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Screening of Novel Lead Compound from *Carissa Carandas* against Breast Cancer using *In-Vitro* and *In-Silico* Methods

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Abstract
Breast cancer is one of the perilous cancers and it is the second death causing cancer that occurs mostly in women. This work aimed at determining the active compound from *Carissa carandas* fruit, which proved to control the growth of cancer cells and this fruit is abundantly found in Tamilnadu, India. The ethanolic fruit extract was subjected to phytochemical and GC - MS analysis. Free radical scavenging and anticancer activity of fruit was analysed by DPPH (1, 1-Diphenyl-2- picrylhydrazyl) and MTT (3- [4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assays respectively. The IC50 concentration of the ethanolic extract was 86.7308 (µg/ml) against MCF-7 cell lines. Drug likeliness was analysed based on Lipinski's rule of five, where 11 out of 24 compounds were selected as ligands. IDC (Invasive Ductal carcinoma) breast cancer protein- Aromatase was selected as target which is an estrogen synthesizing enzyme. It was docked with ligands and the efficient lead molecule against this target was selected based on lowest binding energy value which was found to be 2-Furancarboxaldehyde (-114.56 Kcal/mol). This showed that unripe fruits of *Carissa carandas* could be exploited to get promising lead molecules against IDC breast cancer.

Keywords
Anticancer, Antioxidant, Aromatase, Breast cancer, *Carissa carandas*

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INTRODUCTION:
Breast cancer is the most common type of cancer, diagnosed in women and it is a disease in which malignant (cancer) cells form in the tissues of the breast [1]. There are several types of breast cancer. IDC(Invasive Ductal Carcinoma) is the most common type of breast cancer and it begins in the milk ducts of the breast and penetrates the wall of the duct, invading the fatty tissue of the breast and possibly other regions of the body [2,3].Estrogen is produced locally from circulating inactive steroids and plays major roles in the proliferation and development of breast cancer (hormone-dependent) in postmenopausal women and aromatase promotes the production of estrogen [4, 5, 6]. The level of expression of the Estrogen receptor in invasive ductal carcinoma is more. It is concluded, IDC is an estrogen positive receptor carcinoma [7], so Aromatase inhibitors appear to be more effective in postmenopausal women than in premenopausal women due to the fact that the major source of

estrogen biosynthesis in postmenopausal women is adipose tissue [8]

In this study, *Carissa carandas* unripe fruits were screened for antioxidant and anticancer properties. C.*carandas* is a tropical evergreen flowering shrub that belongs to the family of Apocyanaceae [9]. The plant is native to India and other Asian countries. [10]. The major bioactive constituents of this plant, are alkaloids, flavonoids, Saponins, cardiac glycosides, triterpenoids, phenolic compounds and tannins [11] and its fruits are frequently administered as astringent, and antiscorbutic [12] The leaves and unripe fruits of *C. carandas* is found to be a most potent antioxidant and anticancer activities[13,14,15] In the present study, iGEM Dock software was used for the docking process and it shows lead compound with lowest binding energy by ligand and target interactions[16]. With all aspects of above references, the present study is attempted to identify novel promising lead compound from *Carissa carandas* fruit against ER-positive breast cancer (IDC) causing protein aromatase (estrogen-synthesizing enzyme) by using an in-vitro and bioinformatics approaches.

MATERIALS AND METHODS:

COLLECTION OF *Carissa Carandas* FRUIT (UNRIPE FRUIT):

Unripe fruit of *Carissa carandas was* procured in the month of December to January 2018, 2019 from local market, Thiruvallur district, Tamil Nadu. The fruit was washed under running tap water and green fruits were cut into small pieces [17] and then shade dried for about two weeks. Dried fruit was grinded into fine powder [18].

ETHANOL EXTRACTION:

Powdered form of dried fruits was subjected to ethanol extraction. In that powder of dried fruits, 25 grams of powder was subjected to extraction using 250ml of ethanol (95%) as a solvent at 78◦C for 6-7 hours [12, 19, 20]. After the time that mentioned above, the crude extract was collected from round bottom flask and non-soluble macro particles is discarded, which is remains in the thimble. Extract obtained after soxhlet extraction was stored in airtight container in refrigerator at 4◦C for phytochemical and *in vitro* analysis [15, 17, 21].

PHYTOCHEMICAL ANALYSIS OF *Carissa carandas:*

Phytochemical analysis of ethanol extract of unripe fruits (Carissa carandas) was performed by following standard protocols to confirm the presence of phytochemical constituents such as alkaloids, carbohydrates, flavonoids, glycosides etc [11,17,22,23].

CHARACTERIZATION OF SECONDARY METABOLITES USING GC-MS ANALYSIS

Gas chromatography–mass spectrometry (GC-MS) is an analytical technique that combines the features of chromatography and mass spectrometry to identify different phytochemical compounds within a test sample.

GAS CHROMATOGRAPHY:

A Shimdzu GC-2010 Plus gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15m Alltech EC-5 column (250µ I.D., 0.25µ film thickness). A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35°C, hold for 2 minutes, then ramp at 20°C per minute to 450°C and hold for 5 minutes. The helium Carrier gas was set to 2 ml/minute flow rate (constant flow mode) and directly connected with capillary column metal quadrupole mass filter pre- rod mass spectrometer operating in electron ionization (EI) mode with software GCMS solution ver. 2.6 was used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 1000 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 1000 at 1 second per scan [24,25].

Identification of the components of the compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instruments software. GC/MS metabolomics Database was used for the similarity search with retention index [24,25].

EXTRACT ANTIOXIDANT ACTIVITY

Free radical scavenging ability by DPPH radical (1,1-diphenyl-2-picryl hydrazyl):

The effect of given samples on DPPH radical was estimated according to the standard procedure. Two mL of 6 $\times 10^{-5}$ M methanolic solution of DPPH were added from 10 to 50 µl (different concentration) of an ethanolic solution (10 mg ml^{-1}) of the sample. Absorbance measurements commenced immediately. The decrease of absorbance at 515 nm was continuously recorded in a spectrophotometer for 16 min at room temperature. Methanolic solutions of pure compound [quercetin] were tested at 1 mg/ml concentration [26]. The scavenging effect (decrease of absorbance at 515 nm) was plotted against the time andthe percentage of DPPH radical scavenging ability of the sample was calculated from

the absorbance value at the end of 16 m in duration as follows:

All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the following formula [27]

$IP = [(A_{C(0)} - A_{A(t)} / A_{C(0)})] \times 100$

Where $A_{C(0)}$ is the absorbance of the control at $t = 0$ min; and

$A_{A(t)}$ is the absorbance of the antioxidants at $t = 16$ min.

ANTICANCER ACTIVITY OF *Carissa carandas* FRUIT BY MTT ASSAY:

Preparation of cell suspension and cytotoxicity assay:

A subculture of MCF-7 cells in Dulbecco's Modified Eagle's Medium (DMEM) was trypsinized separately, after discarding the culture medium. To the disaggregated cells in the flask 25 mL of DMEM with 10% FCS was added. The cells suspended in the medium by gentle passage with the pipette and the cells homogenized .1 mL of the homogenized cell suspension was added to each well of a 24 well culture plate along with different concentration of samples (0 to 400 µg/mL) and incubated at 37°C in a humidified CO2 incubator with 5% CO2. After 48 hrs incubation the cells were observed under an inverted tissue culture microscope. With 80% confluence of cells cytotoxicity assay was carried out by MTT assay.The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide (MTT). After 48 h incubation the wells were added with MTT and left for 3 h in room temperature. All wells were removed the content using pipette and 100µl SDS in DMSO were added to dissolve the formazan crystals, absorbances were read in Read Well touch microplate reader at 570 nm [28,29].

IN-SILICO STUDIES:

Target selection involves identification of molecular protein targets that are involved in disease progression. Its structure was retrieved from PDB Database [16]. The compounds identified from GC-MS were retrieved from ZINC database and the 3D structures of the compounds were obtained and PUBCHEM provides information on the biological activities of small molecules and drug likeness properties of ligand compounds were predicted by Lipinski rules [30].

DOCKING:

iGEMDOCK is a suite of automated docking and screening tools. In the present study, the following procedure [16] were followed: The protein structure file was obtained from Protein Data Bank (http://www.rcsb.org/) and the ligand files were obtained from online compound database (ZINC) . In order to get least binding energy and other details, docked posed and post-analysis were carried out [31].

RESULTS AND DISCUSSION:

PHYTOCHEMICAL ANALYSIS OF *Carissa carandas* FRUIT:

The different phyto constituents have different degrees of solubility in different types of solvents depending on their polarity. By using the ethanol solvent, different classes of phytochemicals were been extracted. The presence of alkaloids, tannins, steroids, glycosides, saponins, phenols, flavonoids, and carbohydrates in *Carissa carandas* fruit was evidently confirmed by the preliminary phytochemical screening test. Various studies have shown that many plants are rich source of antioxidants. For instance, vitamins A, C, E, and phenolic compounds such as flavonoids, tannins, and lignins, found in plants, all act as antioxidants[32] and The leaves and unripe fruits of *C. carandas* is found to be a most potent antioxidant and anticancer activities[13,14,15].

GC-MS ANALYSIS OF *CARISSA CARANDAS* FRUIT EXTRACT:

The ethanolic extract of *Carissa carandas* fruit showed 24 secondary metabolites and eleven peaks in the chromatogram (Fig 1). The maximum peak was shown by Butanedioic acid (RT=6.293), Malic Acid (RT=5.811), 2-Furancarboxaldehyde (RT=5.538), Quinic acid (RT=12.192), Pentadecanoic acid (RT=18.690), cis - Vaccenic acid (RT=23.491).

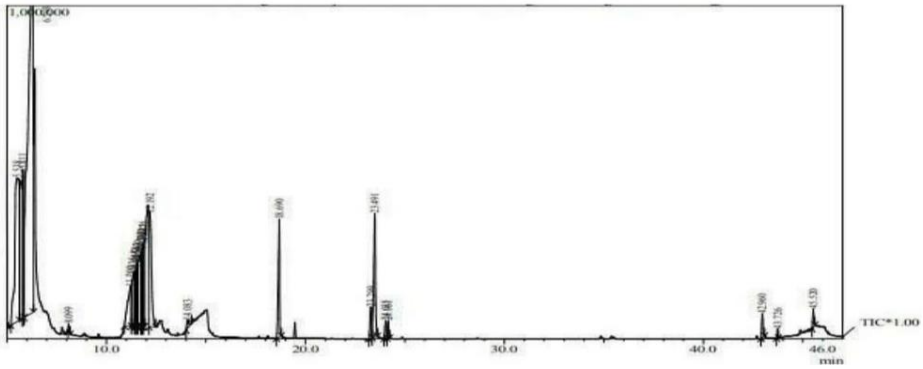

Figure 1: GC-MS Chromatogram of the ethanolic extract of *Carissa carandas*

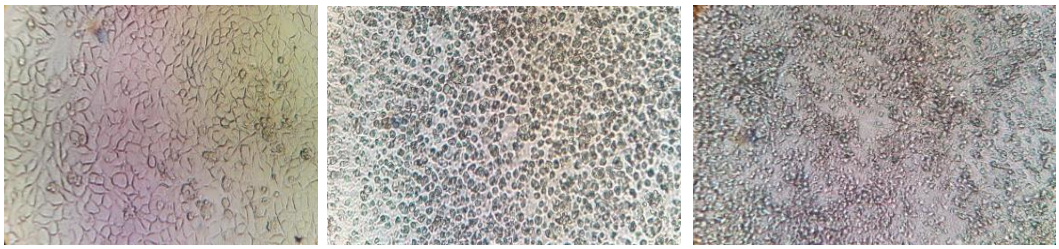

Figure 2- MTT Assay (with different Concentration control, 100 µg/ml, 200µg/ml)

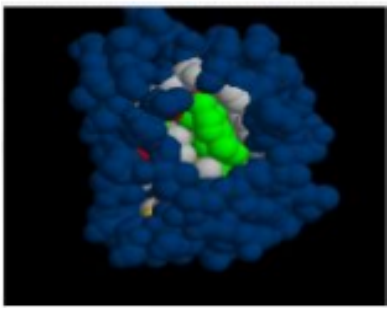

Figure 3– Docking pose of the 2-Furancarboxaldehyde with aromatase receptor.

ANTIOXIDANT ACTIVITY (DPPH ASSAY):

Free radical scavenging ability of the ethanolic extract was determined by a stable DPPH radical (1,1-diphenyl-2-picryl hydrazyl).Samples were diluted to produce 10, 30,50 µg/ml and OD were obtained as 0.453,0.552, 0.563 and inhibition activity shown as 72.60%, 88.46%, 90.22% (Table 1). As concentration increases, percentage of antioxidant activity also found to increase (Fig 2). Various studies show that *C. carandas* is found to be a most potent antioxidant as it exhibited exceptional reducing power, scavenging activity against Nitric oxide, DPPH and peroxide radicals [19,20].

Table 1: Free Radical Scavenging Activity of Ethanolic Extract By DPPH Assay

Concentration(µl)	Optical Density	Inhibition (%)
0	0.624	0.00
10	0.171	72.60
20	0.111	82.21
30	0.072	88.41
40	0.068	89.10
50	0.061	90.22

Table 2: Anticancer Effect of Ethanolic Extract on MCF 7 Cell Line

Sample Concentrations (µg/ml)	MCF-7 Cell Viability (%)
0	100.00
1.5625	93.46
3.125	87.88
6.25	84.65
12.5	78.71
25	62.36
50	45.61
100	27.83
200	20.02

Table 3: Compounds That Followed Lipinski Rule of Five

S.NO	Compounds Obtained from GC - MS	LIPINSKI PARAMETERS			
		MOLECULAR MASS (Da)	H BOND DONOR	H BOND ACCEPTOR	LOG P
1.	2-Furancarboxaldehyde	112	1	2	1.2
2.	Malic Acid	120	3	1	3.8
3.	2-Pyrrolidinone	115	2	2	1.0
4.	Citronellol epoxide	172	1	2	1.7
5.	Decanal	156	0	1	3.6
6.	Cyclohexanol	171	2	3	0.8
7.	1,2,3,5-Cyclohexanetetrol	148	4	4	1.5
8.	Decanoic acid	172	1	2	4.1
9.	Quinic acid	192	4	6	2.4
10.	semicarbazone	123	2	2	0.2
11.	Trifluoroacetyl-lavandulol	250	0	5	4.7

ANTICANCER EFFECT OF SAMPLE ON MCF 7 CELL LINE (MTT ASSAY):

The *in-vitro* cytotoxicity activity studies showed that MCF-7 cell lines were less prone to cytotoxicity when tested with test sample (Table 2). The cytotoxicity effect was observed in tested sample of different concentrations under 48 hours treatment, it also revealed that increase in concentration of drug showed increase in cytotoxicity over the MCF-7 cell lines (Fig 2). The IC_{50} concentration of the ethanolic extract was found to be 86.7308 (µg/ml) against MCF-7 cell lines.

***IN SILICO* STUDIES:**

Using Lipinski drug filter, the compounds obtained from GC-MS analysis of *Carissa carandas* fruit extract were analyzed and used for further studies. Out of twenty-four compounds from GC-MS analysis, eleven compounds followed the Lipinski's five rule [30] (Table 3).

MOLECULAR DOCKING RESULTS:

The interactions of estrogen binding receptor with the ligands are analyzed by the use of iGEMDOCK software. The lowest binding energy of -114Kcal/mol given by 2- furancarboxaldehyde (Fig 3). Within other studies [33] of iGEMdocking, value that obtained for 2-furancarboxaldehyde is considered as good lead compound, so it could be efficiently used as lead compound against Breast cancer. Thus, the docking studies of the plant showed that compounds of *Carissa carandas* fruit had the high specificity and efficiency towards the target protein (Aromatase). Result of the present study ensures that, the lead molecule may act as aromatase inhibitors in hormone dependent breast cancer (IDC) because of least binding energy value.

CONCLUSION:

In conclusion, the result obtained in the study demonstrates that *Carissa carandas* fruit can be used as good source of antioxidant and anticancer agent. *In-silico* techniques strongly support and help to identify the novel and potent inhibitors through mechanism of Ligand-Receptor interaction. From the docking of active compounds, 2-furancarboxaldehyde has shown strong interaction with breast cancer protein Aromatase with the binding energy of -144 Kcal/mol. Moreover, this lead compound also satisfies Lipinski rule of five. So, it could be efficiently utilized as promising novel lead compound against Invasive Ductal Carcinoma breast

cancer. In future research, the work can be further developed, and clinical trials can be done to test its effectiveness and for social benefit. Thus, the isolation and modification of novel product from *Carissa carandas* fruit as well as their analogs and subsequent evaluation of their bioactive properties will leads to the discovery of novel promising drug from fruit of *Carissa carandas*.

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