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# Evaluation of efficacy of *Nelumbium Speciosum* willd. seed extract on Anti-breast cancer activity in Cell line model

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***Abstract***

*The aim of the study is to evaluate the effect of Nelumbium Speciosum willd. seed extract (NSSE) in the treatment of breast cancer using MCF-7 cell lines. The phytochemical profile of the hydro-alcoholic extract (90% ethanol and 10% water) of NSSE revealed the presence of major secondary phytoconstituents. The extract was tested for its anticancer activity on the MCF-7 cell line using an MTT assay. The MTT assay showed that at a concentration of 100µg/ml, significant anticancer activity had been produced with an $IC_{50}$ value of 25.73µg/ml, compared to Standard 5 Fluorouracil with an $IC_{50}$ value of 3.17µg/ml, respectively. In the LCMS analysis, major bioactive constituents for breast cancer were identified. In silico docking studies were performed for two major compounds, namely formononetin, myricetin against two receptors, namely estrogen receptor α and estrogen receptor β receptors. Among those two compounds, formononetin showed the strongest binding energy against all two receptors. From this, formononetin displayed the highest binding energy of -8.3kcal/mol on ER α, -7.2kcal/mol on ER β, while myricetin displayed the highest binding affinity of -7.3kcal/mol on ERα and -7.9kcal/mol on ERβ receptors. Our studies contribute to the potential of natural medicine as a viable and effective alternative. Hence, natural remedies hold great promise in the realms of healthcare and cancer therapy.*

***Keywords:*** *Nelumbium Speciosum* willd. wild seed extract; MCF-7 cell line; MTT assay;Hormonal receptors.

## 1 Introduction

Cancer is a global disease that causes abnormal cells to develop uncontrollably, leading to malignant cells spreading to foreign parts of the body(Chanda et al., 2013). Breast cancer is a prevalent type of cancer, with an estimated 300,590 new cases in the United States in 2023(Singh et al., 2020). Treatments include surgery, radiation, chemotherapy, and other methods, but they often result in adverse effects(Chanda et al., 2013). Investigating plant secondary metabolites can help identify potent compounds as novel anticancer agents with minimal side effects. Lotus seed, a significant economic crop in Asia, contains carbohydrates, proteins, vitamins, and minerals(Yuliani et al.,2020;Yap et al.,2021). Secondary metabolites, such as alkaloids and terpenoids, have anti-cancer, neuro-protective, anti-diabetic, anti-inflammatory, antioxidant, and immunomodulatory properties(Siegel et al.,2023). Lotus seed is also used in innovative food products, such as replacing wheat in bread recipes. This study investigates the cytotoxic potential of an hydro-alcoholic seed extract of *Nelumbium Speciosum willd.* on the MCF-7 human breast cancer cell line(Shahzad et al.,2020;Zeng et al.,2013)).

## 2 Materials and methods

### 2.1 Collection and identification of plant material

*Nelumbo speciosum* wild. seed were obtained from the pond situated at kattukuppam, Pondicherry in the month of January, 2024. The collected *Nelumbo nucifera* were identified and authenticated as plants by Dr. N Surendar , Botanist,Department of Botany, Periyar Arts College, Cuddalore, India.The specimen was deposited in the department with a voucher number 203/PAC/2024.

### 2.2 Preparation of plant material

The Seeds, viz., 500g of *Nelumbium Speciosum willd.,* were cleansed thoroughly with distilled water after washing them with the regular tap water to remove the impurities. Then, the fresh seeds were dried in air for about 2 weeks at normal room temperature in a shaded area without exposing them to direct sunlight.Then the dried leaves were finely powdered with the help of a grinding machine and the resulting coarse powder was collected and stored in an airtight container.

### 2.3 Extraction of *Nelumbium Speciosum willd.* seeds.

The extraction processes for the powdered material of *Nelumbium Speciosum willd.* seeds were carried out individually using Soxhlet apparatus by employing petroleum ether as a solvent. To defatt the substances present in the powdered material, this process is employed. Hydro-alcoholic extract was performed after petroleum ether extract. This process was carried out using ethanol and water in a proportion of 90:10 for 48 hours at a normal room temperature. To get rid of the complete solvent, an hydro-alcoholic extract of *Nelumbium Speciosum willd.* was transferred to the petri dish at room temperature and subjected to evaporation. The remaining crude extract was collected and stored separately after the solvent had been completely removed. 1 gram of an equal amount was pipetted out of the *Nelumbium Speciosum willd.* extract in equal parts (1:1) for additional investigation for phytochemical screening, *in vitro* cytotoxicity evaluation (MTT assay), LCMS, and molecular docking.

### 2.4 Phytochemical screening

The seed extract of the *Nelumbium Speciosum willd.* (NSSE) was exposed to chemical screening to determine the existence of major secondary phytoconstituents, whereas the qualitative determination of alkaloids, flavonoids, tannins, phenols, saponins, terpenoids, and other constituents was performed using the standard procedure as mentioned by(Parthasarathi et al.,2021;Pungot et al., 2020).

#### 2.4.1. MTT assay

The *in vitro* cytotoxic potential of an isolated extract of *Nelumbium Speciosum willd.* was identified with the help of *MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide* assay with certain alterations. In brief, MCF-7 cell lines were sowed in 96-well plates at a concentration of $1 \times 10^5$ cells/ml and kept for incubation up to 24hours at $37^0$C with 5% of $CO_2$. After removing the cell proliferation medium, the obtained NSSE extract was added at various doses (6.25, 12.5, 25, 50, and 100 µg/ml) together with the reference standard 5-fluorouracil (5-FU). The mixture was then kept under incubation at $37^0$C along with 5% of $CO_2$ for 24 hours. Once the incubation was completed, the samples were completely removed from each well, and then the cells were thoroughly washed with the help of phosphate-buffered saline (PBS, pH 7.4). Then, 20µl of 0.2% (w/v) of MTT solution was added to each well and again incubated at $37^0$C with 5% of $CO_2$ for 4h in the dark. After that, a dark purple formazan was formed after incubation. The purple formazan was dissolved in 100µl of *Dimethyl sulfoxide* (DMSO), and its absorbance value was measured at 570nm with the help of a microplate reader. The cells which are not treated were considered as controls, while the DMSO reagent assisted as a blank.

Then the following formula was used to calculate the cell viability(Kaur et al., 2019).

***% of viability = (Sample absorbance/ Control absorbance) × 100***

**2.4.2. Liquid chromatography - mass spectroscopy (LCMS)**

The NSSE extract was then exposed to LCMS (Shimadzu, LCMS 2050) analysis. The seed extract was subjected to chromatographic separation on a Phenomenex Phase C-18 column (100 × 1.0mm) using 0.1% formic acid in water as solvent A and methanol: acetonitrile (95:5) as solvent B. The chromatographic separation was operated at a column temperature of $40^0$C at a flow rate of 0.3ml/min. The overall runtime was found to be 35 minutes. All the analytes were eluted in a runtime period of 0.9 to 34.86 minutes. The acquisition method was said to be MS, with a minimum range of 70m/z and a maximum of 1000m/z with a scanning rate of each spectrum per second. ThermoXcalibur software was used for the identification of isolated constituents.

**2.4.3. Molecular docking**

***2.4.4. Preparation of ligand***

The LCMS compounds of NSSE were chosen as docking ligands. The molecular structure of those compounds was retrieved from the primary source of PubChem database.

***2.4.5. Preparation of protein***

The crystal protein structure of the two receptor proteins (ERα, ERβ) was derived from a protein data bank. The PDB codes for ERα, ERβ were 7UJO, 1QKM respectively. The protein shapes were washed and hetero atom was removed utilizing BIOVIA Discovery Studio (version 21.1).

***2.4.6. Molecular docking***

*In silico* docking studies were performed on the Pyrex software (version 0.8) to examine the binding kinetics of the target as well as compounds from NSSE properly. Protein structures have been transformed into molecules using the software. In addition, the ligand molecules were converted to PDBQT layout and the receptor grid has been prepared with default parameters and without any constrains(Nurkalbi et al.,2021; Mamta et al.,2018). Each ligand was analyzed for docking score and receptor binding affinity, respectively.

**3. Results**

**Phytochemical studies**

The phytochemical study of a 90% hydro- alcoholic extract of *Nelumbium Speciosum willd.* extract indicated the presence of various secondary metabolites, including alkaloids, flavonoids, phenols, tannins, cardiac glycosides, steroids, terpenoids, quinones, and proteins. In this extract, the existence of alkaloids, flavonoids, phenols, tannins and cardiac glycosides found to be in greater intensity, as shown in Table1.

Table1. Phytochemical Screening of Hydro-alcoholic extract *Nelumbium Speciosum willd.*

| Phyto-chemicals | Alkaloids | Flavonoids | Saponins | Tannins | Phenols | Cardiac Glycosides | Steroids | Terpenoids | Quinones | Proteins |
|---|---|---|---|---|---|---|---|---|---|---|
| Observation | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

++ Sign indicates strong color, + indicates weak color and - Sign indicates absence of phytochemical constituent

**MTT Assay on MCF-7 Breast Cancer cell line**

The cytotoxicity of the resulted seed extract of *Nelumbium Speciosum willd.* was determined using the MTT assay. The morphological modifications of MCF-7 cells were noted after 24 hours, which are represented in (Fig 1). Figure 2 showed that in a concentration dependent manner the viability of the MCF-7 cells get decreased.The $IC_{50}$ value for NSSE extract was 25.73µg/ml, while the reference standard 5- FU was 3.17µg/ml against MCF-7 cell lines. As a result, the isolated NSSE extract showed reduced cell viability in MCF-7 compared to untreated control group (Fig 2). The result also displayed that the extract of NSSE had strong anticancer activity against the selected MCF-7 breast cancer cell line (Vijayarathna et al., 2012; Akshaya et al.,2021)

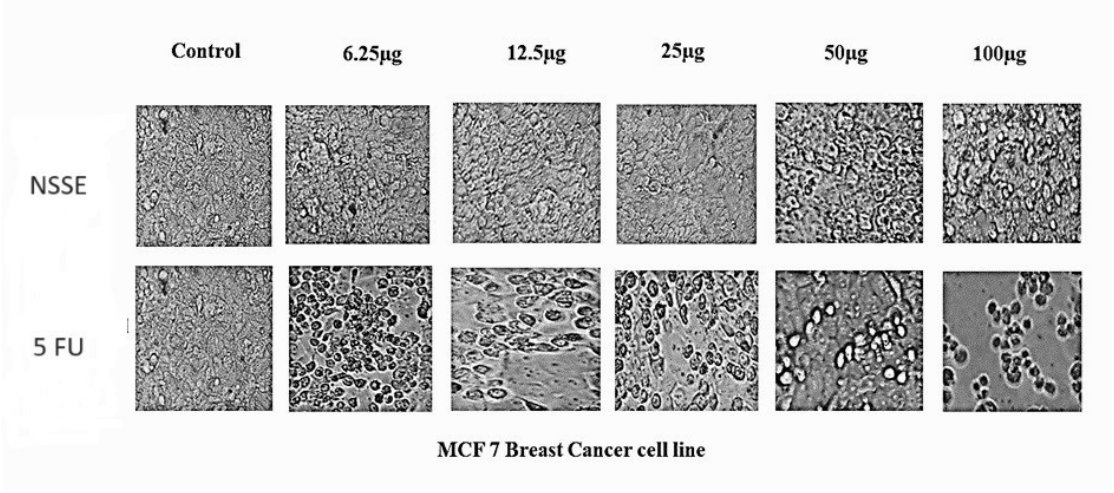


Fig 1. Represents the morphologies of human breast cancer cell line MCF-7 treated with STD (5-fluorouracil) and test (Hydroalcoholic extract of NSSE) for 24 hours.

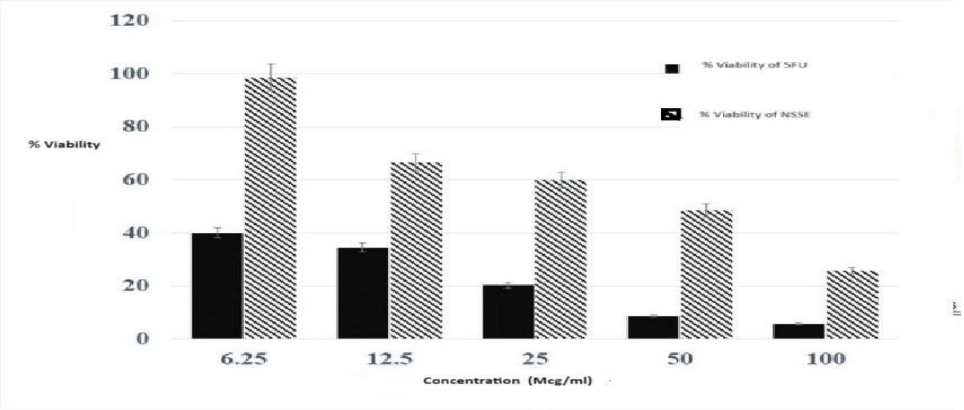


Fig 2 Graph of NSSE compared to Std. 5-FU

**LCMS analysis of *Nelumbium Speciosum* willd. seed extract**

The LCMS chromatogram of the prepared extract of *Nelumbium Speciosum willd.* was shown in (Fig 3) and the identified compound was shown in (Table2). The LCMS analysis reveals the retention time of the identified compounds. It was observed that the different compounds were obtained at different retention times.

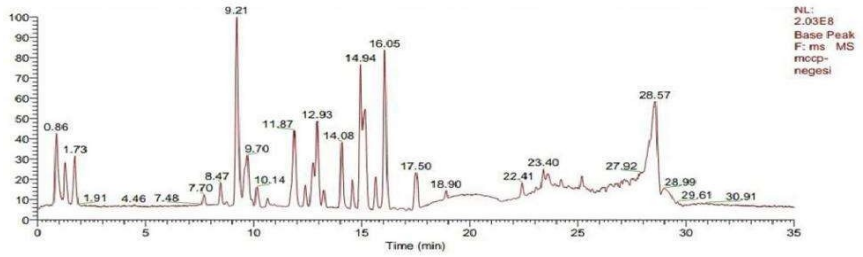


Fig 4. LCMS Chromatogram of *NSSE*

Table2. Secondary metabolites identified in Hydroalcoholic extract *Nelumbium Speciosum willd.* by LCMS analysis:

| S.No. | Identified compound | Retention time (min) | Molecular formula | Molecular weight |
|---|---|---|---|---|
| 1 | 5-hydroxy-6,7-dimethoxy-2-phenyl-4H-chromen-4-one | 18.325 | $C_{17}H_{14}O_5$ | 298.0829 |
| 2 | Chrysin | 14.93 | $C_{15}H_{10}O_4$ | 254.0567 |
| 3 | Formononetin | 17.685 | $C_{16}H_{12}O_4$ | 268.072 |

| | | | | |
|---|---|---|---|---|
| 4. | Apocynin. | 15.292 | $C_9H_{10}O_3$ | 166.0623 |
| 5 | 6-Hydroxy-4,4-dimethyl-7-nitro-2-chromanone. | 7.704 | $C_{11}H_{11}NO_5$ | 237.0628 |
| 6 | 1,3,5-Norcaratriene. | 0.956 | $C_7H_6$ | 90.04662 |
| 7. | (2R)-5-hydroxy-7-methoxy-2-phenyl-3,4-dihydro-2H-1-benzopyran-4-one. | 14.086 | $C_{16}H_{14}O_4$ | 270.0881 |
| 8 | Norhaman. | 8.395 | $C_{11}H_8N_2$ | 168.0682 |
| 9 | 7-hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one | 13.536 | $C_{16}H_{12}O_4$ | 268.0724 |
| 10 | 7-Oxo-7H-furo(3,2-g) chromen-4-yl decanoate. | 17.766 | $C_{21}H_{24}O_5$ | 356.1609 |
| 11 | Kaempferol | 11.915 | $C_{15}H_{10}O_6$ | 286.0465 |
| 12 | Myricetin | 9.187 | $C_{15}H_{10}O_8$ | 318.0366 |
| 13 | Naringenin chalcone | 12.942 | $C_{15}H_{12}O_5$ | 272.0673 |

**Docking studies of selected compounds**

The phytochemicals obtained from the NSSE were analysed by comparing their binding affinity with the selected receptors. Two ligand compounds were chosen namely myricetin, formononetin were docked against two receptors: Estrogen α, Estrogen β. The molecular structure of the two ligands were depicted in (Fig 4).

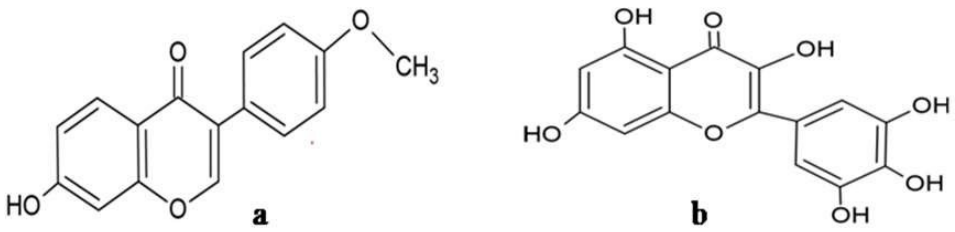


Fig 4. Represents the molecular structure of the two ligands (a) formononetin (b) myricetin chosen for the docking studies.

Upon docking the chosen phytochemicals with the Estrogen α receptors, formononetin demonstrated the highest binding energy of -8.3 kcal/mol among the two compounds as shown in (Table 3). Particularly, the compound formononetin had one hydrogen bonding with the amino acid residue (LYS B: 449), as shown in (Fig 5), while the compound myricetin showed an unfavourable donor-donor bond with the amino acid (ARG D: 394) (Fig 6), so it is considered to be less stable. So, as a result of the interaction between the phytochemicals and the ER α receptors, formononetin has been identified as a lead compound based on the binding energy and the interactions.

Table3: Docking of various bio-active components identified in Hydroalcoholic extract *Nelumbium Speciosum willd.* into the target enzymes namely ERα, ERβ to understand the binding affinity.

| S.NO | Chemical compound | Docking score (Kcal/mol.) | |
|---|---|---|---|
| | | ERα | ERβ |
| 1 | Myricetin | -8.1 | -8.1 |
| 2 | Formononetin | -8.3 | -8.3 |

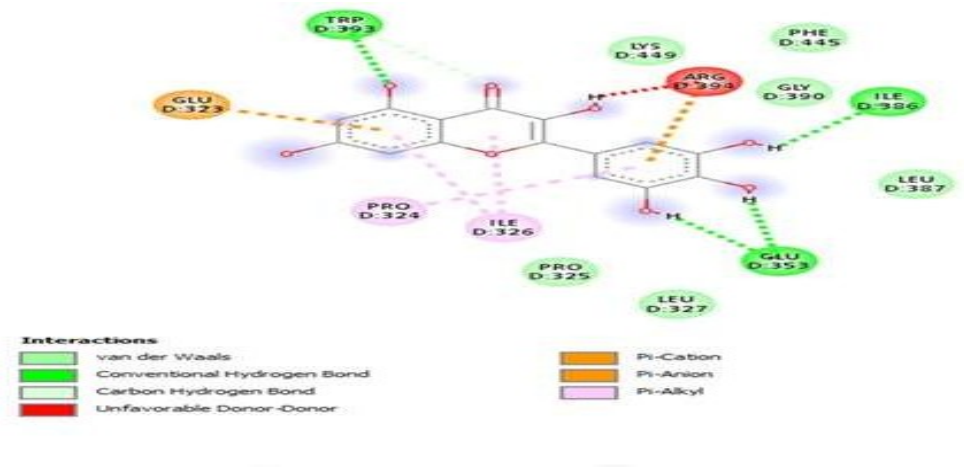


Fig 5. 2D Interaction of Formononetin with Estrogen α receptors.

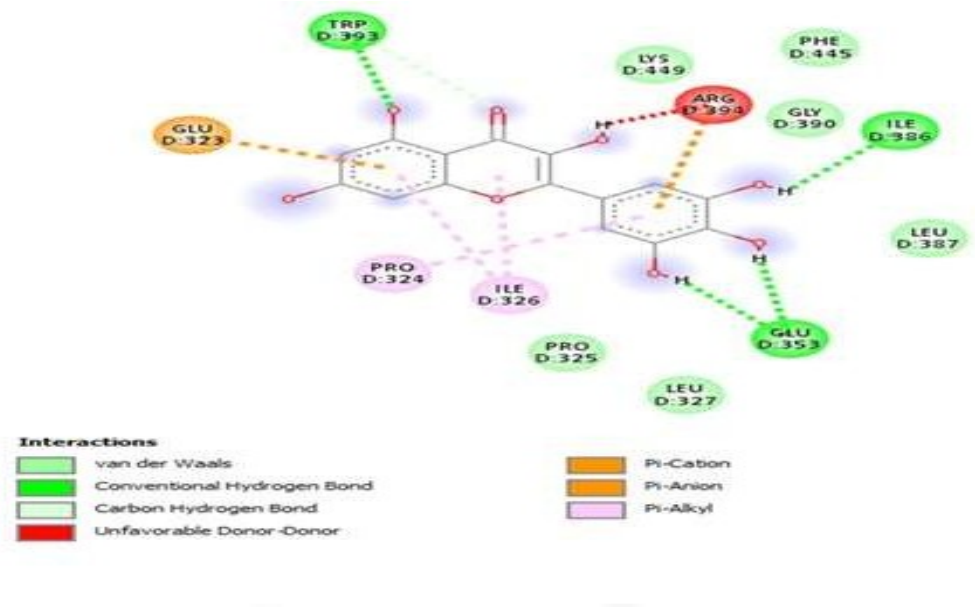


Fig 6. 2D Interaction of myricetin with estrogen α receptor showed unfavourable donor-donor bond with the amino acid residue (ARG D: 394), which indicates that the compound myricetin was less stable with ER α receptor.

From the results of interactions between the phytochemicals with estrogen β receptors, it was identified that the compound myricetin and formononetin have the highest binding energies of -8.1 kcal/mol. and -8.3 kcal/mol. as shown in (Table 3). Particularly, the compound myricetin exhibited three hydrogen bonding with the amino acid namely (VAL A: 280, GLU A: 305, HIS A: 279), whereas formononetin only had one hydrogen bond with the amino acid (LYS A: 401) as shown in (Fig 7). So, as a result of the interaction between phytochemicals and ER β, the compounds formononetin and myricetin are found to be lead compounds based on the binding score.

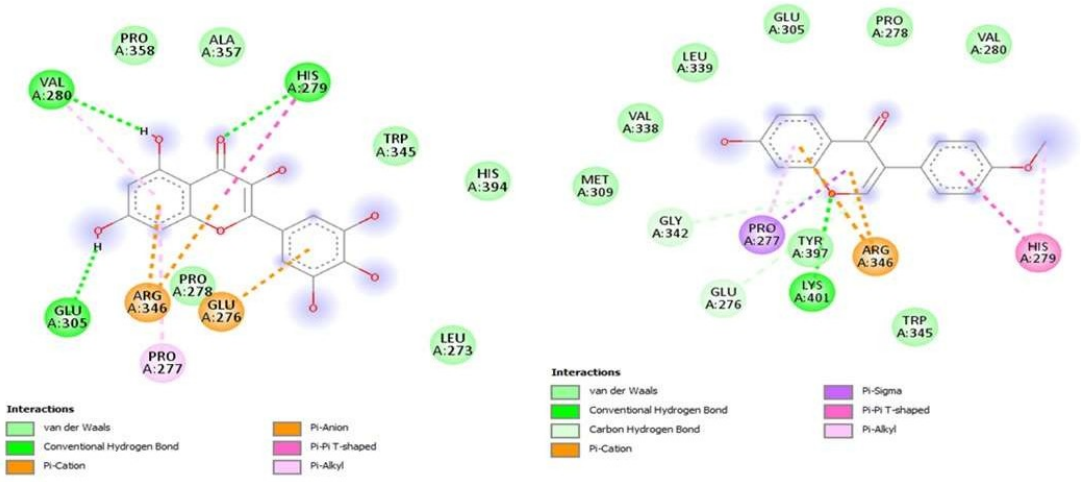


Fig 7 2D interaction of myricetin and formononetin with Estrogen β receptors.

As a final result of docking interactions with the three receptors, formononetin showed the highest binding energy on the ER α receptors; both formononetin and myricetin show a high binding affinity with ER β as predicted by the Pyrex software. From this result, formononetin and myricetin from the NSSE were identified as lead compounds, which can be used to breast cancer by targeting both estrogen receptors.

**4. Discussion**

This study investigates the cytotoxic effects of *Nelumbium Speciosum willd.* seed extracts on MCF-7 breast cancer cell lines. The plant is known for its ability to inhibit proliferation and cytotoxicity in various cancer cells. The cytotoxic activity of *Nelumbium Speciosum* willd. seeds was tested on several cell lines, with an $IC_{50}$ value of 19.25µg/ml. The study aims to understand breast cancer therapy using MCF-7 cells.The Soxhlet method was used to extract *Nelumbium Speciosum* willd. seed, which was found to contain a rich profile of bioactive compounds, including flavonoids, alkaloids, tannins, phenols, glycosides, terpenoids, steroids, quinones, and proteins. These compounds contribute to its anti-cancer properties. The cytotoxic activity of NSSE on MCF-7 cells was calculated using the MTT assay, which converts MTT into colored compounds. The cytotoxic profile was compared with the standard drug 5-FU, with the test showing an $IC_{50}$ value of 25.73µg/ml at 100µg/ml.

The phytochemicals, flavonoid, alkaloids, and phenols, effectively inhibit cell concentration, leading to reduced viable cells with increasing NSSE concentration. Secondary metabolites accumulate, causing stress and cell death, demonstrating NSSE's potent cytotoxic effect on MCF-7 cell lines (Alishah et al., 2017;*Marwa et al.,2020*)**.**

These findings collectively highlight the significance of the NSSE as a potential source of anti-breast cancer extract and warrant further exploration for its therapeutic applications in cancer treatment. After getting the promising results from the MTT assay, the extract has been processed for further studies like high resolution liquid chromatography mass spectroscopy. The LCMS study was performed using Thermal Xcalibur software and these studies have explored the existence of various bioactive components present within the NSSE. This study revealed 9 major compounds these components were mostly accountable for the anti-cancer activity. One recent studies depicted that phytochemical constituents like formononetin, genistein, daidzein, kaempferol, emodin, quercetin, isoliquiritigenin, resveratrol, epigallocatechin were identified as being more effective against various breast cancer cell lines by restricting the cell growth, and this restriction happened through various mechanisms such as inhibiting proliferation, inhibiting angiogenesis, inhibiting migration, increasing apoptosis, decreasing cell growth, etc.(Alishah et al., 2017). The LCMS report of our current study has shown the presence of these major phytochemicals which are shown in (Table2) and the presence of these major phytoconstituents indicate the promising nature of NSSE extract against the selected cancer cell line, MCF-7. Further, *In silico* docking studies have been performed with the help of three target proteins, namely ERα, ERβ receptors. ER have been chosen for this reason because almost 70% of human breast cancer patients were established to be as hormone receptor positive and their cell structure contains a positive expression of estrogen receptors. These receptors play a crucial role in the growth and spread of rapidly proliferating cancer cells. Primarily, estrogen and its receptors mainly involved in the development and rapid progression of breast cancer cells. So, these ER receptors are found to be valuable biomarkers for the drug to produce its action. From the LCMS data, mainly two major compounds have been isolated for molecular docking studies: formononetin and myricetin. The compounds formononetin and myricetin were chosen because, they belong to the flavonoid group and also because they may resemble the actions of quercetin, kaempferol and genistein belong to the same flavonoid group of compounds that have been previously reported for their anti-breast cancer activity (Ling et al.,2007;Alotaibi et al.,2022). So, two compounds have been docked against two receptors to understand the binding interaction of the compounds with the receptor of interest. The two major receptors we used in our docking study were estrogen α, estrogen β.

The molecular docking studies have revealed that among the two compounds, two specific compounds,

myricetin and formononetin, exhibit substantial binding energies with key receptors, namely estrogen receptors (ERα and ERβ). From this, myricetin displayed impressive binding energies values of -7.3Kcal/mol on ERβ , while formononetin exhibited similarly robust binding affinities with -8.3Kcal/mol on ERα, -7.2Kcal/mol on ERβ, docking. More binding affinity is indicated by greater negative values for glide energy and glide score. The hydrogen bond score displays van der Waals interactions and atomic coordinates (bonding type and shape).

According to (Singh et al., 2020), the stronger the hydrogen bonding and, thus, the larger the ligand's binding affinity with the protein receptor, the more negative the hydrogen bond scale. Both myricetin and formononetin have negative glide scores ranging from -7.2 to -8.3. Strong hydrogen bonding suggests a higher affinity for binding to and inactivating cancer protein active sites. Moreover, docked conformations of myricetin and formononetin showed hydrogen bond interactions. The active finder for formononetin revealed the active site order (Lys B: 449, Lys A: 401, MET A:759), and the same as for myricetin, the active finder showed the active site order ( Val A: 280, Glu A: 305, His A: 279, GLN A:725, GLU A:695, ILE A:699). Then the final results of molecular docking suggest that myricetin and formononetin may effectively interfere with the activity of estrogen receptors. The compound formononetin showed the highest binding energy on ER α, whereas both formononetin and myricetin showed the best binding energy on ER β receptors, which are often implicated in the progression of hormone-related cancers. This implies that these compounds could potentially serve as valuable candidates for further investigation and development as therapeutic agents for cancer treatment.

## 5. Conclusions

A study using in vitro MCF-7 cell line models reveals potential extracts in breast cancer therapy. In silico docking research and LCMS analyses were used to understand the plant extract's characteristics and action. The extract showed strong anticancer effects, similar to 5-fluorouracil. This study highlights the potential of natural medicine as a viable alternative to chemotherapy and conventional medicine, highlighting the importance of further research in healthcare and cancer therapy.

## Author contribution

All the authors are equally contributed in this study

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## Competing interests

The authors have no competing interest to declare that are relevant to the content of this article.

## Ethical clearance number

Not necessary and none declared .

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