –10 µg spot^{-1}.

3D overlay of HPTLC densitogram of the calibration spots of the standard DHCL and samples and 3D display as illustrated in Fig. 2 below. The HPTLC chromatograms were taken at 620 nm, and all the spectra were compared.

The identity of the band of DHCL in the sample extracts was confirmed by overlaying its UV absorption spectra with that of the standard DHCL (Fig. 3) and comparing the TLC chromatograms (Fig. 4). The overlayed densitometric TLC chromatogram (Fig. 3) shows similar information to that of Table 2.

HPTLC procedure was checked with two solvents mentioned in the method section and petroleum ether: toluene: methanol (4:5:1) gave a good resolution and well-defined peak at R_f value of 0.69 (Fig. 3 and 4). Other chromatographic conditions like chamber saturation time, sample application volume and positions, the distance between tracks, and detection wavelengths were optimized to get reproducible R_f values and better resolution for the target molecule. As shown in Fig. 4, the TLC chromatogram has identical spots for the root extract with the reference standard at R_f = 0.69.

In silico Study on Anti-breast Cancer Activity

Selection and Preparation of Protein and Ligands

The crystal structure of 3ERT with 4-hydroxytamoxifen has an acceptable resolution (1.9 Å), the overall quality factor is 98.3193 (Fig. 4S, Supplementary data), and the Q-mean z score value is -1.03 (Fig. 3S, Supplementary data). As indicated on the protein's Ramachandran plot, residue in most favoured regions is 91.2 %, residues in additional allowed regions are 8.8 %, and no residue in generously allowed and disallowed regions. The coordinates of the co-crystalized 4-hydroxytamoxifen identified the binding pocket of 3ERT in a radius of 12.8 Å. 70 residues are recognized as the binding pocket residues (37), including Leu346, Ala350, Asp351, Glu353, Arg394, and Thr347.

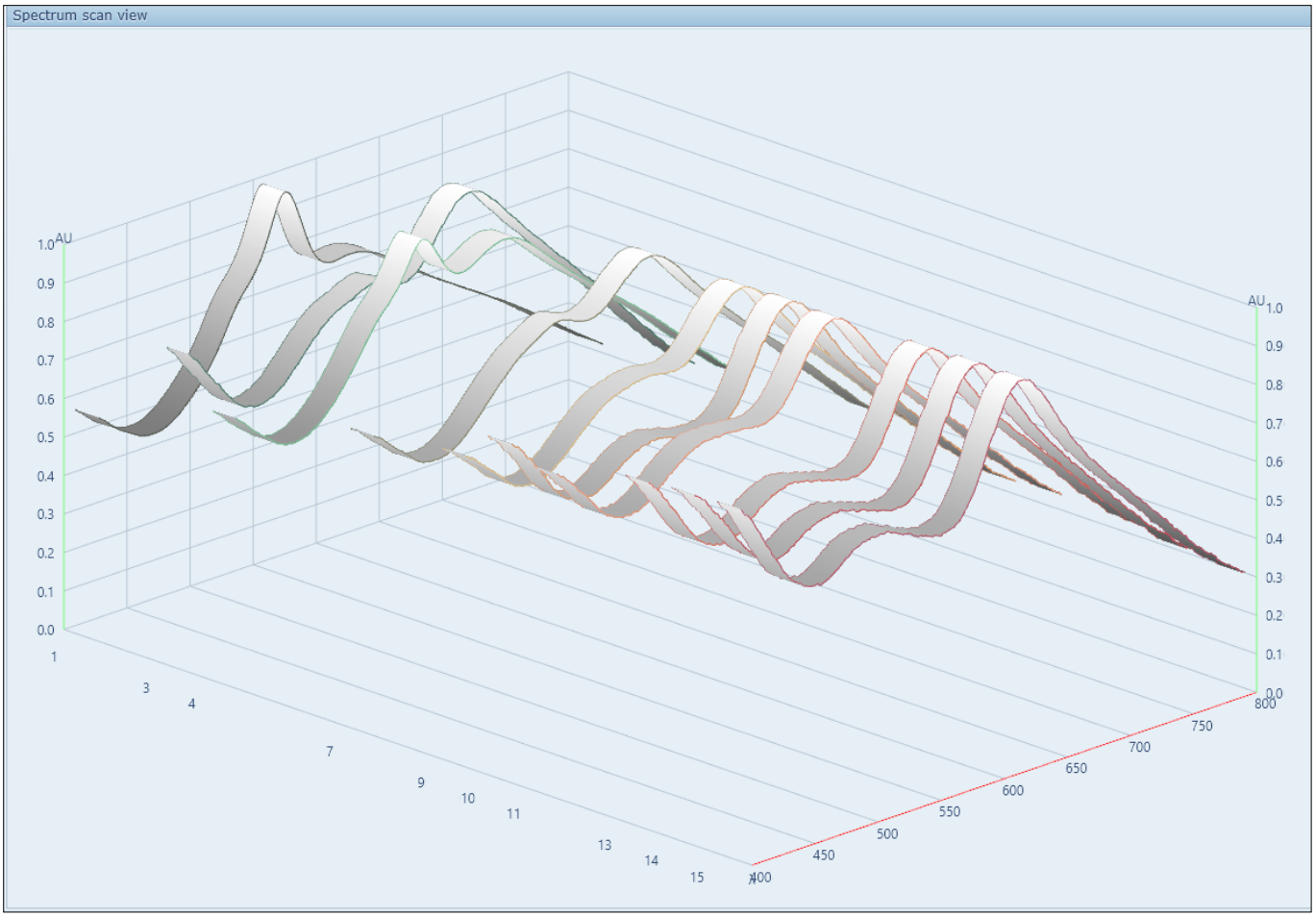

Fig. 2. Three-dimensional overlay of HPTLC densitograms of the calibration spots of the standard DHCL and sample extrcats of *A. integrifolia*. (AIR- Hex), 10(AIA – Acet), and 14(AIA-MeOH) are sample and others are standard DHCL

ADME Prediction

The Lipinski rule of 5 helps distinguish between drug-like and non-drug-like molecules. It predicts a high probability of success or failure due to drug-likeness for molecules complying with 2 or more of the following rules (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp# anchor tag): molecular mass less than 500 Da, high lipophilicity (expressed as Log P less than 5), less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors, molar refractivity should be between 40–130. All values for DHCL contribute to its drug-likeness property. The molecu-

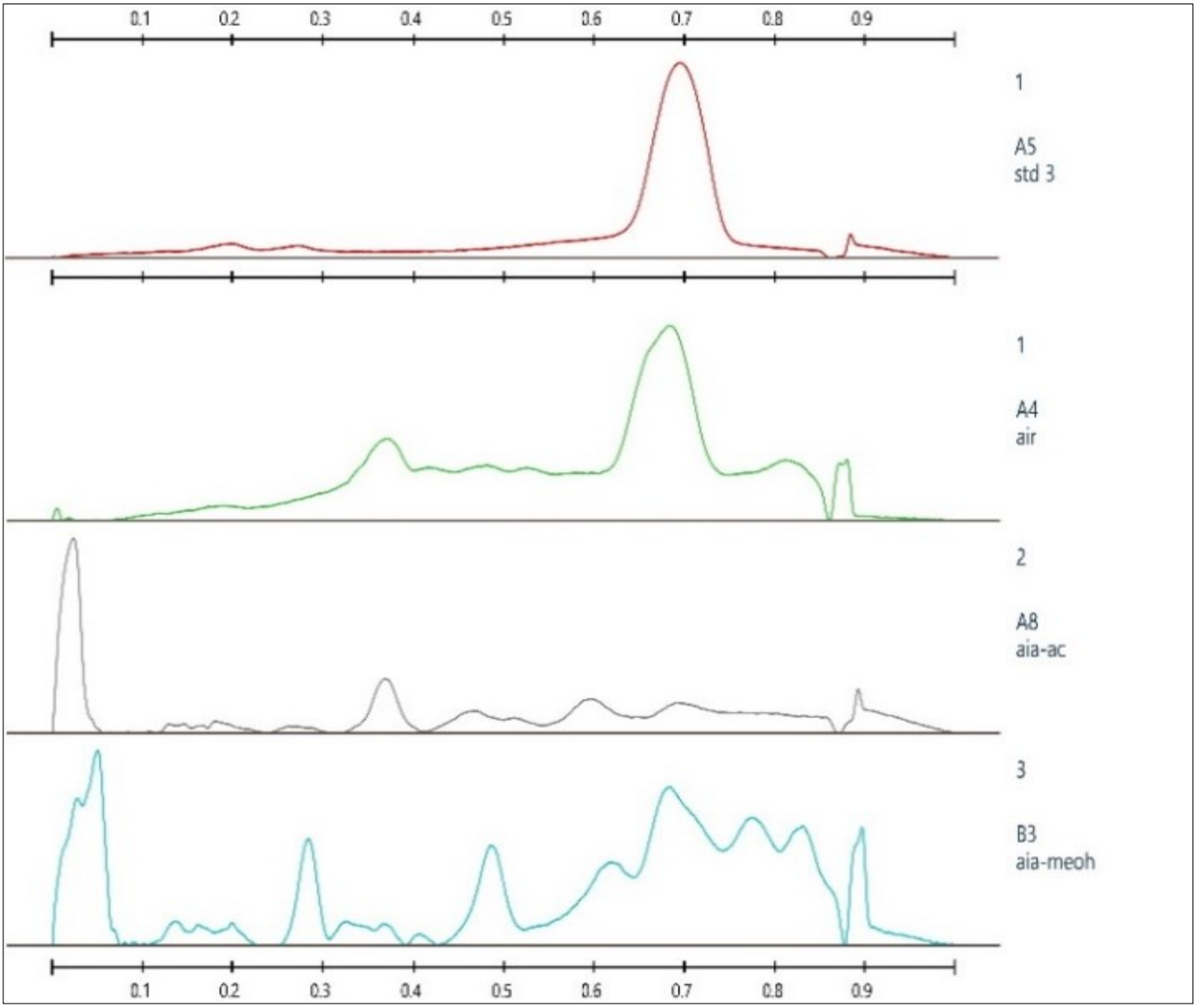

Fig. 3. Overlaid Densitometric TLC chromatograms for methnaol (AIA- MeoH), acetone (AIA acet) and hexane (AIR-Hex), sample extract from *A. integrifolia* and standard reference (bottom to top) using petroleum ether – toluene- methanol (4:5:1, v/v, 10.0 mL) solvent system.

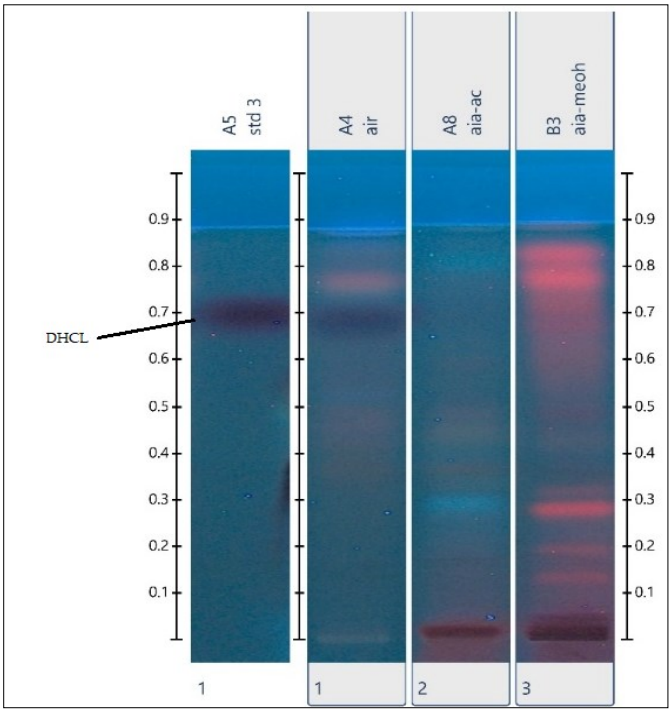

Fig. 4. TLC chromatogram for hexane, acetone and methnaol extract samples from *A. integrifolia* and standard reference using petroleum ether – toluene-methanol (4:5:1, v/v, 10.0 mL)

Table 3. Lipinski rule evaluation for the ligands (DHCL, reptoside and control drug)

Rules	DHCL	Reptoside	Tamoxifen
Molecular Mass	230.00	390.00	371.00
hydrogen bond donor	0	4	0
hydrogen bond acceptors	2	10	1
Log P	3.069749	2.506439	5.082190
Molar Refractivity	69.703995	90.631187	115.476479
Evaluation of Lipinsik Rule	Suitable with no violation	Suitable with one violation	Suitable with one violation

lar mass of 230.00, no hydrogen bond donor and two hydrogen bond acceptors, log P value of 3.0697, and molar refractivity make it follow the Lipinski rule with no violation. The reference drug found one violation which is a log P value of 5.082190, which is a bit greater than five 100 % HIA shown for both DHCL and Tamoxifen (Control) (Table 4), indicating that there is no solubility issue associated if used as a drug for the purpose indicated. This indicated easy oral administration as a drug, as it is expected to be soluble in water. Pa value for DHCL was found to be 0.768, which is above 0.700, in the range of most likely to be active for anti-

neoplastic activity. This value is comparable to the reference drug. The Pa value for reptoside was found to be less than 0.700. Insignificant Pi = 0.005 for DHCL, comparable to the reference drug Tamoxifen (Pi = 0.004), also supports the activity for the purpose indicated.

Molecular Docking Result

Table 4. Human Intestinal Absorption (HIA) test result of ligands (DHCL, reptoside and control drug)from preADME web-based application

PubChem ID	Name of ligand	HIA(%)
73174	DHCL	100.00%
44584096	Reptoside	21.49%
2733526	Tamoxifen (Control)	100.00%

DHCL found multiple binding with Thr347, Ala350, and Asp351 among the residues identified in the binding site determined before the selection and preparation steps. Multiple interactions indicate a stronger interaction and contribute to the overall binding energy. The binding energy (BE = -8.1 kcal/mol) comparable to the reference drug. Inhibition constant (Ki = 1.36 mM) was found to be lesser, indicating a lower inhibition concentration for the intended activity. RMSD in the range of 2 Å also indicates the best fitting of the computing model used for the interaction study. ADME prediction values of no hydrogen donor and two hydrogen bonding acceptors for DHCL suggest less likelihood for hydrogen bonding to be involved in the interaction with the receptor molecule. The absence of hydrogen bonding interaction may be due to the absence of a hydrogen bond donor. Its role may be compensated partly by the larger number of multiple interactions with some of the residues in the binding site of the receptor molecule, as shown in Table 6 and Fig. 5.

Table 5. PASS Test result of DHCL and reference drug of ligands (DHCL, reptoside and control drug)

PubChem ID	Name of ligand	Pa	Pi	Activity
73174	DHCL	0.768	0.005	Antineoplastic (breast cancer)
44584096	Tamoxifen	0.817	0.004	Antineoplastic (breast cancer)
2733526	Reptoside	0.685	0.029	Antineoplastic

Pa: Probability of activeness, **Pi**: Probability of inactiveness.

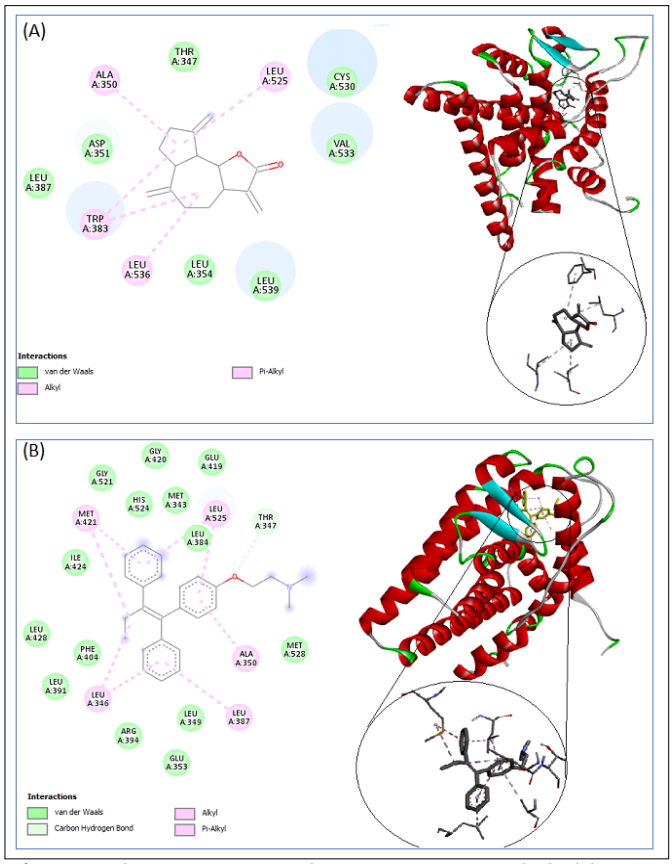

Fig. 5. 2D and 3D interaction image from Biovia Discovery studio for (**A**) DHCL,

Discussion

Anti-cancer activities were determined through pharmacological investigations and ethnobotanical studies (21, 22, 38) on *Ajuga integrifolia* and its synonyms. We used the rapid HPTLC method to quantify one of the active components specifically for anti-breast cancer treatment (9). Both the extraction and estimation procedures are simple and rapid, involving few steps in extraction, and the estimation is possible even without derivatization. Estimation follows a reported procedure by Vijayakanna *et al.* (9). The quantitative determination was done in duplicates and would be better if done in triplicate. The other limitation of the estimation is that we could not validate the method as we have a significantly smaller number of reference standards. TLC comparisons were also managed with the isolated DHCL. The identity of DHCL in the sample extracts was confirmed by overlaying their UV absorption spectra with those of the respective reference standards acquired using Camag TLC scanner III. The presence of DHCL was not reported so far from *A. integrifolia.* The chromatogram from

Table 6. Molecular docking result for ligands (DHCL, reptoside, and control drug) with ERα(PDB ID: 3ERT)

Name of Ligand	Binding Energy (Kcal/mol)	Inhibition Constant (Ki) mM	RMSD (Å)	Interacting Amino Acid Residues
DHCL	-8.1	1.16	2.115	**Thr347, **Ala350, **Asp351, **Leu354, ***Trp383, Leu387, Leu525, **Cys530, **Val533, Leu536, **Leu539
Reptoside	-7.2	5.27	8.267	Met343, Leu346, Thr347, Leu349, Ala350, **Asp351, Glu353, **Trp383, Leu384, Leu387, Met388, Leu391, Arg394, Phe404, Met421, Ile424, Leu428, **Leu525, Met528
Tamoxifen (Control)	-9.7	0.0077	1.426	Met343, Leu346, **Thr347, Leu349, Ala350, Glu353, Leu384, Leu387, Leu391, Arg394, Phe404, Glu419, Gly420, Met421, Ile424, Leu428, Gly521, His524, **Leu525, Met528

* Indicates the number of multiple interactions with similar residues

HPTLC indicates the presence of other sesquiterpenes also. For further quantification of DHCL and other sesquiterpenes, HPLC and other hyphenated methods should be attempted.

Poor absorption or permeation is expected when there are more than five hydrogen bond donors, ten hydrogen bond acceptors, molecular weight > 500, and log p > 5 (39). According to Lipinski's rule of five, Tamoxifen as control and reptoside for comparison, all have a molecular weight of less than 500 g/mol. The number of hydrogen bond donors and hydrogen bond acceptors is less than 5 and 10, respectively, except for 10 for hydrogen bond acceptors for reptoside. The ligands also have a log P value of less than 5, except for the reference drug log P = 5.082190. Following these data, all qualify drug-likeness properties, specifically DHCL with no violation, and reptoside and Tamoxifen, each with one violation (Table 3).

For larger molecular mass drugs, it will be difficult to penetrate the cell membrane as it interferes with the diffusion process (39). Similarly, higher hydrogen bond donor and accepter values indicate greater hydrogen bond formation possibilities and hinder the possibility of the drug reaching the target receptor. The optimum values are needed only for better interaction with the receptor (40). Larger values of log p complicate the passage of drugs through the cell membrane as it is associated with a drug molecule's hydrophobicity (41). A greater log p-value for a drug molecule means it is more hydrophobic. A drug ligand should not be too hydrophobic as it might be retained on the lipid bilayer. This makes the drug molecule widely distributed all over the body and reduces bond selectivity to the target receptor (27).

From the HIA test result (Table 4), DHCL and Tamoxifen can be absorbed well in the human intestinal tract. 70–100 % HIA implies efficient absorption (27). Absorption efficiency plays a role in the ease of reaching breast cancer cells for the intended ERα inhibitory activity (27). The lower log P value shown in Table 3 is related to the higher HIA value indicated in Table 4 for both DHCL and Tamoxifen. Based on the PASS test result (Table 5), Tamoxifen drug DHCL and Tamoxifen have a Pa value greater than 0.700. This indicates that DHCL has a predictable anti-breast cancer (anti-neoplastic) activity either computationally or on a lab scale. In the case of reptoside, its action is not that predictable as the Pa value is slightly less than 0.700. DHCL alone or in combination with other compounds suppresses the proliferation of lung cancer cells. It also modulates growth regulatory and angiogenesis signaling pathways at about 5 mM concentration (25). Ki for DHCL was found to be less than 5 mM, and even the estimated amount per gram of extract is larger for such an activity.

The docking result (Table 6) shows that DHCL has a comparable binding affinity to the reference (Tamoxifen) drug. The more negative the binding energy indicates, the more potent the inhibition. The binding energy is a cumulative effect of the different forms of energy existing in the complex formed among the receptor protein and the ligand (42). All interactions are not similarly weighed and contribute to the total binding energy. The types and number of interactions determine the magnitude of the binding affinities (27). As in the case of the reference drug considered, DHCL also shows multiple interactions for many residues on the receptor protein in the range of up to 3.9 Å (43), as shown in Fig. 6. The absence of hydrogen bonding is more or less balanced with the larger number of multiple interactions for DHCL. The binding energy data obtained from the molecular docking study (Binding energy = -8.1 kcal/mol) suggests DHCL is a promising inhibitor for estrogen receptor interaction in the breast cell and can be used for breast cancer treatment. As shown in Table 6, the inhibition constant (Ki) for DHCL is smaller than the refer-

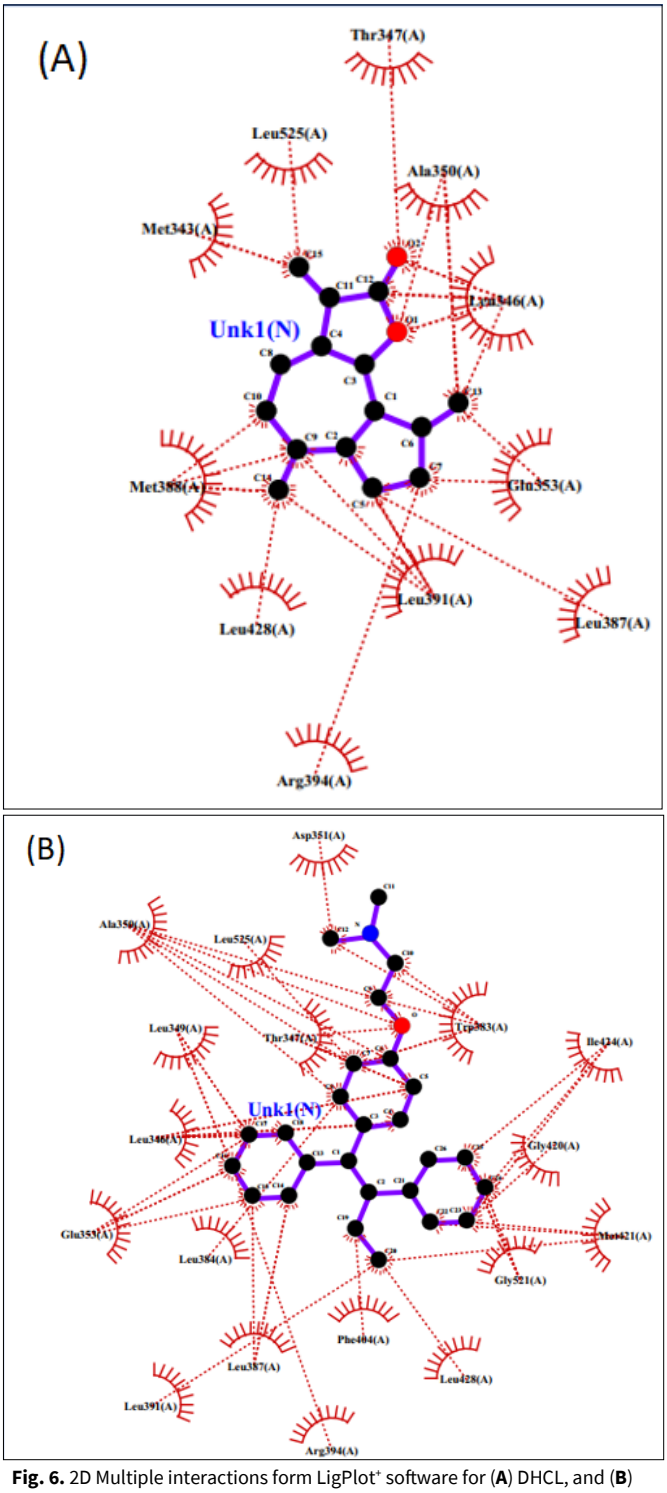

Fig. 6. 2D Multiple interactions form LigPlot⁺ software for (**A**) DHCL, and (**B**)

ence drug. This indicates that the intended inhibition can be accomplished even at a lower concentration. Tamoxifen is also effective in treating and preventing breast cancer at a low dose and topical administration (44). It binds to ER and partially inhibits its activity (18). The binding affinity and Ki values for reptoside are not closer to the reference as DHCL. The inhibition activity and concentration will not be as attractive as DHCL. The *in silico* study result helps to come up with a possible target protein for the anti-breast cancer effect of DHCL.

Residues involved in the interaction of DHCL and Tamoxifen are almost similar, apart from some differences. Most residues that interact with DHCL experience multiple interactions (Table 6 and Fig. 6). This may contribute to the comparable binding affinity of the two compounds to ERα receptor proteins and results in comparable inhibitory activity for the proliferation of breast cancer cells. This is another agreement with the mechanism suggested by Peng *et al.* (10) and Kuo *et al.* (12). The molecular docking results were not supported with molecular dynamic simulation, because of the higher computational cost demanded.

Conclusion

Apart from using *Ajuga integrifolia* for the anti-cancer activity, the presence of DHCL was not reported in the root sample of this plant. It is the first report which shows the presence of DHCL in the root of *A. integrifolia*. DHCL content in the root of *A. integrifolia* was estimated to be 16.5 ± 0.25 mg/g of crude extract using the TLC-densitometric method. From the molecular docking study, DHCL was found to be a promising inhibitor for estrogen receptor interaction in the breast cell and can be used for breast cancer treatment. This can support the use of *A. integrifolia* for anti-cancer purposes as described in ethnobotanical and pharmacological studies. Further study demands checking the presence of other sesquiterpenes for similar activity and molecular dynamic simulation study for the stability of the interactions so far studied. Further, *in vitro* studies are recommended following the PASS prediction result and the identified receptor protein from the molecular docking study.

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Authors contributions

FBT conceptualized the study and drafted the manuscript; YHG and TBA participated in methodology; MGT and RKB supervised the whole steps of the study and manuscript writing and editing; MGT participated in the validation of the methods; YHG, TBA, MGT, AB, ANS, and RKB participated in writing- review & editing manuscript. All the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

Supplementary data

Fig. 1S. ^{13}C NMR for Compound 1

Fig. 2S. DEPT – 135 for Compound 1

Fig. 3S. Quality comparison for 3ERT.pdb and Qmean value

Fig. 4S. Ramachandran Plot for 3ERT. PDB with summary statistics

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