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# Anticancer Potential of *Citrus Limon*-Derived Purified Phytochemicals Using Chromatography Implication in Progesterone and Estrogen Receptor-Dependent Breast Cancer

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**Abstract:** Breast cancer, one of the most common global health issues, is influenced significantly by estrogen and progesterone signaling, with treatments often centered on endocrine-based regimens that include progesterone and estrogen modulators. Historically, a wide range of illnesses and ailments have been treated with medicinal herbs. The Rutaceae family's *Citrus limon* contains a plethora of therapeutically valuable phytoconstituents: sugar, polyphenols, pectin, polysaccharides, and monoterpenes. Citrus peels exhibit a wide range of biological activities like antiviral, antibacterial, antifungal, anticancer, and antidiabetic most valuable ones. The current study was conducted to investigate the anticancer potential of phytoconstituents from *Citrus limon* against the breast cancer target proteins ERa (estrogen receptor alpha) and PR (progesterone receptor) using the technique of molecular docking after purification of phytochemicals. Drug likeliness was predicted using SwissADME while Molinspiration and PASS servers were used to estimate the bioactivity and anticancer activity of phytochemicals, respectively. Among seven phytoconstituents analyzed, humulene demonstrated the highest binding energy (−13.749 kcal/mol) against the targets of breast cancer. Phytochemicals complied with all ADME criteria and were verified as novel therapeutic agents. Humulene and valencene have the potential to treat breast cancer.

**Keywords:** in silico, ADME, molecular docking, *Citrus limon*, anticancer

## 1. Introduction

Fruit and vegetable waste underwent a conversion from linear to circular bioeconomy in past years. A significant quantity of waste was produced in industrial operations producing fruit concentrates, juice, and canned goods as a result of the high consumption of fruits and vegetables [1]. *Citrus limon* is one of the most popular citrus fruits because of its positive health effects. A significant quantity of trash is generated during fruit processing [2]. Citrus fruit waste contains valuable compounds that have medicinal applications [3]. Rich in polyphenols, sugar, pectin, polysaccharides, and monoterpenes, citrus peel, and seed-pressed pulp are regarded as waste products of citrus fruit [4].

The concept of medication and food homology originates from Chinese traditional medicine [5]. Nutraceuticals are emerging as a promising therapy with fewer side effects. These substances combine pharmacological and nutritional benefits that enlist them safer for long-term consumption and have no detrimental effects [6]. Research indicates that the flavonoid content in citrus lemon peel contributes to its significant anticancer potential [7]. Drug discovery advancements throughout the 20th century led to the development of medications like antibiotics and vaccines [8].

A significant medicinal plant belonging to the Rutaceae family and Aurantioideae subfamily is *Citrus limon*. This genus contains a variety of species, such as *Citrus limon* (lemon), *Citrus paradisia* (grapefruit), and *Citrus sinensis* (orange) [9]. The lemon is widely recognized throughout the world and is grown in many tropical and subtropical nations, such as Brazil, China, Japan, Mexico, and Turkey. About 5% of *Citrus limon*'s content is citric acid, which gives the fruit its sour flavor [10, 11]. It ranks third among the citrus species in importance after orange and mandarin. Flavonoids, glycosides, coumarins, sitosterol, and essential oils are abundant in lemon peels, which can be extracted and utilized in a variety of pharmaceutical and cosmetic goods [12]. Citrus peel also contains eriodyctiol, hesperidin, hesperetin, naringin, apigenin, diosmin, quercetin, diosmin, homoorientin, luteolin, orientin, vitexin, citropten, scopoletin, and isorhamnetin [13]. Phytochemicals in *Citrus limon* peel have antibacterial,

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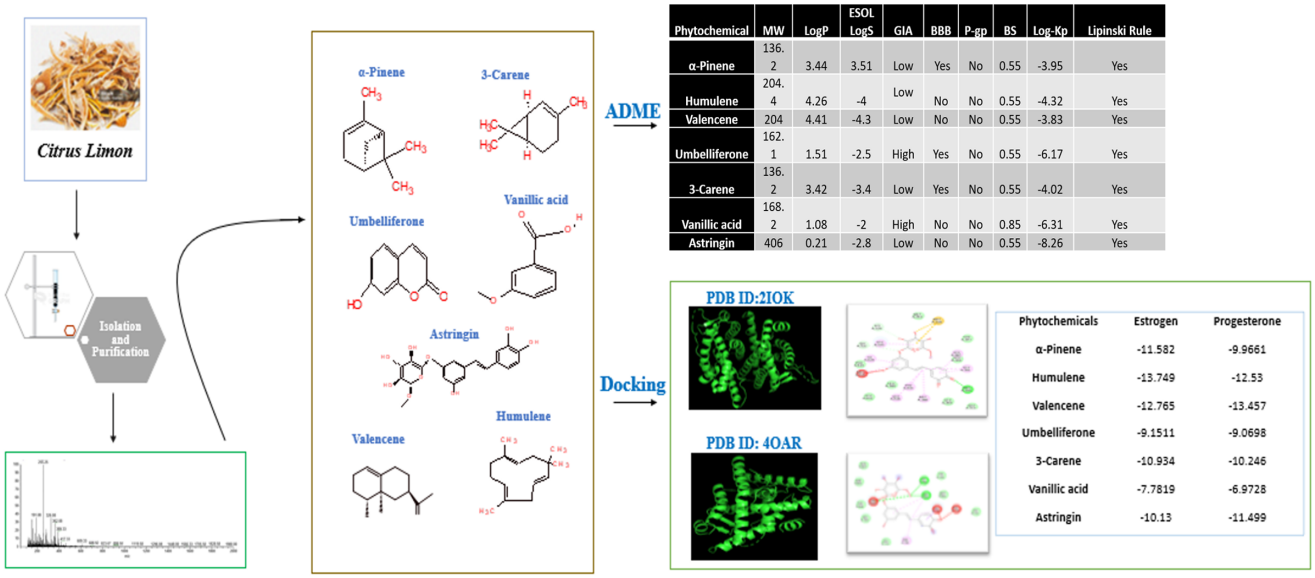


| Phytochemical | MW | LogP | ESOL LogS | GIA | BBB | P-gp | BS | Log-Kp | Lipinski Rule |
|---|---|---|---|---|---|---|---|---|---|
| α-Pinene | 136.2 | 3.44 | 3.51 | Low | Yes | No | 0.55 | -3.95 | Yes |
| Humulene | 204.4 | 4.26 | -4 | Low | No | No | 0.55 | -4.32 | Yes |
| Valencene | 204 | 4.41 | -4.3 | Low | No | No | 0.55 | -3.83 | Yes |
| Umbelliferone | 162.1 | 1.51 | -2.5 | High | Yes | No | 0.55 | -6.17 | Yes |
| 3-Carene | 136.2 | 3.42 | -3.4 | Low | Yes | No | 0.55 | -4.02 | Yes |
| Vanillic acid | 168.2 | 1.08 | -2 | High | No | No | 0.85 | -6.31 | Yes |
| Astringin | 406 | 0.21 | -2.8 | Low | No | No | 0.55 | -8.26 | Yes |

**Figure 1. Graphical abstract**

anticancer, anti-oxidant, anti-allergic, antidiabetic, anti-parasitic, and anti-inflammatory properties in addition to their nutritional benefits [14]. It also offers other therapeutic advantages for the skin, weight loss, digestion, eyes, scurvy, piles, peptic ulcers, respiratory conditions, gums, and urinary diseases. In addition, it is utilized to treat menstruation disorders, diabetes, obesity, oxidative stress, parasite infections, allergies, and cardiovascular diseases [15].

A class of disorders known as cancers is defined by the unchecked proliferation and dissemination of aberrant cells. Death may occur if the spread of cancer cells, known as metastasis, is not stopped at this point [16]. Lung cancer has been surpassed by female breast cancer as the most prevalent cancer diagnosed globally. In 2020, there were an anticipated 2.3 million recent incidences of breast cancer, which represents 11.7% of all new cancer cases. Of those cases, 684,996 cases resulted in death [17]. The etiology of breast cancer involves hormonal regulation and genetic, environmental, and nutritional factors that contribute to its pathogenesis. It is a biologically diverse group of diseases classified into biological subtypes based on immunohistochemistry and gene expression profiling [18]. During its development stage, breast cells become abnormal and proliferate uncontrollably, forming a tumor [19]. Chemotherapy, radiation, and surgery are standard treatments for breast cancer (BC), but their effectiveness is limited by poor prognosis, metastasis, recurrence, and drug resistance [20, 21]. Several bioactive compounds derived from medicinal plants have an anticancer effect on breast cancer cell lines [19].

The use of in silico techniques offers a platform for evaluating the efficacy of possible treatments against molecular targets, assisting in the identification of the most promising ones for additional in vitro and in vivo testing [22]. Because it can handle the complexity, high cost, and time-consuming nature of drug development projects, in silico studies have proven to be particularly useful in screening anticancer drugs in recent years. Anticancer drug discovery extensively utilizes molecular docking and PASS Online to predict anticancer activity [23].

This study aims to use in silico analysis to investigate the anticancer potential of purified constituents of *Citrus limon*, elucidate the dynamics of the molecular targets, and evaluate the components computationally.

## 2. Materials and Methods

### 2.1. Extract preparation of limon peel

The *Citrus limon* peel was removed and let it dry at room temperature for three days. A little over 40 g of crushed powder was steeped in 0.1 L of 80% methanol for a full day with time-to-time stirring. The solvent was eliminated using a rotary evaporator. Prior to being eventually gathered in tiny microfuge tubes, the residues were allowed to air dry [24].

### 2.2. Isolation and purification of compounds

#### *2.2.1. Column chromatography*

Column chromatography was used to separate the extract into its component fractions using two grams of the extract. The solvent system was employed with methanol, ethanol, and water as the mobile phase, while the stationary phase was silica gel (60–120 mesh). Glass wool was stocked into the lowest portion of the glass column during the column chromatography setup with the use of a glass rod. The sample was made by adsorbing 2.0 g of the extract to 10 g of silica gel and letting it dry. Then, the dried powder was carefully stacked on top of the column, followed by glass wool to prevent the solvent system from splattering when the column was filled. The extract was eluted using a 1:1:1 Methanol: Ethanol: Water solvent solution. Bottles were used to collect the eluted fractions [25].

#### *2.2.2. Thin-layer chromatography*

The dimensional ascending method was applied to the TLC analysis. The 20 × 20 cm TLC plate, which was coated with silica gel 60G F254 (Merk, India), was cut into a 14 × 3 cm form using scissors. The plate was gently marked 1.5 cm from the top and bottom using a pencil. The sample was spotted on the TLC plate on the pencil-marked bottom line using glass capillaries. After drying the plate in the fume hood, the sample was loaded once more until a black spot was achieved. Next, roughly 20 milliliters of the solvent Methanol: Ethanol: Water (1:1:1) were added to the chamber. The plate was positioned on top of the chamber liner.

Plates were used to find the areas after the run and were dried in the fume hood. Following the drying of each plate, the spots were found using UV light at 254 and 366 nm. The retention factor (Rf) represented the mobility of the active ingredient [13].

*2.2.3. Characterization of selective phytoconstituents by LC/MS*

An LC-MS device with an ESI interface was used to carry out the chemical screening of the limon extract. The Acquity C18 column (100 × 4.6) × 5 µm was utilized for chromatographic separation. A: B was used in the isocratic elution condition for the mobile phase. Methanol concentration: 2 mM 0.6 mL/min of ammonium acetate: formic acid (65:35:0.1) is the flow rate that is maintained. Voltage of 30 V, capillary voltage of 3.5 V, desolvation gas of 900 L/h, desolvation temperature of 400 °C, cone gas of 50 L/h, source temperature of 150 °C, and collision energy of 22 V were the conditions for the LC-MS. All of the information was controlled utilizing the database in order to validate the chemicals [26].

## 2.3. In silico studies

*2.3.1. Pharmacokinetics assessment*

Canonical SMILE was obtained, which is the first step via the PubChem site (https://pubchem.ncbi.nlm.nih.gov/) of purified compounds. Using SwissADME (http://www.swissadme.ch/), ADME was estimated in terms of its absorption, distribution, metabolism, and excretion [27]. The bioactivities of phytochemicals were predicted using the Molinspiration database, which is accessible online at (http://www.molinspiration.com/). The following bioactivities were predicted for the compounds: nuclear receptor ligand, kinase inhibitor, protease inhibitor, ion channel modulator, and enzyme inhibitor [28].

*2.3.2. PASS prediction test*

PASS (prediction of activity spectra for substances) Online analysis was used to predict the biological activity of limon purified compounds in order to assess the potential anticancer properties of the citrus's active ingredients. Using the website (http://www.way2drug.com/passonline/index.php), the analysis was performed by selecting Go for prediction and clicking on Predict New Compound. The *Pa* (positive activity) and *Pi* (probable inactivity) values showed the analysis's findings [29].

*2.3.3. Drug-target interaction*

The STITCH (Search Tool for Interactions of Chemicals; www.stitch.emblde) webserver was utilized to logically choose potential drug targets for the generation of a drug-target network [30].

*2.3.4. Protein-protein interactions*

The interactions of human ER-α and PR were analyzed using the STRING database, which includes both predicted and experimentally validated protein-protein interactions. ER-α and PR were queried to generate a graphical network of interactions with other proteins, identifying significant physical as well as functional associations. Interaction types such as gene fusion, expression, co-occurrence, homology, literature support, experimental data, and predicted links were assessed that leads to a score-based evaluation of each interactor [31].

*2.3.5. Molecular docking*

The target proteins used in this study were estrogen receptor alpha/ERa (PDB ID: 2IOK) and progesterone receptor/PR (PDB ID: 4OAR), which were obtained from the Protein Data Bank (https://www.rcsb.org). The molecular docking analysis was compared with control such as doxorubicin (CID: 31,703) and letrozole (CID: 3902). The molecular docking was performed using ArgusLab software. Discovery Studio software to determine the binding pose between the receptor and ligand. The result of molecular docking was indicated by the free energy value-binding affinity of the bond formed [32].

## 3. Results

Refer to Figure 1 for a detailed overview of the study. Methanol extraction was carried out for dried and powdered *Citrus limon* peel. About 25 g of the extract was subjected to column chromatography on a silica gel (100–200 mesh, Merck) pack. The effect of polarity on the extraction and the extracted phytochemicals is demonstrated by the methodical order in which the solvents were chosen. The results were obtained as Rf values of phytoconstituents regarding the TLC analysis to detect compounds utilizing solvents (ethanol, methanol, and water). Polyphenols were found in water with Rf values of 0.58 and 0.92; coumarins with one spot were found in ethanol extract with Rf values of 0.43. Conversely, two spots, Rf 0.85 and Rf 0.91, were found in the methanol extract for sesquiterpenes in the extract. Terpenes were also pointed with two spots in ethanol with Rf of 0.32 and 0.68, respectively.

LC-MS was used to assess the bioactive components in *Citrus limon*. Based on the findings, the whole chromatogram is displayed in Figure 2. A total of seven bioactive chemicals in the citrus are demonstrated in Figure 3. The peak area represented the relative abundance of each constituent. This investigation therefore demonstrated that α-Pinene, Humulene, Valencene, Umbelliferone, and 3-Carene were the most abundant compounds of Citrus. Astringin and vanillic acid were the next most abundant compounds as in Table 1. The structure of all the constituents was validated using NIST and drawn in the ACD/ChemSketch tool.

The SwissADME online software application was employed in this work to assess the ADME characteristics of *Citrus limon*'s phytochemicals. As a result, the ADME properties of the phytoconstituents were examined and shown in Table 2. The two most important chemical characteristics in a drug's absorption are its lipophilicity and its solubility. The Lipinski Rule of Five is based on the physicochemical properties of the tested substances and is used to predict the oral bioavailability of a medicine. These properties include clogP must be less than five and molecular weight (MW) not more than 500 g/mol. The number of hydrogen bond acceptors (HBA) must be greater than ten. The number of hydrogen bond donors (HBD) must be greater than five. Later on, additional relevant criteria were introduced, such as polar surface area (PSA) $< 140$ Å2 and number of rotatable bonds (nRotb) $\leq 10$. Purified constituents pass the Lipinski rule of five, hence proved to be a good drug.

Molinspiration Cheminformatics determined the following distribution of biological activity parameters: Strong biological activity is indicated by a value greater than zero; moderate biological activity falls between −0.5 and 0; and inactivity falls below −0.5. The prediction results are shown in Table 3. α-Pinene is inactive ion channel modulator while moderately effecting the activity of protease and enzyme inhibition. Humulene and valencene were strongly interacting with nuclear

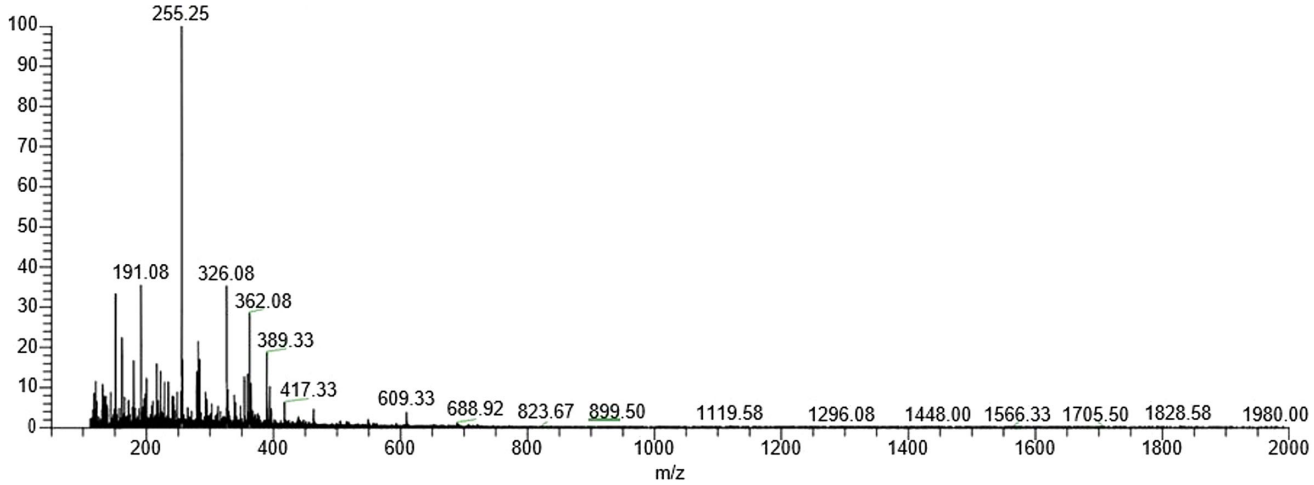


**Figure 2. Total chromatogram of purified constituents from *Citrus limon***

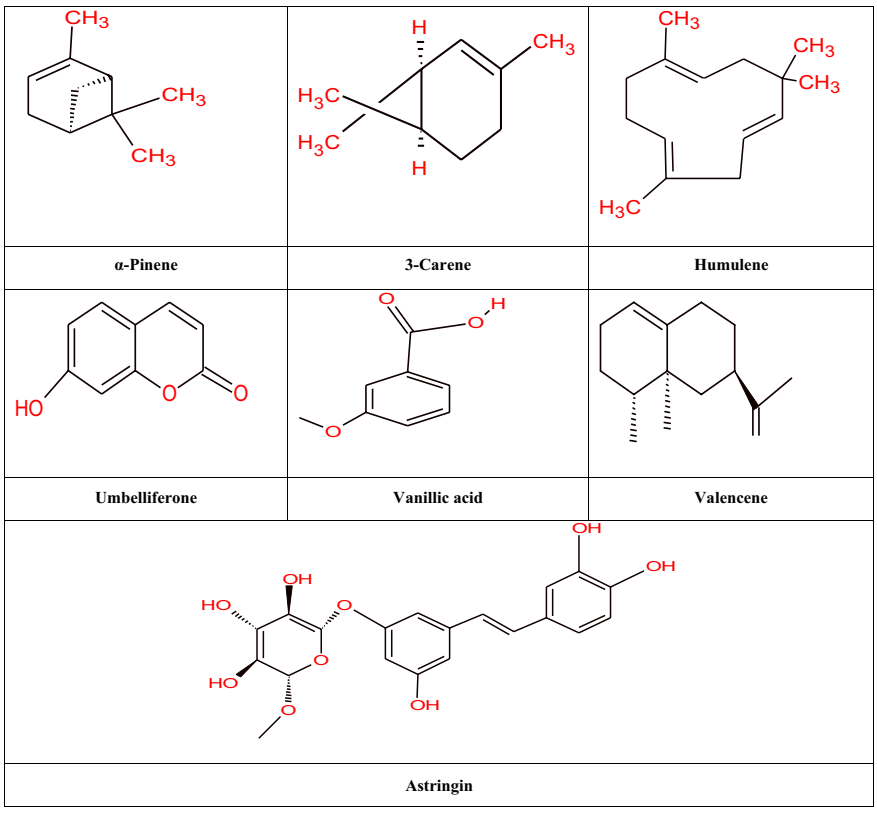


**Figure 3. Chemical compositions of purified phytoconstituents**

receptor ligand. Valencene is strongly attracting to enzyme inhibition. 3 carene a polyphenol is also inactive in G protein-coupled receptor like Humulene with value of −0.67. Umbelliferone is inactive in case of G protein-coupled receptor while moderately effecting the enzyme inhibition capacity with inactivity in case of Nuclear receptor ligand.

Limon phytochemicals have anticancer capabilities, such as humulene as anti-neoplastic ($Pa = 0.835$), astringin as anticarcinogenic ($Pa = 0.889$), 3-carene as anti-dyskinetic ($Pa = 0.815$), and the anti-mutagenic effects of umbelliferone ($Pa = 0.898$). Higher action is shown by α-pinene as a metastasis inhibitor ($Pa = 0.875$). However, doxorubicin displayed a greater anticancer effect than letrozole because of its higher *Pa* value. It is commonly understood that the probability of a chemical exhibiting biological activity is represented by its *Pa* value. The higher the *Pa* value, the more likely the action is to occur. Each biological activity's *Pa* value demonstrates that doxorubicin has greater antitumor properties. Additionally, Table 4 shows that every bioactive material being studied has been demonstrated to have anticancer properties.

A statistical approach was utilized to model drug-protein interactions using the STITCH database. Data on almost 5 million

**Table 1. Identified compounds in *Citrus limon* extract**

| Phytochemicals | M/Z[ADDUCT][M+H] | RT (min) | M.W. (g/mol) | Relative intensity | Chemical formula | Class |
|---|---|---|---|---|---|---|
| α-Pinene | −135.23 | 2.25 | 136.23 | 65.4 105.3 121.8 | $C_{10}H_{16}$ | Terpene |
| 3-Carene | 137.23 | 2.95 | 136.23 | 54.1 94.6 109.2 | $C_{10}H_{16}$ | Monoterpene |
| Humulene | 205.35 | 3.35 | 204.35 | 87.2 150.2 203.1 | $C_{15}H_{24}$ | Sesquiterpene |
| Umbelliferone | −161.14 | 3.64 | 162.14 | 49.1 112.3 148.4 | $C_9H_6O_3$ | Coumarin |
| Vanillic acid | 167.15 | 3.96 | 168.15 | 33.2 76.2 100.5 | $C_8H_8O_4$ | Benzenoids |
| Valencene | 205.35 | 4.17 | 204.35 | 69.2 131.4 199.3 | $C_{15}H_{24}$ | Sesquiterpene |
| Astringin | −405.4 | 4.85 | 406.4 | 198.6 276.5 321.9 | $C_{20}H_{22}O_9$ | Stilbenes glycosides |

**Table 2. Predicted drug likeliness of purified phytochemicals of *Citrus limon***

| Phytochemical | MW | Log-P | ESOL Log-S | GIA | BBB | P-gp | BS | Log-Kp | Lipinski rule |
|---|---|---|---|---|---|---|---|---|---|
| α-Pinene | 136.2 | 3.44 | 3.51 | Low | Yes | No | 0.55 | −3.95 | Yes |
| Humulene | 204.4 | 4.26 | −4 | Low | No | No | 0.55 | −4.32 | Yes |
| Valencene | 204 | 4.41 | −4.3 | Low | No | No | 0.55 | −3.83 | Yes |
| Umbelliferone | 162.1 | 1.51 | −2.5 | High | Yes | No | 0.55 | −6.17 | Yes |
| 3-Carene | 136.2 | 3.42 | −3.4 | Low | Yes | No | 0.55 | −4.02 | Yes |
| Vanillic acid | 168.2 | 1.08 | −2 | High | No | No | 0.85 | −6.31 | Yes |
| Astringin | 406 | 0.21 | −2.8 | Low | No | No | 0.55 | −8.26 | Yes |

**Note:** MW: Molecular Weight Log-P: Lipophilicity consensus log, ESOL Log-S: Water solubility, GIA: Gastrointestinal absorption, BBB: Blood-brain barrier, P-gp: P-glycoprotein, BS: Biostability Log-Kp: Skin permeation.

**Table 3. Molinspiration study score for bioactivity of phytochemicals**

| Phytochemicals | GPCR-L | ICM | KI | NRL | PI | EI |
|---|---|---|---|---|---|---|
| α-Pinene | −0.94 | −0 | −2 | −0.74 | −1.4 | −0.4 |
| Humulene | −0.67 | −0 | −1 | 0.14 | −0.6 | −0.1 |
| Valencene | −0.26 | −0 | −1 | 0.17 | −0.6 | 0.19 |
| Umbelliferone | −1.22 | −1 | −1 | −0.92 | −1.3 | −0.4 |
| 3-Carene | −0.94 | −0 | −2 | −0.47 | −1.2 | −0.2 |
| Vanillic acid | −1.15 | −1 | −1 | −1.02 | −1.3 | −0.7 |
| Astringin | −0.03 | 0.1 | −0 | 0 | −0.1 | 0.04 |

**Note:** GPCR: G protein-coupled receptor ligand, ICM: Ion channel modulator, KI: Kinase inhibitor, NRL: Nuclear receptor ligand, PI: Protease inhibitor, and EI: Enzyme inhibitor.

interactions between 430,000 chemicals and 9.6 million proteins, gathered from 2031 genomes, are integrated into STITCH. The main methods used to predict protein-drug interactions are experimental data and keyword mining of the literature. We utilized STITCH, which is based on STRING v10, to build a network based on binding affinities (*Ki* of protein-drug interactions with thickness of edges between nodes, rising as *Ki* value increases). The STITCH predictions for the drug-gene interactions of the seven phytochemicals that bind strongly are displayed in Figure 4. Most of the genes interacted with umbelliferone with cytochrome and myeloperoxidase CYP2A7 and MPO, respectively. UGT1A10 refers to UDP-glucosyltransferase and interacts with vanillic acid, while α-Pinene binds effectively to the CER1 protein from the

**Table 4. The *Citrus limon* active and control chemicals' PASS prediction findings**

| Phytochemicals | Pa | Pi | Activity |
|---|---|---|---|
| α-Pinene | 0.875 | 0.054 | Anti-metastatic |
| Humulene | 0.835 | 0.008 | Anti-neoplastic |
| Valencene | 0.888 | 0.002 | Carminative |
| Umbelliferone | 0.898 | 0.002 | Anti-mutagenic |
| 3-Carene | 0.815 | 0.005 | Anti-dyskinetic |
| Vanillic acid | 0.964 | 0.002 | Chlordecone reductase inhibitor |
| Astringin | 0.889 | 0.003 | Anti-carcinogenic |
| Doxorubicin | 0.96 | 0.004 | Anti-neoplastic |
| Letrozole | 0.605 | 0.041 | Anti-neoplastic |

Cytokine Knot superfamily. Humulene has a high binding affinity to ERA1 of the Farnesyl trans-transferase family.

Estrogen receptor alpha and progesterone from humans exhibited noteworthy interactions with different proteins Figure 5. With a maximum value of association of 0.961 as given in Table 5, ER-α and PR exhibited the most significant interactions with NCOA2. Both ER-α and PR exhibit closed interaction and both have health impacts in the case of cancer. ER-α and PR were secondarily interacting with NRIP1SP1. NCOR-1 was thirdly found to be the most interacting gene with ER-α and PR, with an interaction score of 0.94. The lowest interaction was seen with the protein of NCOA1, with an interaction score of 0.9.

It was discovered that the Avian Sarcoma Virus has the JUN gene as its conversion product from proto-oncogene to oncogene. SRC non-receptor tyrosine kinase plays an important role in differentiation, proliferation, motility, and adhesion of cells.

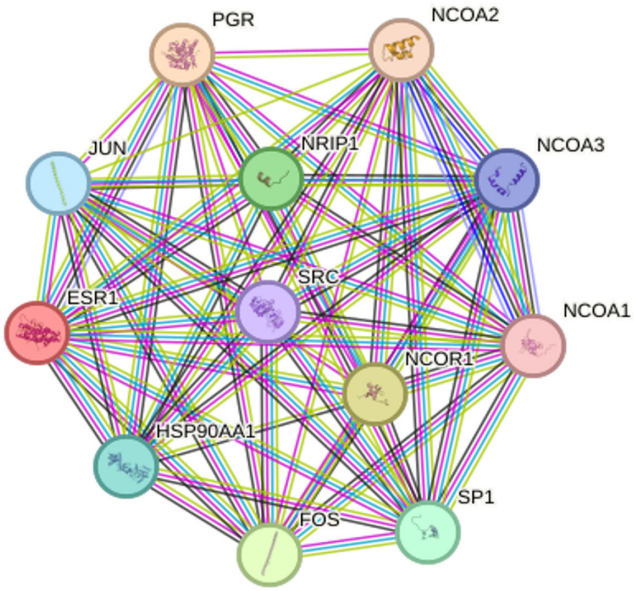


**Figure 5. The query protein and its important interactors are graphically presented**

Molecular docking was used to assess how *Citrus limon* bioactive chemicals interacted with the target protein. Molecular docking operates on the basis of scoring or calculating the free energy involved in the creation of ligand-protein interactions between proteins and ligands. The resultant link and indicated

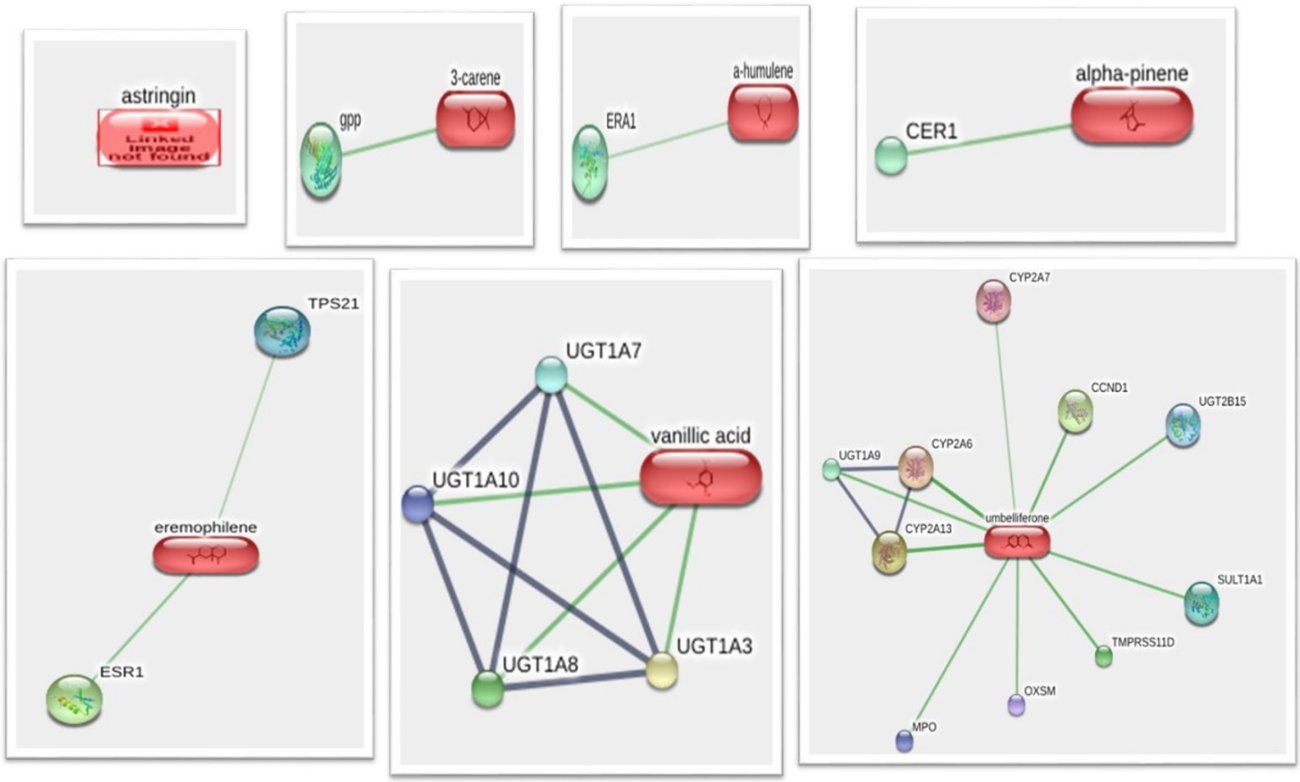


**Figure 4. Gene-drug network built with STITCH. The drug-protein binding affinity determines the scale for the edge width of protein-drug interactions.**

**Table 5. Scores of evidence channels of human ER-$\alpha$ and PR with its interactors**

| Interactors | Neighborhood | Gene fusion | Homology | Coexpression | Score |
|---|---|---|---|---|---|
| NCOR1 | No | No | No | Yes | 0.94 |
| FOS | No | No | No | No | 0.93 |
| NRIP1SP1 | No | No | Yes | No | 0.95 |
| HSP90AA1 | No | No | No | Yes | 0.91 |
| JUN | No | No | No | No | 0.917 |
| NCOA1 | No | No | Yes | No | 0.9 |
| NCOA2 | No | No | No | Yes | 0.961 |
| SRC | No | No | Yes | No | 0.918 |
| NCOA3 | No | No | No | No | 0.901 |

**Note:** NCOR1: Nuclear receptor corepressor 1 FOS: Proto-oncogene c-FOS NRIP1: Nuclear receptor interacting protein SP1: Transcription factor Sp1 HSP90AA1: Heat shock protein HSP-90 JUN: Transcription factor AP-1 NCOA1: Nuclear receptor co-activator SRC: Proto-oncogene tyrosine protein kinase.

stability increase with the ligand's spontaneity of binding to the receptor. Targets of this study were PR and ER-α. Receptors called ERa and PR are implicated in estrogen signaling, which is linked to hormone-dependent breast cancer. The value of the binding affinity or free energy indicates the outcome of molecular docking. The bioactive chemicals from the citrus were studied, with letrozole and doxorubicin as controls. The results of the molecular docking analysis indicate that the ER-α lowest binding energy (− 13.749 kcal/mmol) was observed when it interacted with humulene, while Vanillic had the highest binding (−7.7819 kcal/mmol) energy, which was less than when it came to doxorubicin (− 8.5 kcal/mmol), respectively. As Table 6 elucidates.

Additionally, the significant interaction with PR was demonstrated by doxorubicin (−10.1 kcal/mol), which was followed by valencene (−13.457 kcal/mol) and humulene (−12.53 kcal/mol). Because of the strong contact, the binding between the ligand and the receptor is more stable than lower binding energy. LEU-391 for ERa and MET-388 are two examples of the sites or amino acid residues where humulene and vanillic acid have the lowest binding affinity against target proteins and interact as hydrogen bonds, as shown in Figure 6. The interacting sites between the bioactive compounds under investigation and the control ligand demonstrate similarities in both potential and mechanism as compared to the control action of the drug. The strong interactions of valencene with PR and humulene with ER-α were primarily driven by H-bond formation, the most robust type of molecular interaction.

**Table 6. Receptor-ligand interaction between respective docking scores**

| Phytochemicals | ERa | PR |
|---|---|---|
| α-Pinene | −11.582 | −9.9661 |
| Humulene | −13.749 | −12.53 |
| Valencene | −12.765 | −13.457 |
| Umbelliferon | −9.1511 | −9.0698 |
| 3-Carene | −10.934 | −10.246 |
| Vanillic acid | −7.7819 | −6.9728 |
| Astringin | −10.13 | −11.499 |
| Doxorubicin | −8.5 | −10.1 |
| Letrozole | −9.5 | −7.8 |

**Note:** ERa: Estrogen receptor alpha PDB ID: 2IOK whereas PR: Progesterone receptor PDB ID: 4OAR

## 4. Discussion

Natural substances have been extensively used for their potential in drug development, containing bioactive compounds beneficial for human health. In plant-based chemotherapy, naturally occurring antimicrobial compounds hold significant potential [33]. An enormous amount of waste and byproducts are produced annually during the processing of citrus fruits into juices. Citrus peels have a wide range of biological activity, including antiviral, antibacterial, anticancer, antifungal, and antidiabetic properties [34]. The peels can be used in value-added forms and in a number of practical ways to lessen the management of solid waste. About half the weight of the processed fruit is wasted when lemons are processed, which is a major waste for the lemon juice industry [35]. Traditional medicines, including formulas and herbs, have been reported to treat tumors by inhibiting growth, enhancing other therapies, and alleviating symptoms to improve quality of life [36].

Pure medications that are extracted from plants or manufactured industrially may be chosen for their strong efficacy in treating human illnesses. At similar concentrations or doses of the active component, they seldom exhibit the same level of action as the raw extraction [37] while purified chemicals by applying suitable method of purification give more therapeutic effect as compared to whole extract [38]. Nitrogenous compounds especially derived from natural source act as anticancerous agent [39]. Chromatography being fundamental in phytochemistry for isolating pure compounds as well as for standardizing and ensuring phyto-therapeutic potential [40].

Globally, breast cancer is the most prevalent and deadly type of cancer that affects women. It is the complex, heterogenous disease characterized by significant genetic and clinical variability [41]. It manifests as a painless lump in the breast site [42]. Breast cancer staging is based on tumor size, metastasis, nodal involvement, and key biomarkers including estrogen receptors (ER), progesterone receptors (PR), and ERBB2 receptors [43]. The etiology of breast cancer is not fully understood, but major risk factors include sex, age, socioeconomic status, hormonal replacement therapy, reproductive factors, genetic susceptibility, dietary habits, and obesity [44].

Two-thirds of tumors in breast cancer are hormone-dependent (estrogen and progesterone receptor positive) and estrogen signaling plays a key role in this relationship [45]. Around 70% of patients have hormone-dependent breast cancer, where the ER is expressed by tumor cells known as luminal A and B. Estrogens are the main signals that drive the growth and spread of tumor cells in various cancers. The

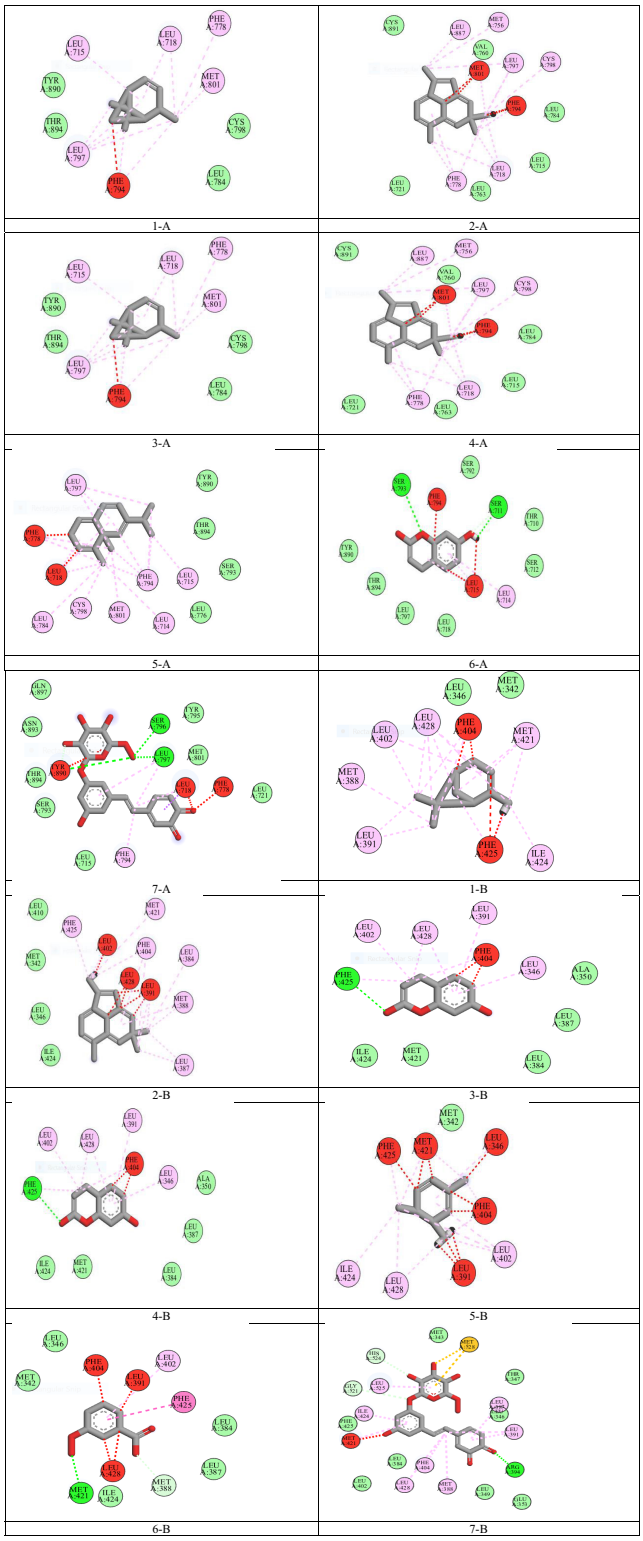


**Figure 6. Visualization of the relationship between bioactive substances and the progesterone receptor (PR), estrogen receptor (Era).** **Note:** A: progesterone receptor; B: estrogen receptor alpha; 1: α-Pinene; 2: Humulene; 3: Valencene; 4: Umbelliferone; 5: 3-Carene; 6: Vanillic acid; 7: Astringin

primary mechanisms via which estrogens operate on cells are the nucleus ERα, ERβ, and the membrane G protein-coupled ER (GPER, also known as GPR30) [46]. Since the ERα is thought to be the receptor mainly responsible for the development of breast cancer, it is essential to target it in breast cancer treatments [47].

In silico approach demonstrates that lowest binding affinity with significant interaction was seen in case of Humulene with binding energy of −13.749 kcal/mol. Valencene, a sesquiterpene extracted from citrus, has a binding energy of −13.457 kcal/mol when bound to the progesterone receptor. The stability of a docked complex is indicated by the formation of hydrogen bonds between the ligand and target residues, with umbelliferone forming hydrogen bonds at PHE-404 in ER-α and PHE-794 in PR. Purified phytoconstituents from the peel extract of *Citrus limon* were found to bind to the estrogen and progesterone receptors more successfully, demonstrating an effective response when used clinically to treat breast cancer.

## 5. Conclusion

The analysis of the component composition in *Citrus limon* revealed the presence of various bioactive components of different classes, like polyphenols, sesquiterpenes, and coumarins. Chromatographically purified seven phytochemicals —α-Pinene, Humulene, Valencene, Vanillic Acid, Astringin, and 3-Carene—show anticancer efficacy. The anticancer properties of the bioactive compounds under investigation were shown to be anti-metastatic, anti-mutagenic, and anti-neoplastic according to PASS analysis. Using molecular docking and other techniques, the anticancer potential of Citrus was further examined, with results describing that purified chemical showed the lowest but strongest binding affinity to receptor proteins. While these in silico results are promising, it is important to remember that additional experimental research—including validations in vitro and in vivo—is necessary to support and convert these computational predictions into useful applications. In order to determine the safety, effectiveness, and ideal dosage of Citrus for use as an anticancer, thorough testing and clinical trials are necessary.

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## Ethical Statement

This study does not contain any studies with human or animal subjects performed by any of the authors.

## Conflicts of Interest

The authors declare that they have no conflicts of interest to this work.

## Data Availability Statement

The data that support this work are available upon reasonable request to the corresponding author.

## Author Contribution Statement

**Asad Nawaz:** Methodology, Writing – review & editing. **Sadia Falak:** Resources, Supervision, Project administration. **Muhammad Saqlain:** Formal analysis, Writing – review & editing. **Mazhar Abbas:** Conceptualization, Methodology, Resources, Data

curation, Supervision, Project administration. **Waqas Haider:** Methodology, Software, Investigation, Writing – original draft, Visualization. **Maha Gul Zafar:** Validation, Writing – original draft, Writing – review & editing. **Marium Nadeem:** Validation, Writing – review & editing. **Ayesha Farooq:** Writing – original draft, Writing – review & editing.

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