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Research Article
Identification of Potential Anticancer Bioactive Compounds from Fractions of *Alpinia monopleura* Rhizome Extract

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Abstract

Background and Objective: Cancer is a malignant disease in body tissue where abnormal cells grow excessively and uncoordinated. Chemotherapy treatment still has weaknesses because apart from killing cancer cells, it also affects normal cells with fast proliferation rates, such as hair follicles, bone marrow and digestive tract cells, producing typical chemotherapy side effects. *Alpinia monopleura* has secondary metabolite content such as phenolic and flavonoid compounds as anticancer activity. This study aimed to investigate the cytotoxic activity of *A. monopleura* extract and its fractions and determine the phytoconstituents in the most active fraction against three distinct cancer-related protein targets. **Materials and Methods:** The *A. monopleura* extract and fractions were tested for cytotoxic against HeLa, MCF-7 and WiDr cell lines by using MTT assay. Then, the most active fraction was identified as its components by LC-HRMS and followed by molecular docking. **Results:** The most active cytotoxic effect was fraction 2 in HeLa cells, while fraction 4 in MCF-7 and WiDr. Several compounds have been successfully identified as contributing to their cytotoxic activity, proven by molecular docking investigation. It was found that compounds from fraction 2- Dehydroepiandrosterone, 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one and 2-(3,4-dimethoxy phenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one-exhibited higher binding energies than Erlotinib, the native ligand with the cervical cancer target protein. **Conclusion:** Meanwhile, fraction 4 compounds had lower binding energy than the native ligands for each colon cancer and breast cancer protein target. Therefore, compounds from *A. monopleura* are promising for developing novel anticancer agents.

Key words: *Alpinia monopleura*, anticancer, cytotoxic, LC-HRMS, molecular docking

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is a malignant disease in body tissue where abnormal cells grow excessively and uncoordinated[1]. Cancer was the major cause of death before 70 years in 2019 according to the World Health Organization (WHO). Cancer causes 9.6 million deaths every year; an estimated 70% of cancer deaths occur in developing countries, including Indonesia. According to Globocan 2020, new cases of cancer in Indonesia were 396,314 cases with 234,511 deaths. The highest cancer in women is breast cancer with 65,858 cases, followed by cervical cancer with 36,633 cases. The highest cancer in men is lung cancer with 34,783 cases, followed by colorectal cancer with 34,189 cases[2].

Cancer primarily arises from damage or mutations in proto-oncogenes, which are responsible for coding proteins that promote cell proliferation and differentiation. It can also result from alterations in tumor suppressor genes, which code for proteins that inhibit cell growth and stimulate apoptosis[1]. Each cancer cell exhibits unique characteristics during its growth and development, leading to the formation of a tumor. This characteristic of cancer cells is called a hallmark and until now, various mechanisms for inhibiting the hallmark of cancer are still being developed[3].

The six hallmarks of cancer (6 characteristics of cancer cells) are growth signal autonomy, cancer cells can produce their growth factors and growth factor receptors and in their proliferation, cancer cells do not depend on normal growth signals; evasion growth inhibitory signals, meaning that cancer cells do not recognize and do not respond to growth inhibitory signals. This situation is often caused by mutations in several genes (protooncogenes) in cancer cells. Evasion of Apoptosis Signals: Cancer cells are not sensitive to apoptotic signals due to mutations in apoptosis regulator genes and signal genes. Unlimited replicative potential cancer cells have specific mechanisms to keep their telomeres long, allowing them to continue dividing and cancer cells have unlimited replicative potential. Angiogenesis is the formation of blood vessels, where cancer cells can induce angiogenesis, namely the growth of new blood vessels around cancer tissue. The formation of new blood vessels is necessary for cancer cell survival and expansion to other body parts (metastasis). Invasion and metastasis occur when cancer cells move from primary to secondary or tertiary locations. The mutation process increases enzyme activity in cancer cell invasion (MMPs). Also, mutations allow reduced or lost adhesion between cells by cell adduct molecules, increasing attachment, degradation and migration[3].

The causes of cancer itself are genetic factors, carcinogenic factors (chemicals, radiation, viruses, hormones and chronic irritation) and behavioral or lifestyle factors (smoking, unhealthy eating patterns, alcohol and lack of physical activity)[4]. Surgery and radiotherapy are the most effective treatments for localized and non-metastatic cancer. However, these methods are less effective when cancer has spread throughout the body. For treating metastatic cancer, the current preferred options are cancer drugs, including chemotherapy, hormones and biological therapy. These treatments are effective because they can travel through the bloodstream and reach every organ in the body. Chemotherapy is a common treatment for cancer, but it has some significant drawbacks. While, it effectively kills cancer cells, it also impacts normal cells that rapidly divide, such as those in hair follicles, bone marrow and the digestive tract. This results in the typical side effects associated with chemotherapy. Therefore, there is a pressing need to develop new treatments that can selectively target and kill cancer cells without harming healthy cells[5].

One plant frequently utilized in cancer therapy is *Alpinia monopleura*. The genus Alpinia comprises approximately 250 species found across tropical and subtropical regions, making it the largest genus within the Zingiberaceae family. Alpinia is primarily recognized for its ethnomedicinal applications in various countries, including Indonesia, India, Vietnam, China and Japan. Numerous pharmacological studies have been conducted on Alpinia, revealing a wide range of bioactivities, such as anticancer, antioxidant, antimicrobial, antiviral, anti-inflammatory, antinociceptive, antiparasitic, neuroprotective, antihypertensive and analgesic activities[6,7].

Alpinia plants, especially *Alpinia monopleura*, can be found easily in the Sulawesi Area. This plant is endemic to Alpinia in Sulawesi. It is widely distributed and abundant and widely used by Southeast Sulawesi people. It is also known as Wundu Watu. Empirically, the people of South Konawe use Wundu Watu rhizomes to reduce body aches and as a cooking spice. Previous research stated that the secondary metabolite content in the Wundu Watu plant is alkaloids, saponins, flavonoids and steroids[6,7]. Phenolic and flavonoid compounds have been proven to have anticancer activity[8]. Docking experiments are necessary to predict which compounds in Alpinia extract have the potential to be anticancer agents[9].

Thus, this study aimed to investigate the cytotoxic activity of *Alpinia monopleura* extract and its fractions against MCF-7, WiDr and HeLa cell lines and the chemicals that might contribute to their anticancer activity.

MATERIALS AND METHODS

Study area: The research was conducted from April to October, 2024 at the Faculty of Pharmacy, Halu Oleo University, Indonesia.

Samples and preparation: *Alpinia monopleura* rhizomes were obtained from Ranomeeto District, South Konawe Regency, Southeast Sulawesi Province (4°2'48.7"S 122°44'23.8"E). The rhizomes are wet sorted, washed, dried at 50°C, dry sorted and ground to a powder.

Extraction and fractionation: Dry powder of *A. monopleura* rhizomes (500 g) was macerated with methanol (Merck) for 3 days. The filtrate was concentrated (Buchi 2412V0 RII Vertical Rotary Evaporator System, Europe) at 50°C with a speed of 60 rpm. The extract was fractionated using vacuum liquid chromatography (VLC) with a diameter of 10 cm using the stationary phase Silica Gel GF254 (Brand) and the mobile phase hexane:ethylacetate (9:1, 8:2, 5:5 and 2:8) and 100% methanol. The analysis of the separation results was performed using thin layer chromatography (TLC) Silica Gel GF254 (Brand) in the mobile phase hexane:ethylacetate (7:3).

Cell culture: HeLa, MCF-7 and WiDr cell line (ATCC, USA) were cultured in a complete medium composed of DMEM supplemented with 1% of p/s (penicillin/streptomycin), 1% of amphotericin B and 10% of FBS (fetal bovine serum) in an incubator at 37°C (5% CO_2) until confluence.

Cytotoxicity assay: The MCF-7, WiDr and HeLa cells were seeded in 96 well-plates until reaching 70% of confluence, respectively. Then, the old medium was discarded, washed twice with phosphate buffer saline and treated with extract and fractions from *A. monopleura* for 24 hrs. Then, the medium containing samples was replaced with MTT solution and incubated for 4 hrs. Doxorubicin was used as a positive control. Thereafter, the crystal formazan formed was extracted by DMSO solution and a microplate reader measured the optical density (OD). The cell viability (CV) of cells was calculated by using the equation as follows[10,11]:

$$\text{CV (\%)} = 1 - \frac{\text{OD control}}{\text{OD sample}} \times 100$$

where, OD control and OD sample were an OD of the wells containing the cells without treatment and the wells containing extract and fractions of *A. monopleura*, respectively.

LC-HRMS analysis of metabolites in active fractions: The LC-HRMS was performed using liquid chromatography (Thermo Scientific™ Vanquish™ UHPLC Binary Pump) and Orbitrap high-resolution mass spectrometry (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ High-Resolution Mass Spectrometer). The column used was Thermo Scientific™ Accucore™ Phenyl-Hexyl 100 mm × 2.1 mm ID × 2.6 µm with MS-grade water containing 0.1% formic acid (A) and MS-grade methanol containing 0.1% formic acid (B) as mobile phase for liquid chromatography with flow rate 0.3 mL/min in gradient manner. Firstly, it was set with eluent A at 95% and eluent B at 5% for 16 min, continued by eluent A at 10% and B at 90% for 4 min and finally, eluent A at 95% and B at 5% for 5 min. The volume of injection was 3 µL with the temperature set at 40°C. Nitrogen was used for sheath, auxiliary and sweep set at 32, 8 and 4 AU, respectively, with spray voltage at 3.30 kV. The capillary temperature was set at 320°C and the auxiliary gas heater was set at 30°C, with a scan range of 66.7 to 1000 m/z with a resolution used 70,000 for full MS and 17,600 for dd-MS2, in positive and negative ionization modes[12,13].

Molecular docking simulation: The molecular docking analysis was performed using AutoDock Vina 1.2.4 to evaluate binding interactions with three distinct cancer-related protein targets. For cervical cancer, the epidermal growth factor receptor (EGFR, PDB ID: 1M17) was selected as the target protein, with a grid box configured to dimensions of 42 × 40 × 40 Å^3, centered at coordinates (x = 21.697, y = 0.303 and z = 52.093). For colorectal cancer, human Leukotriene A4 hydrolase (PDB ID: 3U9W) was utilized with a grid box of 36 × 22 × 20 Å^3, centered at coordinates (x = 29.909, y = 1.546, z = 1.893). The breast cancer studies employed the human estrogen receptor alpha (PDB ID: 3ERT) with a grid box of 40 × 40 × 40 Å^3, centered at coordinates (x = 30.282, y = -1.913, z = 24.206). All grid box parameters were optimized based on the respective co-crystallized ligand positions. The docking protocol underwent rigorous validation through analysis of Root Mean Square Deviation (RMSD). This validation process involved superimposing the binding orientations of co-crystallized ligands before and after docking simulations. The protocol was deemed valid when the RMSD value was less than or equal to 2 Å, ensuring the reliability and reproducibility of the docking parameters for subsequent analysis of test compounds[14].

RESULTS AND DISCUSSION

Extraction and fractionation of *Alpinia monopleura*: The *Alpinia monopleura* extract obtained was 15 g (3% of yield),

followed by fraction 1 (1.16 g), fraction 2 (1.12 g), fraction 3(3.32 g) and fraction 4 (8.26 g). The extract and fractions continued to be tested for their cytotoxic activity in various cell cancers.

Cytotoxic activity: The treatment of cancer, such as chemotherapy, has various side effects, including nausea and vomiting, neutropenia, rash and redness, nephrotoxicity and cardiotoxicity. Therefore, utilizing natural plants as a source of novel agent of anticancer agents is essential[5].

Alpinia monopleura extract and fractions were tested against HeLa, MCF-7 and WiDr cell lines. It was found that the *A. monopleura* extract and fractions had anticancer potency with the ability to inhibit the proliferation of cell cancer (Fig. 1a-c). According to the United States National Cancer Institute, the cytotoxic activity is classified if IC_{50} values less than 20 µg/mL means highly cytotoxic, 21 to 200 µg/mL means moderate cytotoxic, 201 to 500 µg/mL means weakly cytotoxic and more than 500 µg/mL means no cytotoxic[15].

The IC_{50} of extract and fraction 1 to 4 against HeLa cells were 348.02±35.05, 68.23±0.74, 58.98±1.09 and 96.34±2.80 µg/mL, respectively. The extract was classified as weakly cytotoxic and the fractions were categorized as moderately cytotoxic in HeLa cells, compared to cisplatin as positive control, which was highly cytotoxic with IC_{50} of 2.09±0.17 µg/mL[15]. The most active was found in Fraction 2 against HeLa cells.

Moreover, the IC_{50} of extract and fraction 1 to 4 against MCF-7 cells were 368.77±6.55, 681.16±59.55, 276.00±6.32, 276.17±12.00 and 192.25±1.61 µg/mL, respectively. The IC_{50} of the control positive, Doxorubicin, was 1.91±0.15 µg/mL. Fraction were considered weakly cytotoxic, except for fraction 4 as most pot, which was moderately toxic against MCF-7. Meanwhile, fraction 1 did not possess cytotoxic activity in MCF-7[15].

In addition, the IC_{50} of extract and fraction 1 to 4 against WiDr cells were 374.05±8.47, 797.85±13.03, 254.22±4.93, 236.25±10.53 and 209.55±1.76 µg/mL, respectively. At the same time, the IC_{50} of Doxorubicin was 2.05±0.11 µg/mL. All extracts and fractions were weakly cytotoxic except for fraction 1, considered noncytotoxic[15].

LC-HRMS analysis of bioactive compounds in the most active fractions in *A. monopleura*: By LC-HRMS, several compounds have been identified from fraction 2 and fraction 4, which matched a fragmentation pattern in the mzCloud database or standards, as shown in Table 1 and 2. The listed compounds were screened by using LC-HRMS and were shown at the indicated retention time, measure mass compared to calculated mass indicated the accuracy of compounds showed by delta mass and matching the MzCloud score[12,13].

In fraction 2, compounds such as 2-(3,4-dimethoxy phenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one, 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, glycitein, apocynin, syringic acid, vanillin, Dehydroepiandrosterone (DHEA), carvone and (-)-caryophyllene oxide might involved for its anticancer activity (Fig. 2). The compound 2-(3,4-dimethoxy phenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one, known as eupatilin, is one of the compounds found in Fraction 2 with anticancer potency. Eupatilin acts as an anticancer by inducing apoptosis by regulating apoptotic proteins, including BAX and BCL2 and inducing mitochondrial depolarization[16]. The compound 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one, known as pectolinarigenin, has anticancer activity by down-regulating the PI3K/AKT/mTOR pathway leading to G2/M phase cell cycle arrest, autophagic and apoptotic cell death[17]. Glycitein induces apoptosis and cell cycle arrest by ROS-related MAPK/STAT3/NF-κB signaling pathways[18]. Apocynin promotes anticancer by decreasing intracellular production of reactive oxide species (ROS), thus inhibiting the NF-κB transcriptional activity. In addition, the apocynin also inhibits the Akt phosphorylation through IKK activation, thus lowering c Myc, cyclin D1 and iNOS levels[19]. Syringic acid acts as an anticancer by inhibiting cancer cell proliferation, suppressing inflammation, inducing apoptosis and altering autophagy through mTOR via AKT signaling pathway upregulation[20,21]. Vanillin provides anticancer by inhibiting cell cancer migration related to metastasis by inhibiting the activity of phosphoinositide 3-kinase (PI3K). Moreover, vanillin is also an antimutagenic through a DNA repair pathway[22,23]. Dehydroepiandrosterone (DHEA) is an endogenous steroid precursor hormone with anticancer properties by downregulating the WNT signaling pathway, which is involved in cancer cell proliferation, survival and progression[24]. Carvone induces intrinsic apoptosis through decreased Bcl2 and Bax and the release of cytochrome C, which induces Caspase expression and PARP cleavage. Moreover, the cell cycle arrest at G 2 /M via its action on cyclin-dependent kinase 1 (CCK-1)[25]. The (-)-Caryophyllene Oxide promotes apoptosis, the proliferation of cancer cells, reduces the tumor angiogenesis and metastasis marker levels through activation of mitogen activated protein kinase (MAPK) pathway and inhibition of PI3K/AKT/mTOR/S6K1 and STAT3 signaling[26].

In fraction 4, compounds including 4-Coumaric acid, (+)-(6)-Gingerol, 5-pentyl resorcinol, Eugenol, 6-Methoxymellein, betaine, oleamide, and kynurenic acid provide anticancer activity (Fig. 3). The 4-Coumaric acid

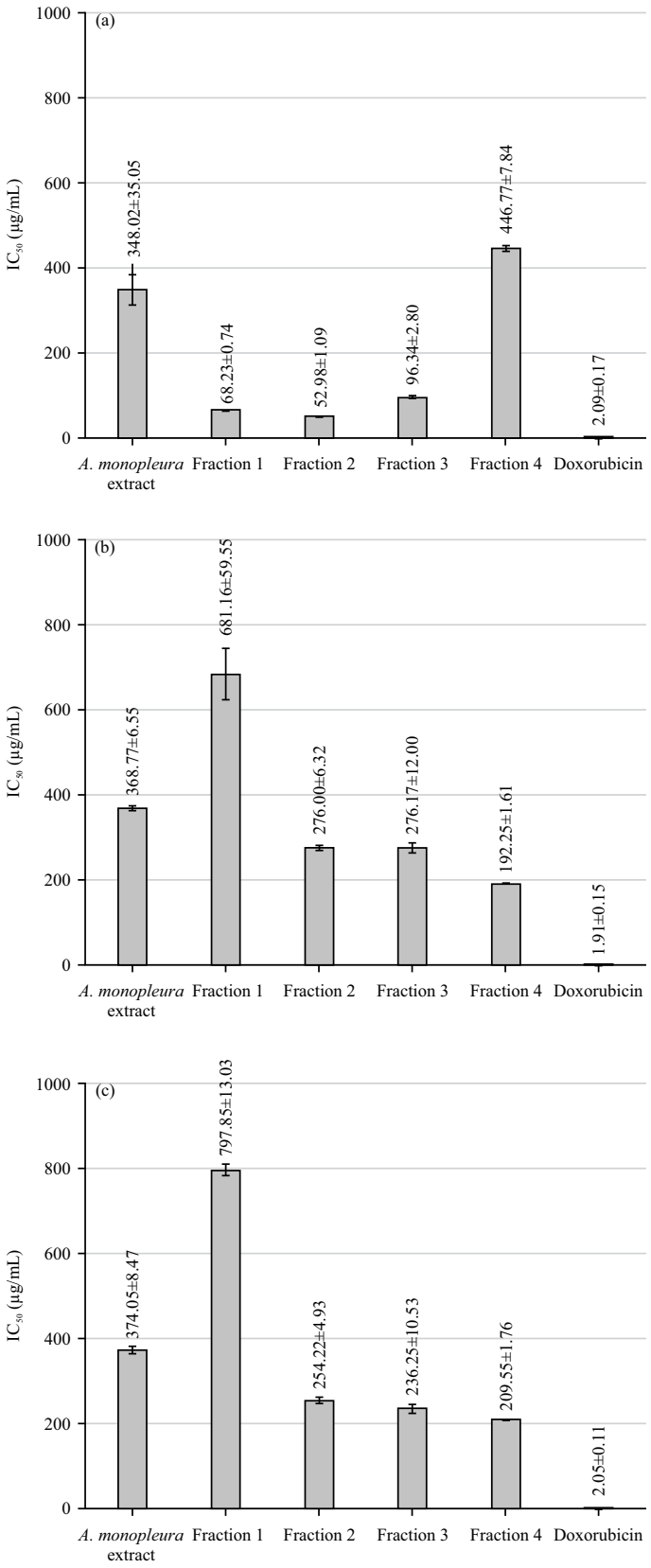

Fig. 1(a-c): IC_{50} of *Alpinia monopleura* extract and fractions, (a) HeLa cell lines, (b) MCF-7 cell lines and (c) WiDr cell lines
Data is presented as Mean±SD (n = 3), x-axis: Extract and fractions

Table 1: Metabolites in the fraction 2 from *A. monopleura* according to LC-HRMS with databases mzCloud

Compound	RT (min)	Measure mass ($M+H^+$)	Calculated mass	Formula	Area (Max.)	Delta mass (ppm)	mzCloud score
Flavonoid							
5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl) -7-methoxy-2,3-dihydro-4H-chromen-4-one	10.534	317.1012	316.0939	$C_{17}H_{16}O_6$	41,096,728.91	-2.43	81.7
2-(3,4-dimethoxyphenyl)-5,7-dihydroxy -6-methoxy-4H-chromen-4-one	10.844	345.0963	344.0891	$C_{18}H_{16}O_7$	428,608,130.20	-1.55	91.5
5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl) -4H-chromen-4-one	10.457	315.0858	314.0784	$C_{17}H_{14}O_6$	873,558,995.22	-2.08	92.2
Glycitein	10.289	285.0753	284.0681	$C_{16}H_{12}O_5$	479,884,317.25	-1.27	97.7
Phenolic							
Apocynin	6.785	167.0703	166.0630	$C_9H_{10}O_3$	46,571,572.93	-0.02	80
Syringic acid	4.686	199.0599	198.0526	$C_9H_{10}O_5$	14,384,036.14	-1.29	90.6
4-Hydroxybenzaldehyde	4.516	123.0439	122.0367	$C_7H_6O_2$	1,764,630,648.52	-0.59	96.9
Vanillin	5.264	153.0544	152.0472	$C_8H_8O_3$	253,552,248.50	-1.17	97.3
NP-014113	16.232	347.2574	346.2501	$C_{22}H_{34}O_3$	63,613,424.39	-1.96	98.3
2-hydroxy-6-[(8Z,11Z)-pentadeca-8,11,14-trien -1-yl]benzoic acid	15.273	343.2263	342.2190	$C_{22}H_{30}O_3$	100,164,668.96	-1.55	98.4
Alkaloid							
Indole	13.439	118.0652	117.0579	C_8H_7N	95,867,057.96	0.44	71.4
2-Amino-1,3,4-octadecanetriol	9.761	318.2999	317.2926	$C_{18}H_{39}NO_3$	35,730,477.42	-1.37	77.5
N,N-Diisopropylethylamine	1.966	130.1589	129.1516	$C_8H_{19}N$	126,243,536.02	-1.41	79
Triethanolamine	0.766	150.1124	149.1051	$C_6H_{15}NO_3$	13,868,602.18	-0.75	91
2,2,6,6-Tetramethyl-1-piperidinol	9.175	158.1538	157.1465	$C_9H_{19}NO$	120,442,206.85	-0.94	93
Hexadecanamide	14.496	256.2559	255.2559	$C_{16}H_{33}NO$	45,774,726.15	-1.21	93.6
Stearamide	15.592	284.2947	283.2874	$C_{18}H_{37}NO$	182,117,789.25	-0.29	97.7
Tributylamine	7.098	186.2214	185.2141	$C_{12}H_{27}N$	27,256,084.04	-1.36	98.3
N,N-Diethyl-3-methylbenzamide	9.385	192.1381	191.1308	$C_{12}H_{17}NO$	1,396,573,367.03	-1.23	99.8
Dibenzylamine	5.780	198.1275	197.1202	$C_{14}H_{15}N$	97,958,051.92	-1.37	99.9
Steroid							
Dehydroepiandrosterone	12.518	289.2159	288.2086	$C_{19}H_{28}O_2$	192,440,440.76	-1.23	60.5
Corticosterone	12.898	347.2209	346.2135	$C_{21}H_{30}O_4$	118,345,299.00	-2.66	60.9
6ß-Hydroxytestosterone	11.892	305.2105	304.2032	$C_{19}H_{28}O_3$	219,390,015.43	-2.25	71.8
Methyldienolone	13.133	287.2005	286.1932	$C_{19}H_{26}O_2$	86,452,509.47	-0.2	75.9
5α-Dihydrotestosterone	13.070	291.2315	290.2242	$C_{19}H_{30}O_2$	146,242,790.22	-1.31	77.4
11-Ketotestosterone	10.723	303.1951	302.1878	$C_{19}H_{26}O_3$	131,708,740.70	-1.16	84
Terpenoid							
(1S,6R,11aR,13R,14aS)-1,13-dihydroxy-6-methyl -1H,4H,6H,7H,8H,9H,11aH,12H,13H,14H,14a H-cyclopenta[f]oxacyclotridecan-4-one	9.664	281.1747	280.1674	$C_{16}H_{24}O_4$	43,102,220.79	-0.3	61.3
NP-020535	10.001	279.1589	278.1514	$C_{16}H_{22}O_4$	24,950,053.40	-1.42	61.4
Carvone	5.904	151.1115	150.1043	$C_{10}H_{14}O$	228,825,718.23	-1.13	62.3
6-hydroxy-4a-(hydroxymethyl)-5-methyl - 3-(prop-1-en-2-yl)-2,3,4,4a,5,6,7,8-octahydronaphthalen-2-one	11.137	251.1641	250.1567	$C_{15}H_{22}O_3$	93,818,757.87	-0.82	66.8
Jasmone	10.094	197.1534	164.1198	$C_{11}H_{16}O$	117,175,819.85	-1.82	67.1
4-hydroxy-6-[2-(2-methyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl)ethyl]oxan-2-one	12.569	293.2108	292.2035	$C_{18}H_{28}O_3$	52,983,748.88	-1.26	71.5
5-(4-carboxy-3-methylbutyl)-5,6,8a-trimethyl -3-oxo-3,4,4a,5,6,7,8,8a-octahydronaphthalene -1-carboxylic acid	8.468	351.2163	350.2090	$C_{20}H_{30}O_5$	31,932,604.20	-0.83	71.8
(2E)-5-[(8aS)-2,5,5,8a-tetramethyl-3-oxo-3,4,4a, 5,6,7,8,8a-octahydronaphthalen-1-yl]-3-methylpent -2-enoic acid	12.197	319.2261	318.2188	$C_{20}H_{30}O_3$	1,656,252,585.93	-2.25	73.3
Pulegone	6.821	153.1272	152.1200	$C_{10}H_{16}O$	364,218,565.70	-1.05	74.2
2,4-Dimethylbenzaldehyde	11.920	135.0804	134.0732	$C_9H_{10}O$	63,927,733.82	-0.09	74.3
(9cis)-Retinal	16.191	285.2211	284.2139	$C_{20}H_{28}O$	966,473,091.80	-0.46	77.1
(4aS,5R,6S,8aS)-5-[(3E)-5-methoxy-3-methyl-5-oxopent-3-en-1-yl]-5,6,8a-trimethyl-3,4,4a,5,6, 7,8,8a-octahydronaphthalene-1-carboxylic acid	14.649	349.2368	348.2294	$C_{21}H_{32}O_4$	59,244,028.24	-1.93	78.2

Table 1: Continue

Compound	RT (min)	Measure mass (M+H⁺)	Calculated mass	Formula	Area (Max.)	Delta mass (ppm)	mzCloud score
Aflatoxin B2	12.076	315.0862	314.0784	$C_{17}H_{14}O_6$	74,311,845.33	-2.08	78.4
NP-004038	10.709	265.1797	264.1722	$C_{16}H_{24}O_3$	727,572,401.37	-1.22	80.7
D-(+)-Camphor	6.502	153.1272	152.1200	$C_{10}H_{16}O$	113,070,128.30	-1.08	80.9
1,4-dihydroxy-1,4-dimethyl-7-(propan-2-ylidene) -decahydroazulen-6-one	10.062	253.1796	252.1723	$C_{15}H_{24}O_3$	119,917,226.82	-0.89	84.6
(1R,3S,4S,5R,7R)-4-(3-hydroxybutyl)-5-methyl-10-methylidene-8-oxatricyclo[5.3.0.0Âl,â µ]decan-9-one	10.461	251.1639	250.1568	CHO	204,114,703.41	-0.43	84.8
Fmoc-L-Pentafluorophenylalanine	10.343	253.1796	252.1723	$C_{15}H_{24}O_3$	128,837,525.18	-0.89	85
1,2,3,4-Tetramethyl-1,3-cyclopentadiene	10.138	123.1167	122.1095	C_9H_{14}	128,471,359.33	-0.48	87.3
Aflatoxin G2	10.486	331.0805	330.0734	$C_{17}H_{14}O_7$	140,729,954.96	-1.82	87.4
8-hydroxy-11-(hydroxymethyl)-1,5,11-trimethyltricyclo[6.2.1.0Â?,â]undec-2-en-9-one	10.872	251.1639	250.1567	$C_{15}H_{22}O_3$	188,850,216.88	-0.93	90.3
9-hydroxy-2,10,10-trimethyltricyclo[6.3.0.0^{1,5}] undec-6-ene-6-carboxylic acid	10.177	251.1641	250.1568	$C_{15}H_{22}O_3$	161,116,525.72	-0.43	90.7
(-)-Caryophyllene oxide	11.031	221.1897	220.1824	$C_{15}H_{24}O$	91,922,872.95	-1.62	96
Fatty acid							
Docosapentaenoic acid	14.204	331.2628	330.2556	$C_{22}H_{34}O_2$	38,853,480.99	-0.84	73
9S,13R-12-Oxophytodienoic acid	10.774	293.2108	292.2035	$C_{18}H_{28}O_3$	21,936,384.83	-1.26	80.8
Arachidonic acid methyl ester	16.307	319.2624	318.2550	$C_{21}H_{34}O_2$	104,729,041.97	-2.79	81.7
5-OxoETE	11.645	319.2261	318.2188	$C_{20}H_{30}O_3$	407,789,911.23	-2.29	84
Methyl palmitate	17.115	271.2629	270.2556	$C_{17}H_{34}O_2$	165,968,265.37	-1.14	89.7
α-Eleostearic acid	12.681	279.2318	278.2244	$C_{18}H_{30}O_2$	91,512,134.70	-0.6	93.3
13(S)-HOTrE	18.606	295.2264	294.2192	$C_{18}H_{30}O_3$	14,088,070.58	-1.02	93.9
cis-12-Octadecenoic acid methyl ester	17.153	297.2785	296.2712	$C_{19}H_{36}O_2$	29,174,429.31	-1.13	94.6
1-Stearoylglycerol	15.273	359.3149	358.3076	$C_{21}H_{42}O_4$	36,124,132.91	-1.99	94.9
9-Oxo-10(E),12(E)-octadecadienoic acid	13.070	295.2265	294.2192	$C_{18}H_{30}O_3$	254,089,293.43	-0.91	96.8
Oleamide	14.916	282.2789	281.2716	$C_{18}H_{35}NO$	187,437,740.51	-1.07	99
α-Linolenic acid	14.563	279.2317	278.2244	$C_{18}H_{30}O_2$	152,306,615.14	-0.61	99.5
9(Z),11(E),13(E)-Octadecatrienoic Acid methyl ester	16.330	293.1469	292.2397	$C_{19}H_{32}O_2$	130,984,385.15	-1.91	99.5
Other							
Decanophenone	13.222	233.1897	232.1824	$C_{16}H_{24}O$	126,423,733.90	-1.4	60.5
Acetophenone	6.591	121.0648	120.0575	C_8H_8O	43,594,141.96	-0.02	63.5
NP-007909	8.854	225.1483	224.1409	$C_{13}H_{20}O_3$	195,211,754.34	-1.51	67.9
trans-Anethole	6.633	149.0959	148.0888	$C_{10}H_{12}O$	114,718,407.75	-0.36	85.6
Bis(4-ethylbenzylidene)sorbitol	11.624	415.2109	414.2037	$C_{24}H_{30}O_6$	18,537,787.56	-1.32	99.8

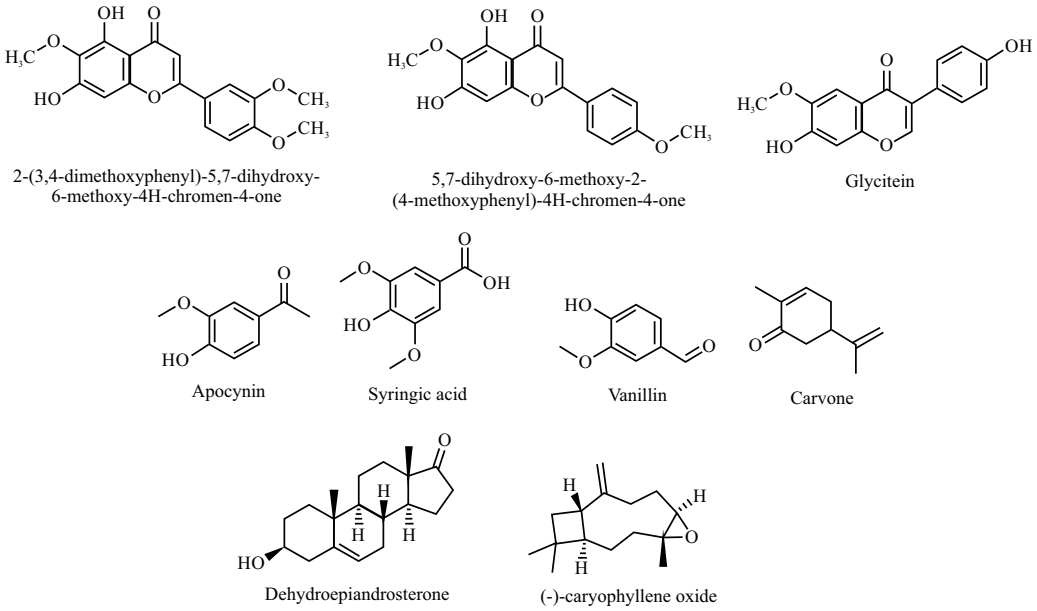

Fig. 2: Compounds from fraction 2 of *Alpinia monopleura* extract

Table 2: Metabolites in fraction 4 from *A. monopleura* according to LC-HRMS with databases mzCloud

Compound	RT (min)	Measure mass ($M+H^+$)	Calculated mass	Formula	Area (Max.)	Delta mass (ppm)	mzCloud score
Phenolic							
4-Octylphenol	8.247	206.1668	207.1741	$C_{14}H_{22}O$	307,866,479.93	-1.55	93.1
4-Coumaric acid	5.146	164.0472	165.0546	$C_9H_8O_3$	219,365,049.11	-0.64	90.2
Phenol, 2-(3,7-dimethylocta-2,6-dienyl)-	5.296	230.1667	231.1739	$C_{16}H_{22}O$	192,970,247.71	-1.65	90.2
(+)-[6]-Gingerol	9.562	294.1829	295.1901	$C_{17}H_{26}O_4$	75,097,923.51	-0.83	92
4-Hydroxybenzaldehyde	4.163	122.0368	123.0441	$C_7H_6O_2$	72,678,494.07	0.28	80.4
7-Hydroxymethyl-naphthalene-1,2-diol	11.509	190.0628	191.1791	$C_{11}H_{10}O_3$	56,192,390.56	-0.98	99.9
5-Pentylresorcinol	5.994	180.1148	181.1221	$C_{11}H_{16}O_2$	40,159,844.86	-1.56	88.7
2-hydroxy-6-[(8Z,11Z)-pentadeca-8,11,	14.981	342.2187	343.2261	$C_{22}H_{30}O_3$	38,912,121.53	-2.48	98.2
14-trien-1-yl] benzoic acid							
Paradol	10.643	278.1880	279.1952	$C_{17}H_{26}O_3$	38,399,428.68	-0.68	98.2
p-Cresol	1.518	108.0577	109.0649	C_7H_8O	23,477,720.35	1.95	81.9
Eugenol	6.042	164.0836	165.0909	$C_{10}H_{12}O_2$	23,266,560.77	-0.67	81.9
Vanillin	4.942	152.0472	153.0545	$C_8H_8O_3$	21,502,254.29	-1.07	78.1
Syringic acid	4.415	198.0523	199.0597	$C_9H_{10}O_5$	17,420,746.36	-2.44	86.6
2,6-di-tert-butyl-4-ethylphenol	9.727	234.1980	235.2054	$C_{16}H_{26}O$	13,005,614.13	-1.76	80.8
6-methoxymellein	6.592	208.0734	209.0807	$C_{11}H_{12}O_4$	11,375,086.35	-0.56	99.5
1-(4-hydroxyphenyl) pent-1-en-3-one	6.091	176.0837	177.0909	$C_{11}H_{12}O_2$	9,121,044.22	-0.36	89.4
Alkaloid							
Betaine	0.851	117.0789	118.0862	$C_5H_{11}NO_2$	476,662,309.78	-0.29	93.1
Epristeride	11.181	399.2769	400.2842	$C_{25}H_{37}NO_3$	370,346,456.22	-1.03	93.1
Oleamide	14.608	281.2716	282.2789	$C_{18}H_{35}NO$	190,985,052.45	-0.97	98.3
Choline	0.777	103.0998	104.1071	$C_5H_{13}NO$	76,867,911.20	0.93	92
Dibenzylamine	5.520	197.1201	198.1274	$C_{14}H_{15}N$	60,305,507.29	-1.75	99.9
Kynurenic acid	17.647	189.0421	190.0492	$C_{10}H_7NO_3$	54,205,518.35	-2.77	99.9
Decarbamoyl-neosaxitoxin	4.045	272.1235	273.1307	$C_9H_{16}N_6O_4$	52,897,981.36	0.58	88.7
Dicyclohexylamine	5.597	181.1829	182.1901	$C_{12}H_{23}N$	34,436,677.46	-0.95	98.2
Oleamide	15.064	281.2716	282.2791	$C_{18}H_{35}NO$	26,766,067.40	-0.97	98.9
PV9	7.752	273.2092	274.2165	$C_{18}H_{27}NO$	23,766,597.07	-0.29	81.9
Hydrocotarnine	6.718	221.1049	222.1123	$C_{12}H_{15}NO_3$	17,123,658.15	-1.16	86.6
3-Succinoylpyridine	4.725	179.0582	180.0654	$C_9H_9NO_3$	15,102,705.22	-0.16	80.8
Diisopromine	16.189	295.2298	296.2369	$C_{21}H_{29}N$	14,115,374.56	-0.63	80.8
Triisopropanolamine	0.815	191.1518	192.1591	$C_9H_{21}NO_3$	11,484,507.72	-1.58	99.5
Stearamide	15.047	283.2872	284.2945	$C_{18}H_{37}NO$	11,000,175.39	-1.05	89.4
Piracetam	0.805	142.0741	143.0814	$C_6H_{10}N_2O_2$	5,091,449.79	-0.91	89.4
Steroid							
Ethylestrenol	12.676	288.2450	289.2523	$C_{20}H_{32}O$	110,697,172.36	-1.16	98.3
Dehydroepiandrosterone	5.306	288.2085	289.2156	$C_{19}H_{28}O_2$	87,559,403.70	-1.48	74.1
Methyldienolone	5.189	286.1929	287.2003	$C_{19}H_{26}O_2$	62,392,386.25	-1.2	65.7
Corticosterone	11.119	346.2138	347.2211	$C_{21}H_{30}O_4$	32,342,724.04	-1.87	98.2
Boldione	6.387	284.1775	285.1846	$C_{19}H_{24}O_2$	25,070,926.77	-0.65	98.9
Progesterone	15.542	314.2241	315.2314	$C_{21}H_{30}O_2$	23,961,756.84	-1.41	98.9
Paravalarine	10.169	343.2506	344.2578	$C_{22}H_{33}NO_2$	14,684,218.01	-1.64	80.8
11-Hydroxyetiocholanolone	12.698	306.2187	307.2263	$C_{19}H_{30}O_3$	12,559,446.21	-2.48	80.8
5α-Androstan-3,6,17-trione	10.693	302.1880	303.1953	$C_{19}H_{26}O_3$	11,857,700.00	-0.63	65.6
Medroxyprogesterone	15.435	344.2346	345.2419	$C_{22}H_{32}O_3$	11,343,481.35	-1.48	99.5

provides an anticancer activity with several mechanisms, including inhibition of cell proliferation and cell cycle, promoting apoptosis, modulation of inflammatory and oxidative stress and sensitivity to the chemotherapeutic drugs[27]. The (+)-[6]-Gingerol promotes apoptosis by reactivating the apoptotic factor p53 through Caspase-3 and PARP pathways, inhibiting cancer cell proliferation, reducing tumors and accelerating DNA destruction of cancer cells[28]. The 5-pentyl resorcinol has antiproliferative activity against human breast cancer cell lines[29]. Eugenol acts as an anticancer by promoting apoptosis, autophagy, cell cycle arrest, inflammation, invasion and metastasis through MAPK/ERK, JNK/STAT3, WnT/β-Catenin pathway, E2F1/surviving and NF-κB signaling cascades[30,31]. The 6-methoxymellein acts as an anticancer by inhibiting the proliferation of breast cancer cells, migration, colony and mammosphere formation. It also decreases the CD44+/CD24-subpopulation and the expression of c-Myc, Sox-2 and Oct4 proteins. The 6-Methoxymellein lowers the nuclear NF-κB p65 and p50 protein expression, thus reducing the expression and secretion of IL-6 and IL-8[32].

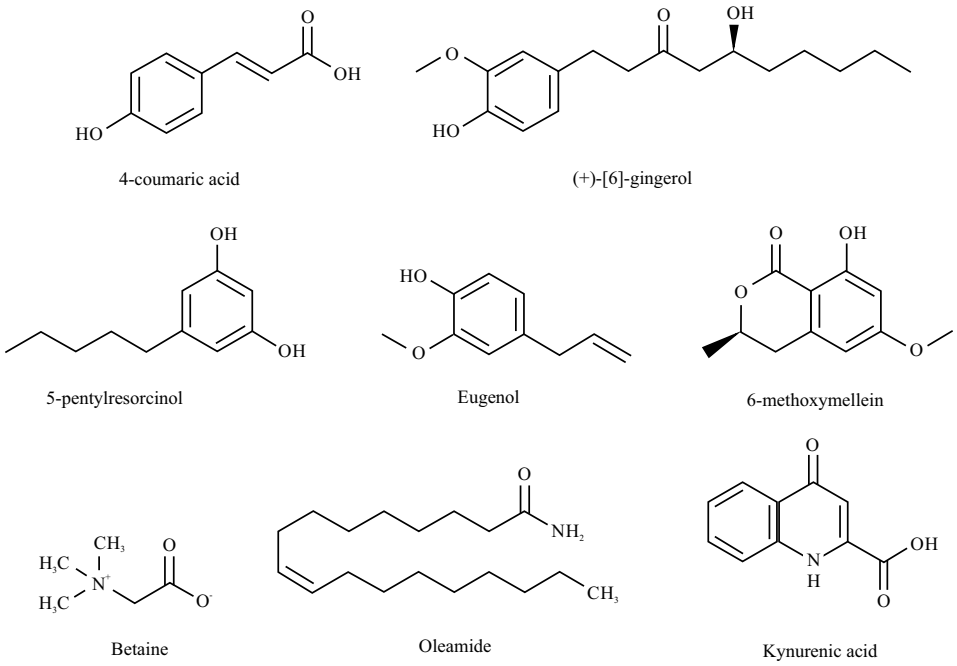

Fig. 3: Compounds from fraction 4 of *Alpinia monopleura* extract

Betaine acts as an anticancer by promoting subpopulation and the expression of c-Myc, Sox-2 and Oct4 proteins. The 6-Methoxymellein lowers the nuclear NF-κB p65 and p50 protein expression, thus reducing the expression and secretion of IL-6 and IL-8[32]. Betaine acts as an anticancer by promoting apoptosis and inhibiting cell proliferation of cancer cells[33]. Oleamide promotes apoptosis by affecting mitochondrial viability and DNA fragmentation and affecting the apoptosis-related proteins Bcl-2 and Caspase-3, as well as inducing cell arrest by increasing p53, p21WAF1/Cip1 and p27Kip1 proteins[34]. Kynurenic acid acts as an anticancer by inhibiting cell proliferation and growth by regulating PI3K/AKT and MAPK signaling pathways[35].

Docking molecular: This simulation highlights the potential interactions between compounds from the active fractions (fractions 2 and 4) of *A. monopleura* rhizome extract and three distinct cancer-related protein targets. To confirm the accuracy of these simulations, we conducted a redocking procedure with the crystal structures of Erlotinib, tamoxifen and N-[3-(4-benzylphenoxy)propyl]-N-methyl-beta-alanine within EGFR, ERα and LTA4H, respectively and evaluated the Root Mean Square Deviation (RMSD). An RMSD value of $\leq$2 Å indicates that the docking parameters effectively reproduced the native ligand's conformation, consistent with X-ray crystallography observations.

In this study, the top phytoconstituents from fraction 2 were identified for each target protein and compared their binding energies with those of the native ligands. The results showed that Dehydroepiandrosterone, 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one and 2-(3,4-dimethoxy phenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one exhibited strong binding affinities for EGFR, with binding energies of -8.959, -8.063 and -8.023 kcal/mol, respectively. Additionally, Dehydroepiandrosterone displayed notable binding scores with ERα and LTA4H (-10.071 and -9.323 kcal/mol, respectively). Table 3 presents those molecules' binding energies (kcal/mol).

The molecules were docked into the erlotinib binding site within the crystal structure of EGFR (PDB ID: 1M17) using AutoDock Vina software. The co-crystallized erlotinib exhibited a moderate-strength hydrogen bond, evidenced by the distance between the hydrogen acceptor and donor. Specifically, this bond occurs between the N1 atom of the quinazoline moiety and the side chains of Thr769[36]. Additionally, there was a weak hydrophobic interaction involving the aromatic ring, as well as two hydrophobic interactions within the aliphatic side chain ($-CH_2-O-CH_3$)[37]. Figure 4 presents a 2D depiction of Erlotinib's interactions at the receptor site.

Based on binding energy, the three compounds from fraction 2 that exhibited higher energy than Erlotinib demonstrated interaction with the EGFR binding site through hydrogen bonding with Thr769 and additional hydrogen bonds. These interactions contributed to stronger binding with the receptor site than Erlotinib achieved. The compounds 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-

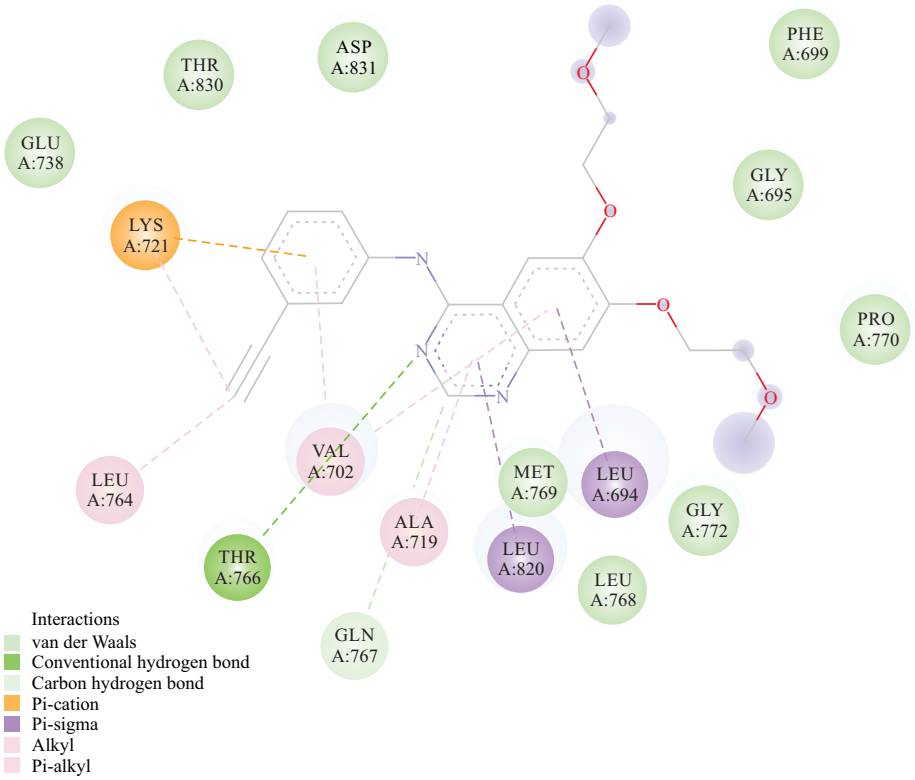

Fig. 4: 2D interactions of Erlotinib at the EGFR binding site display a single type of hydrogen bond

Table 3: Comparison of estimated free energy of binding of the investigated ligands against EGFR, ERα and LTA4H

Compound	Protein	Binding energy (Kcal/mol)
Erlotinib		-6.873
2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one	EGFR	-8.023
5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one		-8.063
Glycitein		-7.77
Apocynin		-5.525
Syringic acid		-5.364
Vanillin		-5.656
Carvone		-5.822
Dehydroepiandrosterone		-8.959
(-)-Caryophyllene oxide		-6.829
Tamoxifen	Erα	-10.501
4-coumaric acid		-6.101
(+)-[6]-gingerol		-6.793
5-pentyl resorcinol		-6.059
Eugenol		-5.625
6-methoxymellein		-7.108
Betaine		-3.898
Oleamide		-6.09
Kynurenic acid		-6.708
N-[3-(4-benzylphenoxy)propyl]-N-methyl-beta-alanine	LTA4H	-9.673
4-coumaric acid		-7.821
(+)-[6]-gingerol		-9.249
5-pentyl resorcinol		-8.019
Eugenol		-7.539
6-methoxymellein		-8.297
Betaine		-4.141
Oleamide		-8.52
Kynurenic acid		-8.326

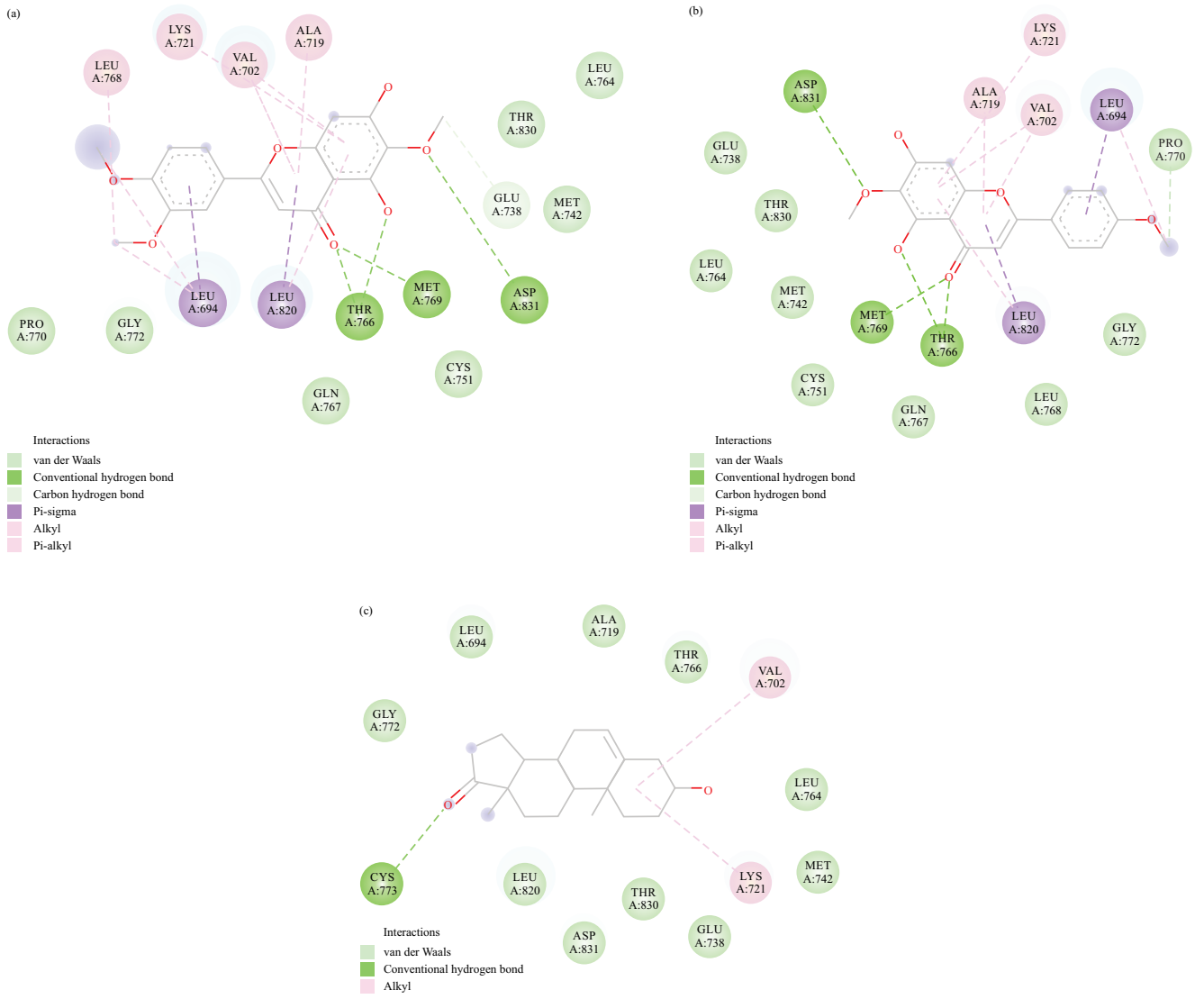

Fig. 5(a-c): 2D interactions of compounds 2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one, 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one and Dehydroepiandrosterone with the EGFR binding site, (a) 2D interactions of compound 2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one with EGFR binding site, (b) 2D interactions of compound 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one with EGFR binding site and (c) 2D interactions of compound Dehydroepiandrosterone with EGFR binding site

Table 4: Summary of the interactions of the three compounds with the highest binding energies in fraction 2 of *A. monopleura* rhizome extract against EGFR

Compound	Hydrogen bond	Hydrophobic Interaction
Erlotinib	Thr766	Leu694, Leu820, Ala719, Val702, Leu764
2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one	Thr766, Met769, Asp831	Leu694, Leu820, Ala719, Val702, Lys721, Leu768
5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one	Thr766, Met769, Asp831	Leu694, Leu820, Ala719, Val702, Lys721
Dehydroepiandrosterone	Cys773	Val702, Lys721

4-one and 2-(3,4-dimethoxy phenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one formed hydrogen bonds between (C = O) and (OH), as well as (C-O) and (OH) with Thr769, in addition to bonds between (C = O) and Met769 and between (R-C-O-R) and Asp831. Table 4 presents the interactions of these molecules against EGFR protein. These interactions resulted in better binding energy scores than Erlotinib. Furthermore, Dehydroepiandrosterone, which had the highest binding energy score among all compounds, formed a single hydrogen bond with EGFR at a different amino acid residue, specifically between (C = O) and Cys773.

Figure 5a illustrates the 2D interactions of the compound 2-(3,4-dimethoxy phenyl)-5,7-dihydroxy-6-methoxy-4H-chromen-4-one with the amino acid residues in the EGFR binding site. The analysis predicts that this compound forms favorable interactions through hydrogen bonds with Thr766, Met769 and Asp831, as well as hydrophobic interactions with Leu694, Leu820, Ala719, Val702, Lys721 and Leu768. These interactions involve the same amino acid residues as those of erlotinib, the native EGFR ligand. Similarly, Fig. 5b presents the 2D interactions of the compound 5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-one with EGFR, showing hydrogen bonds with Thr766, Met769 and Asp831, along with hydrophobic interactions involving Leu694, Leu820, Ala719, Val702 and Lys721. Figure 5c depicts the 2D interactions of Dehydroepiandrosterone with EGFR, highlighting hydrogen bonding with Cys773 (a residue not involved in erlotinib binding) and hydrophobic interactions with Val702 and Lys721, which overlap with erlotinib's interaction profile. Hydrogen bonds are represented in green, while hydrophobic interactions are marked in purple. These docking results align with toxicity tests on HeLa cells, which indicate that fraction 2 is predicted to be more toxic to HeLa cells than fraction 4.

Molecular docking was also performed for phytoconstituents from fraction 4 to predict the binding modes of these compounds with ERα and LTA4H and to compare their binding affinities with those of the respective native ligands. According to the molecular docking data, fraction 4 compounds had lower binding energy than the native ligands. This does not suggest inconsistency between the toxicity test and molecular docking results; rather, it indicates that the compounds active against MCF-7 and WiDr cancer cell lines were minor constituents.

CONCLUSION

The extract and fractions obtained from *Alpinia monopleura* rhizome are beneficial for discovering novel anticancers. Fraction 2 was the most active against HeLa cells, while Fraction 4 was the most active against MCF-7 and WiDr cells. These compounds obtained in both fractions might be developed and studied further to develop novel anticancer agents by focusing on the isolation their active compounds and testing their anticancer activity.

SIGNIFICANCE STATEMENT

"This study discovered the *Alpinia monopleura* rhizome fractions that can be beneficial for anticancer against various cancer cells *in vitro* and *in silico.* This study will help the researchers to uncover the criticals areas of drug discovery from natural plants that many researchers were not able to explore. Thus, a new theory on the utilization of *Alpinia monopleura* plants as potential anticancers may be arrived at.

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