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In Vitro And In Silico Evaluation Of Citrus Limon (L.) Burm. F. Ethanolic Fruit Extract For Its Anticancer Activity

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Abstract

The present investigation was undertaken to evaluate the anti-cancerous activity of ethanolic concentrate of Citrus limonplants growing at two different locations of Southern Tamil Nadu (Site I Nagercoil of Kanyakumari district and Site II Kudunkulam of Tirunelveli District) was determined using the MTT assay tetrazolium reduction assay in MCF-7 cultures and in silico studies.The percentage of cell viability in the Raw 264.7 macrophage cell line was recorded. The ethanolic fruit extract of Citrus limon from plants of site I showed an IC 50 value of 218.72 g/ml, while the ethanolic fruit extract from Citrus limon of Site II yielded an IC 50 value coming to 65.08 g/ml. The ethanolic fruit extract of Citrus limon from Site II was found to have more activity against MCF-7 (Human Breast Adeno Carcinoma) cell lines, exhibited as inhibition of cancer cell proliferation. The present study showed that, all the tested extracts decreased the cell viability of cancer cells only in a concentration – dependent manner when exposed to MCF-7 cell lines culture. The binding capability of the two bioactive compounds Indole-2-one-2,3 –di hydro-N-hydroxy -4 – methoxy-3, 3-di methyl from ethanolic fruit extract of Citrus limon from Site I and Urs-12-en-3-ol, acetate, (3.beta) from ethanolic fruit extract of Citrus limon from Site II were evaluate using molecular docking approach with a cancer target cyclin dependent kinase 4 (CDK4). The compound Indole-2-one, 2,3-di hydro – N- hydroxy -4 – methoxy -3, 3-dimethyl (CD1) possessed a binding energy of -5.46 while the compound Urs-12-en-3-ol, acetate, (3.beta)- (CD2) showed a least binding energy of -7.17 with the target Arg 200 shares two hydrogen bonds with the oxygen atoms of CD2. However both the compounds showed good binding affinity with the target molecule than the control drug, 5-flurouracil. Lesser binding energy during docking shown by CD1 shows that the interaction of the protein and ligand is more. This work has paved a way for future studies of drug designing using compounds from a common plant like Citrus limonso as to fight the ever increasing reports of cancer to benefit mankind.

Introduction

*Citrus limon*is a member of the Rutaceae family, and some commercial Citrus types, such as sweet orange, grapefruit, lime, and lemon, are thought to contain natural chemicals that have a variety of health benefits. Citrus fruits are one of the most important fruits with strong medicinal potential, and they have long formed the foundation of traditional remedies in various Asian countries. Citrus fruits are used as salad dressings, sauces, jams, and vinegar, as well as whole fresh fruits, in addition to being utilised as medicine. Because of the therapeutic and commercial relevance of these compounds, extensive scientific research has been conducted, providing a wide understanding of their chemical composition and bioactivities.Citrus fruits have been recognized as having high contents of bioactive compounds. The pulp and the peel, such fruits contain folate, vitamin C, dietary fiber, and bioactive compounds such as flavonoids [32]. Flavonoids are widely distributed in aromatic plants such as mint and tea but are present in high concentrations in citrus fruits and their peels. Citrus peel has untapped potential as a source of medicinal compounds because it contains carotenes, essential oils, pectin, and a range of polyphenolic compounds [29]. Epidemiological studies have suggested that high consumption of fruits and vegetables (>400 g/d) can reduce cancer risk by ≥20%. The Mediterranean diet is rich in fruit pulp and juice, and the associated high intake of fiber, antioxidants, and polyphenol compounds is linked with a lower cancer risk [10].

1. Materials and Methods

4.1 Cell culture and MTT assay:

RAW 264.7 macrophage cells was used for cell cytotoxicity assay. These cells were obtained from NCCS (Pune, India) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Himedia, India) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 ng/mL streptomycin and incubated at 37°C under 5% CO2. RAW macrophage cells were co-treated with 1µg/mL of LPS and concentrations (25 to 500 µg/ml) of test compound. After 12 h treatment, cell viability was analysed and cell culture medium was used for estimation of nitrite by the Griess reaction. To determine cell viability, the MTT assay was performed. The RAW cells were incubated with MTT solution, final concentration of 0.5 mg/mL, for 4 h. After incubation, the supernatant was removed, the formazan crystals were dissolved in 100 m L of DMSO and absorbance at 570 nm was determined by microplate reader.

The Breast Cancer cell line (MCF-7) were plated separately using 96 well plates with the concentration of 1×10^4cells/well in EMEM media with 1X Antibiotic Antimycotic Solution and 10% fetal bovine serum (Himedia, India) in CO_2 incubator at 37˚C with 5% CO_2. The cells were washed with 200 µl of 1X PBS, then the cells were treated with various test concentration of compound in serum free mediaand incubated for 24 h. The medium was aspirated from cells at the end of the treatment period. 0.5mg/mL MTT prepared in 1X PBS was added and incubated at 37˚C for 4 h using CO_2 incubator. After incubation period, the medium containing MTT was discarded from the cells and washed using 200 µl of PBS. The formed crystals was dissolved with 100 µl of DMSO and thoroughly mixed. The development of colour intensity was evaluated at 570nm. The formazan dye turns to purple blue colour. The absorbance was measured at 570 nm using microplate reader.

4.2 Docking Studies

4.2.1 Retrieval of protein and ligands

The threedimensional crystal structure of the target CDK4 (PDB ID: 6PH8) was downloaded from RCSB Protein Data Bank (https://www.rcsb.org/).

4.2.2 Preparation of protein and ligands

Effective docking requires good quality receptor and ligand coordinates. Hence optimization of the protein was done by separating the macromolecule from the solvent and non-standard residue, adding a polar hydrogen atom, and repairing the force field by adding a Gasteiger charge. The .molecular format of the ligand coordinates is converted into .pdb format by using a chemical toolbox, OpenBabel[20]. AutoDock use a simplified representation of the molecules, which is stored in a modified PDB file format, called PDBQT.

4.2.3 Molecular docking

The selected compounds from *C. limon*was subjected to grid based molecular docking using Autodock. The grid boxes were set with the dimension of X=127; Y=127; Z=127.The docking procedure was done using the Lamarckian genetic algorithm for 100 runs. One best conformation from 10 different conformations generated by autodock was considered. The complex structures showing least binding energy, ligand efficiency, with more number of hydrogen bonds were selected for proficient results.

4.2.4 Analysis of docking

The interaction analysis of protein- ligand complexes and their amino acid position with bond distances and the types of bonds involved were analysed and visualized through Discovery studio (DS) visualizer [6]. Discovery studio visualizer is a feature-rich molecular modeling application for viewing and analysing macromolecular data. DS automatically plots protein-ligand interactions. It creates pictorial representations of protein-ligand interactions for a given PDB file. DS represents the Hydrogen bonds as dashed lines between the atoms involved, and hydrophobic contacts by an arc with spokes radiating towards the ligand atoms they contact. The contacted atoms are presented with spokes radiating back.

2. Results

5.1 Cytotoxicity and Anticancer Activity of citrus lemon ethanol extract

*Citruslimon*is a member of the Rutaceae family, and some commercialCitrus types, such as sweet orange, grapefruit, lime, and lemon, are thought to contain natural chemicals that have a variety of health benefits. Citrus fruits are one of the most important fruits with strong medicinal potential, and they have long formed the foundation of traditional remedies in various Asian countries. Citrus fruits are used as salad dressings, sauces, jams, and vinegar, as well as whole fresh fruits, in addition to being utilised as medicine. Because of the therapeutic and commercial relevance of these compounds, extensive scientific research has been conducted, providing a wide understanding of their chemical composition and bioactivities. Citrus fruits' beneficial benefits can be due to their chemical elements, which include vitamins, dietary fibre, carotenoids, flavonoids, lipids, and essential oils. Citrus fruits' anticancer properties are mostly due to high levels of monoterpenes and flavanones, two classes of chemicals that are thought to reduce the risk of digestive system cancer about half. These compounds lower the chance of cancer in two ways. They inhibit inflammation, which is an essential stimulant for cancer cell growth, and they also interfere with several of the processes required for cancer cell growth, making tumour development more difficult. While Citrus has numerous chemicals that are uncommon in other plants, its individual components have shown anti-cancer efficacy against a variety of cell lines.

The cell viability of ethanolic fruit extract of *Citrus limon* from the two separate selectedlocations (Site I and Site II) was determined using the MTT assay (3- (4,5 dimethylthiazol -2- yl) 2,5 diphenyltetrazolium bromide) tetrazolium reduction assay. For five different concentrations, the percentage of cell viability against the Raw 264.7 macrophage cell line was recorded (Fig. 1& 2).

Cell viability of treated cellswere found to be 86.36 % at 500 g/ml, 90.48 % at 250 g/ml, and 92.99 % at 100 g/ml , 50 g/ml yields 95.71 % cell viability of Raw 264.7 cell lines cultures, while 25 g/ml yields 99.43 % cell viability when exposed to the ethanol extract of *Citrus limon* fruits from site II (Fig A: Plate 1). Cell viability was 83.84 % when using an ethanol extract of *Citrus limon* fruits from site II at 500 g/ml. 87.93% cell viability is achieved at 250 g/ml, 90% at 100 g/ml, 97.10 at 50 g/ml, and 97.90 at 25 g/ml when exposed to the ethanol extract of *Citrus limon* fruits from site I(Plate 1).

Evaluation of anticancer activity of ethanol extracts of *Citrus limon* fruits extracts from plants of from sites I and II against MCF 7 cells exhibited good inhibition of cell viability with increase in concentration of fruit extract.The fruit extract from plants of site I exhibited only 18.43 % cell viability of cancer cells at 500 g/ml, 30.82 % at 250 g/ml, and 43.81 % at 100 g/ml. Cancer cell ell viability was 57.70% at 50 g/ml and 65.56% at 25 g/ml(Fig C: Plate 1).It was observed that cancer cell viability increased as the concentration of extract decreased. At 500 g/ml, of the fruit extract from plants of site II, cancer cell viability was only 27.79 %, 44.41 % at 250 g/ml and 66.77 % at 100g/ml. 50g/ml of fruits extract showed around 82.18 % vitality of MCF-7 cells, while 25g/ml of fruits extract showed 91.84 % cancer cell viability (Fig3& 4: Plate 2).

5.2 Docking Studies

The compoundsIndole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl- (CD1) and Urs-12-en-3-ol, acetate, (3.beta.)- (CD2) from the selected plant was found to possess good anti-cancer activity from the results shown in the present investigation. In the *in silico* studies both the compounds showed good binding affinity with the target molecule than the control drug, 5-flurouracil (Table 1).The residues ILE117 (4.18 Å and 4.31 Å) and GLU115 (1.9 Å) for CD1; ARG29 (2.16 Å), ARG123 (5.31 Å), LEU287 (5.17 Å), HIS65 (5.48 Å and 4.30 Å), PHE127 (4.84 Å) and PHE284 (5.0 Å) showed interaction with the molecule CD2 (Fig. . The key interactions contributing to good binding affinity includes hydrogen bonding interactions, alkyl and pi-orbitals interactions.

Table 1: Docking results of the selected phytochemicals with CDK4

Ligand	Binding Energy	Ligand Efficiency	Inhibition Constant	Electrostatic Energy	RefRMS
5-Fluorouracil	**-2.88**	**-0.32**	**7.79 mM**	**-0.2**	**72.68**
Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl- (CD1)	**-3.51**	**-0.23**	**2.66 mM**	**-0.05**	**72.62**
Urs-12-en-3-ol, acetate, (3.beta.)- (CD-2)	**-5.59**	**-0.16**	**5.53**	**-0.19**	**60.24**

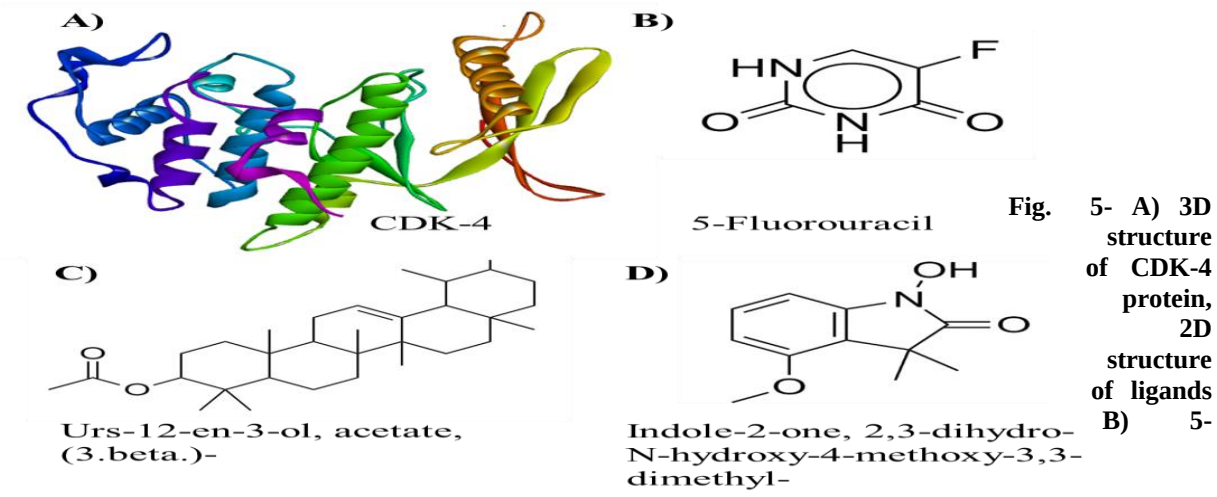

Fig. 5- A) 3D structure of CDK-4 protein, 2D structure of ligands B) 5-Fluorouracil, C) Urs-12-en-3-ol, acetate, (3.beta.) (CD-2), D) Indole-2-one, 2, 3-dihydro-N-hydroxy-4-methoxy-3, 3-dimethyl- (CD-1)

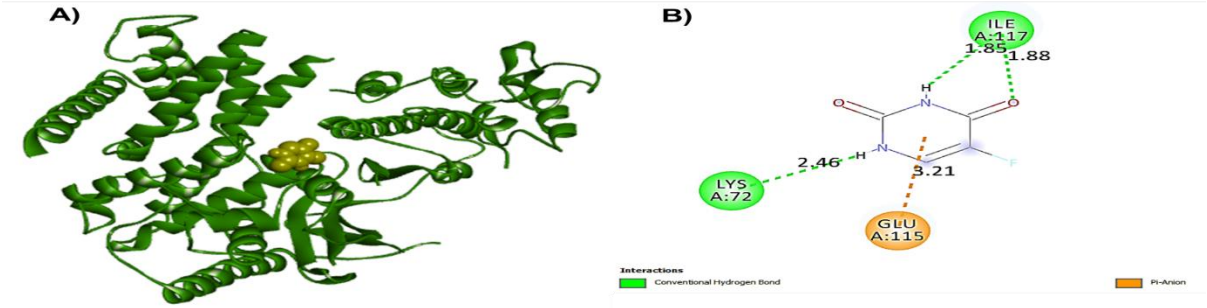

Fig. 6- Interaction of 5-fluorouracil (control) with CDK4. A) 5-flurouracil and CDK4 complex. The protein is represented in pink and green represents the ligand. B) 2D plot of the complex generated by DS visualizer.

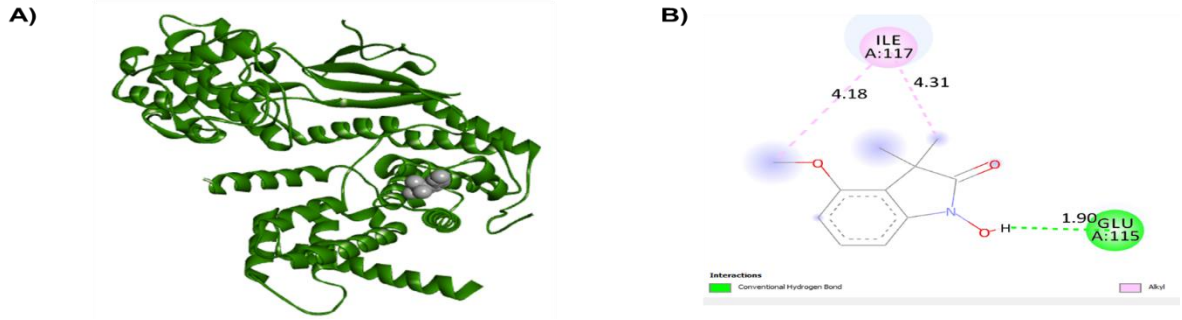

Fig. 7- Interaction of CD1 with CDK4. A) CD1-CDK4 complex. The protein is represented in pink and green represents the ligand. B) 2D plot of the complex generated by DS visualizer.

Both the compounds CD1 and CD2 showed good binding affinity with the target CDK4. Lesser the binding energy better is the interaction of the protein and ligand. From Table 1it is clear that the compound showed good binding affinity than the control, when considering the binding energy. Docked result of the complex displayed in Fig. 5indicates that CD1 form a conventional hydrogen bonds with the residue GLU115, possessing interatomic distance in the preferred range. CD1 also shares two Alkyl hydrophobic bonds with ILE-117. CD2 possess aleast binding energy of -5.59.ARG29 share hydrogen bonds with the oxygen atoms of CD2. Moreover, this shared Alkyl and pi-Alkyl interactions with the compound. The residues ARG123 and LEU287 formed alkyl interactions with CD2. The interaction of the aromatic ring with the positively charged arginine (ARG123) and histidine (HIS65) residues and LEU287, which usually occurs in case of aromatic ligands, is observed.

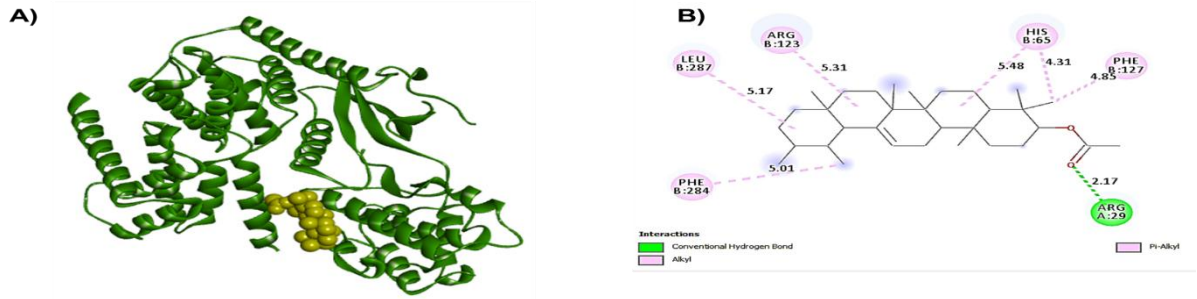

Fig. 8- Interaction of CD2 with CDK4. A) CD2-CDK4 complex. The protein is represented in pink and green represents the ligand. B) 2D plot of the complex generated by DS visualizer. Black labelled residues

3. Discussion

6.1 Anticancer Activity

Most of the cancer medicines are joined by a level of natural enhancements. There is profitable impact of therapeutic plants on malignancy. A few treatments incorporate natural solutions for improve the quality of life. Plant determined regular compounds like flavonoids, terpenes, alkaloids and have gotten wide consideration as of last few years because of their significant pharmacological properties including cytotoxic and malignant growth chemo preventive impacts [28]. The search for hostile to disease specialists from plant sources began during the 1950s with the revelation and advancement of the vinca alkaloids like leurocristine and vincaleukoblastine, and the isolation of the cytotoxic naphtho, dioxol-6(5aH)-one. Natural compounds found

from restorative plants play had a crucial impact in the administration of disease [8]. Plant based drug plays unquestionably discovered a part in disease recuperating (chemotherapy), and the component of association between numerous phytochemicals and malignant growth cells has been concentrated broadly. Specifically, there is developing interest in the pharmacological assessment of different plants utilized in Indian custom arrangement of medication. There are in excess of 2, 70,000 higher plants existing on this planet. However, just a little bit has been studied phyto synthetically. Thus, it is expected that plants can give possible bioactive mixtures to the improvement of new prompts battle malignancy infections [22]. The phytochemical screening of lemon showed the presence of substance mixtures like alkaloids, flavonoids, glycosides, phenolics, steroids, tannins and terpenoids [19]. Therapeutic plants might contain different sorts of substance segments and their organic exercises are not by and large owing to a solitary moiety.The present investigation was undertake to evaluate the cytotoxic action of fruit ethanol extricate from *C.limon* plants growing at two selected sites in Tamil Nadu. The cytotoxicity of ethanolic concentrate of *C. limon* organic product was evaluated against Raw 264.7 and MCF-7 cell lines.

Citrus fruits' beneficial benefits can be due to their Polyphenolic compounds consist of various classes of bioactive compounds including flavonoids, limonoids, coumarins, phenolic acids, terpenoids, tannins, stilbenes, lignans, and carotenoids[1].The anticancer properties of these fruits are contributed by its high levels of monoterpenes and flavanones, two classes of chemicals that are thought to reduce the risk of digestive system cancer about half. These compounds lower the chance of cancer in two ways, either by inhibiting inflammation, which is an essential stimulant for cancer cell growth, and by interfering with several of the processes required for cancer cell growth, making tumour development more difficult. Citrus has numerous chemicals that are uncommon in other plants, its individual components have shown anti-cancer efficacy against a variety of cell lines.

In the present investigation cell viability of RAW cell lines grown in medium containing ethanolic fruit extract of *C. limon*plants grown at two separate locations (Site I and Site II) was determined showed a concentration dependent activity of cell viability, in which maximum cell viability was observed at 500 g/ml of ethanol extract of *C. limon* fruits from site I, which came to be 86.36 % and it was 87.93% at the same concentration of the extract from plants grown in site II. The MCF 7 cell cultures exhibited inhibition in proliferation when grown in medium containing the ethanolic fruit extract from plants of site I and site II. The fruits extract from plants of site I and site II demonstrated only 18.43 % and 27.79 %. cell viability at 500 g/ml anda maximum of 65.56% and 91.84 % at 25 g/ml of the extract respectively. It was observed that the MCF-7 cell lineviability increased as the concentration of extract decreased. Based on the concentration the cytotoxic effect of each extracts varied this may be due to the bioactive mixtures and variety of phytochemicals like phenolics, flavonoids present in the cells which could prompt the cytotoxic action of this restorative plant. [19]. Also, the capacity of flavonoids and phenolic mixtures to fill in as anticancer specialists. The ethanolic fruit extracts from different locations have varying levels of action on MCF -7 cell lines [22]. This activity could be due to a combination of environmental factors and extract chemicals, or it could be a tissue-specific response. The samples' contribution to MCF -7 proliferation is measured in percent cell viability. The ethanol fruit extract of *C. limon*from site I generated an IC_{50} value of 218.72 g/ml, while the ethanol fruit extract from plants of site II yielded an IC_{50} value of 65.08 g/ml. The anticancer activity shown by the fruit extract is due to the phytochemical components present in the fruit extract, including flavonoids and terpenoids, are responsible for the possible cytotoxic effect against breast cancer cell lines. Earlier research on the phytochemical contents of *C. limon* fruits confirmed the presence of flavonoids and terpenoids, which is supported by the current study.

An investigation nanovesiclesfrom *C.limon*hindered malignant cellexpansion in various tumor cell lines. Besides, *C. limon*nanovesicles smother constant myeloid leukemia (CML) tumor development *in vivo* by explicitly arriving at the tumor site by initiating TRAIL-interceded apoptotic cell measures [25]. The aglycones and glycosides of the limonoids and flavonoids present in the *Citrus*concentrate may possibly fill in as a chemopreventive specialist for breast cancer [2].

The ethanolic fruit extracts from different locations have varying levels of action on MCF -7 cell lines. This activity could be due to a combination of environmental factors and extract chemicals, or it could be a tissue-specific response. The samples' contribution to MCF -7 proliferation is measured in percent cell viability. The ethanol fruit extract of *C. limon* from site I generated an IC_{50} value of 218.72 g/ml, while the ethanol fruit extract from site II yielded an IC_{50} value of 65.08 g/ml. The ethanol fruit extract of *C. limon* from site II was found to have strong activity against MCF -7 (Human Breast Adeno Carcinoma) cell lines, as well as proliferation. The majority of chemotherapy drugs is not only cytotoxic to the cancer cells but also is toxic to healthy cells and has immune suppressive side effects. Therefore, the discovery of novel compounds that possess not only cytotoxic activity against cancer cells but also non-toxic to healthy cells and modulating the immune response has become an important goal of research in the biomedical sciences [18]. The present study showed that, all the tested extractsclearly decreased the cell viability in a concentration-dependent manner in both MCF-7 cells and Raw 264.7 cell lines. The anticancer activity of citrus peels was reported either in the form of single molecules as or as a mixture of molecules [32]. For example, the essential oils of lemon and

grapefruit peel exhibited moderated to weak cytotoxicity toward human prostate (PC-3), lung (A549) and breast (MCF-7) tumor cell lines [35]. Moreover, ethanolic extract from orange peel and its fractions exhibited a weak to moderate cytotoxic activity toward HL-60 cells [24]. Our results suggest that higher cytotoxicity of the ethanol extracts may be due to the presence of limonoid. To relate with these results, the phytochemical components present in the fruit extract, including as flavonoids and terpenoids, are responsible for the possible cytotoxic effect against breast cancer cell lines. Earlier research on the phytochemical contents of *C. limon* fruits confirmed the presence of flavonoids and terpenoids, which is supported by the current study.

Increased mortality associated with cancer, along with high treatment cost calls for continuous research into possible anticancer drugs that are more suitable for treating cancer with reduced side effects. Secondary metabolites such as phenols, flavonoids, alkaloids and tannins have been shown to possess anticancer properties [13]. The anticancer properties of the ethanolic extract of lemon against human cancer cell lines MCF7 (breast) and Raw 264.7 were investigated. The cytotoxic effects shown by the extracts on the cell lines were observed to be in concentration dependent pattern [14]. In addition, consistent with the findings of this study, who observed that citrus peel extracts caused inhibition of PC3 xenograft tumor growth in immune-deficient mice even at lower dose. The IC50 values of the petroleum ether and ethyl acetate rind extracts in prostate and breast cancer cells, respectively, when compared to the standard curcumin, were however not significant.

The observed cytotoxicity, yet little selectivity to breast cancer cells due to significant cytotoxicity to normal cells could be as a result of trace amounts of organic solvents in the extracts. Though useful in increasing solubility, some organic solvents may have some cytotoxic interference in cellular based assays. Furthermore, lesser selectivity to MCF-7 compared to Raw 264.7 cells could be attributed to a generally lower cytotoxicity to the breast cancer cells [3]. This could be as a result of the fact that MCF-7 is a hormone-dependent cell line while Raw 264.7 is not. Certain phytohormones have been shown to promote growth of hormone-dependent cancer cells. The phytoestrogen Genistein stimulated the growth of estrogen-dependent MCF-7 cells at low doses in vitro with no such effect on hormone-independent breast cancer cells MDA-MB231[15]. Naringenin, which is found in some citrus fruits and is a precursor 79 to genistein, to be capable of exhibiting some protumor activity [11]. However naringenin is able to prevent growth of breast cancer cells by inhibiting the secretion of Transforming Growth Factor TGF-β1, which is a protein responsible for cell growth and proliferation [34]. Overall, this study has shown that a number of *C. limon* components possess anticancer activity.

In order to understand the anti-cancer property of the phytochemicals from *Citrus*, molecular docking procedure was done with the chosen ligands and the cyclin dependent kinase, CDK4. CDK4 is an important CDK cell cycle regulator that controls the progression of G1 to S phase through complex formation with cyclin-D at the G1 phase [12]. Several cancer types including non-small cell lung cancer, colorectal cancer, melanomas and breast cancers possess low levels of endogenous CDK inhibitors, as well as high levels of CDK4 [27]. Additionally, CDK4 related cyclin “cyclin D1” is generally up-regulated in numerous cancer types such as breast, lung, bladder and gastro intestinal tract cancers [30]. Hence, CDK4 inhibition is identified as a probable strategy for targeted treatment of numerous cancers through cell cycle arrest within the G1 phase [23].

Both the compounds showed good interactions with the target like the control drug. Each shares a single hydrogen bond with interatomic distance in the preferred range, strengthening their interaction with the target. Moreover, they possess weak interactions like alkyl hydrophobic interactions like the control drug, which plays a major role in determining protein structure and function. The aromatic structure of CD2 was found to form pi-alkyl interactions with certain residues of the target. Several analyses based on X-ray crystallography and computational studies reported the pi-cation interaction in protein-ligand complexes [5].

4. Summary and Conclusion

In this post-genomic era, research increasingly focuses on proteomics. Experimental and computational efforts are dedicated to large-scale generation and analysis of information derived from 3D structures of proteins, with the goal of scientific and commercial breakthrough in drug discovery.Cancer is a multifactorial disease where the major causative factors include specific genetic background, prolonged exposure to different environmental stresses and improper diet. All these risk factors will develop molecular changes or mutations ofkey proteins which leads to the initiation of cancer. CDK4, a cyclin dependent kinase is found to over-express in most of the cancers. Hence there is a need for suitable inhibitors targeting the Cyclin D1/ CDK4-mediated pathway. As citrus extract, showed good anticancer activity, we searched for the anti-cancer potential of the selected two phytochemicals.

From the docking results, it isquite clear that the compounds showed least binding energy with the target than the control drug. The type of interaction exhibited and the residues with which it interacted conveyed that they have good binding affinity with the target. Hence compounds CD1 and CD2 from *Citrus*can be considered as a source for the isolation, identification, and development of effective anticancer agents. However the results need experimental confirmation, which will be conducted in near future via molecular biology studies considering the structural features that may possibly make a choice to act as anti-cancer lead molecules in future.

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