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# Anticancer activity of Flavanone Isolated From *Citrus medica* and Its Combination Effect with A Synthetic Drug 2-Deoxy-D-Glucose

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**ABSTRACT**

Cancer research is an on-going field aimed at discovering novel treatments for various stages of the disease. Although chemo and hormonal therapy have been found to be effective in treating cancer, there are still challenges draw a parallel with them, such as therapeutic resistance and repetitiveness, which make the disease difficult to control. Therefore, it is imperative to explore alternative therapies that can provide better treatment outcomes. The present research work towards the potential use of phytochemicals, particularly flavonoids found in *Citrus medica* leaves, as a treatment for breast cancer. Using chromatographic techniques, flavanone, a compound found in citrus extract, was isolated and its structure was characterized using UV, FTIR, HPLC, NMR, and MS analyses, as well as comparisons with literature. The anticancer activity of flavanone was evaluated using a standard MTT test against commonly used breast cancer cell(MCF-7). Additionally, the present study investigated the combination effect of flavanone with a synthetic drug, 2-deoxy-D-glucose (2DG), on MCF-7 cells. The findings reveal that flavanone and the combined flavones with 2DG had $IC_{50}$ values of 57.10 and 34.09 µg/mL, respectively. This fusion study provides promising evidence that the combined effect of flavanone with a synthetic drug may enhance treatment effectiveness by improving drug transport and reducing the required dose. Additional study is required to confirm these results and investigate phytochemicals' potential as a replacement therapy for breast cancer.

**Keywords:** *Citrus medica*, Flavanone, 2-deoxy-D-glucose, MCF-7.

## INTRODUCTION

The use of plants as medicine dates back to ancient times, with traditional folk medicine employing a wide range of plants to treat both mild and severe ailments. More recently, modern clinical approaches have utilized large drugs either directly derived from plants or synthesized

copies and derivatives of plant-based compounds[1]. Even the treatment of cancer, a disease that has a significant impact on people all over the world, including children and adults, relies on drugs such as paclitaxel and camptothecin, which are obtained from plant products[2]. The National Cancer Institute has estimated that by 2040, approximately 30 million cancer-related deaths will be reported. In response to this growing public health concern, researchers are focusing on a range of areas, including genetics and molecular biology, precision medicine, and immunotherapy. However, the multidisciplinary nature of this field presents challenges in terms of early detection and screening, as well as the development of targeted therapies[3,4]. To address these challenges, new drugs and drug combinations are needed that can eliminate the recurrence of cancer and reduce side effects, while also investigating the potential benefits of using natural products as a major source[5].

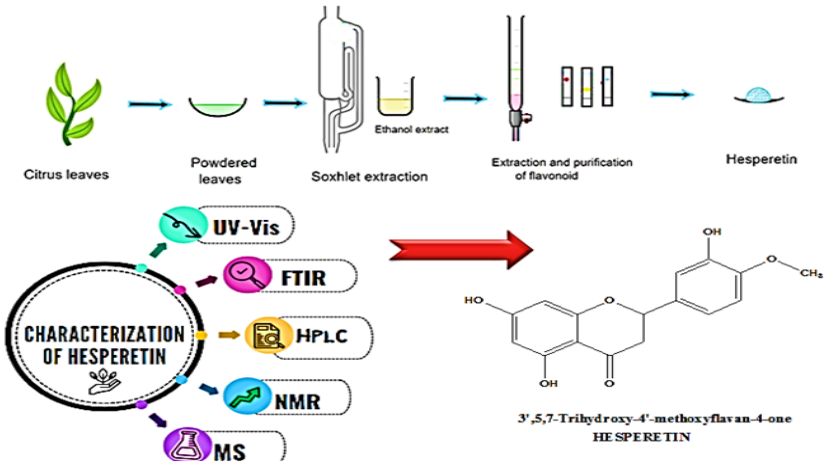


**Fig. 1. Schematic diagram of isolation and characterization process of hesperetin from *Citrus medica* leaves**

Plants have a long history of use as medicine, with traditional folk medicine employing a range of plants to treat various ailments. Approximately one-third of drugs used today are derived directly from plant sources, and these have become a foundation of the medical field. Examples of such drugs include aspirin and morphine, which have inspired the synthesis and manufacturing of compounds on a large scale. However, the uncertainty of other bioactive compounds and their safety has encouraged researchers to focus on the isolation and fractionation of compounds from various plants[6,7]. Given the increasing and alarming statistics of cancer cases, it is critical to have alternative and better drugs available to combat this disease.

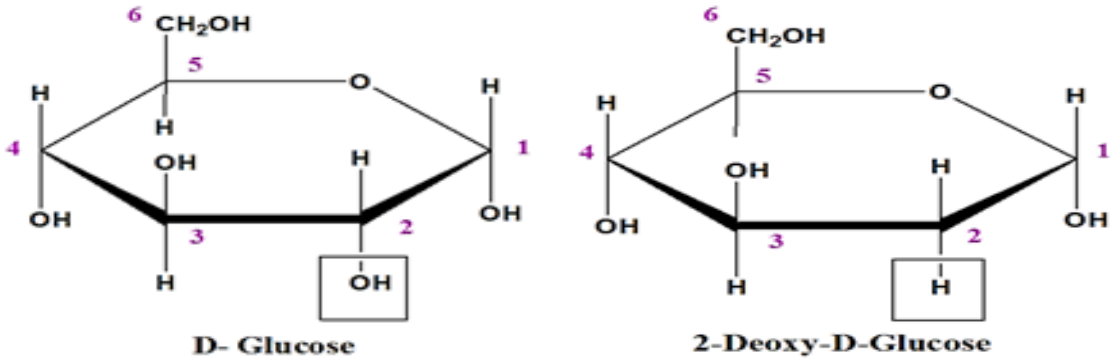


**Fig. 2. Structure of D-Glucose and 2-Deoxy-D-Glucose**

The combined use of synthetic and natural product-based medications has been shown to have potential advantages for improving the biological network. Furthermore, recent studies have thoroughly outlined the advantages of natural products, including their low toxicity and metabolite resemblance[8]. These benefits make the combination of natural and synthetic

drugs a viable area for novel drug discovery research. Many synthetic medications and natural products have been examined in the past for their synergistic effects, particularly in the treatment of pancreatic, colon and breast cancer[9]. In the present investigation, a flavonoid derived from plants was identified and its potential combination with a synthetic medication to 2-Deoxy-D glucose was investigated. 2-Deoxy-D-glucose (2DG) is a synthetic form of modified glucose with hydrogen replacing the hydroxyl group on a glucose molecule. It has been used in recent studies for its potential antiviral and anticancer properties due to its ability to inhibit glycolysis and prevent the growth of viruses and infected cells. By blocking the conversion of glucose into energy, cancer cells are deprived of the energy they need to survive, leading to cell death[10,11]. Additionally, 2DG has been found to have selective toxicity towards infected cells. Research into the use of 2DG as an effective cancer drug is ongoing, with recent studies focusing on its efficiency, safety, and other responses. In this study, the isolated flavonoid was fractionated using chromatographic techniques and validated through spectral studies. The study aimed to isolate bioactive compounds from *Citrus medica* leaves and test their efficacy on breast cancer cell lines, both individually and in combination with the synthetic drug 2DG.

## MATERIALS AND METHODS

The chemicals and reagents used for the testing were obtained from Sigma Aldrich. The plant samples were collected from Pudukkottai, Tamil Nadu, India (10$^{O}$ 22' 26" N Latitude; 78$^{O}$ 45' 40" E Longitude). The plant species were authenticated at the Rabinet Herbarium, St Joseph's College, Tiruchirappalli, India. The chemicals utilized for the anticancer activity were purchased from Gibo (USA) and Sigma Aldrich. The most commonly used cell line MCF-7 represent breast cancer was received from the National Centre for Cell Science, Pune.

### Preparation of CM extract

The fresh and healthy leaves of *Citrus medica* were thoroughly washed with running water. The leaf samples were then dried in the shade until no moisture remained. The leaf samples were ground into a fine powder. One hundred grams of the leaves were used for Soxhlet extraction with 800 mL of ethanol, which was run for 12 hours[12,13]. The extract was subjected to rotary evaporation to remove the excess solvent.

### Isolation of flavonoid from CM extract

The fractionation of the CM ethanolic extract was performed using column chromatography as well as thin layer chromatography. Silica gel for column chromatography 60-120 mesh, was filled into a column chromatography (3 cm diameter, 60 cm length) with hexane as the solvent. Five grams of the concentrated CM extract were placed in the column and diluted with various solvents of increasing polarity. Initially, 100% hexane was used with a ratio of 20:0. Further, the extract was combined with Ethyl Acetate up to a ratio of 0:20. Similarly, ethanol was used to elute the extract with a ratio of 20:0 to 100% ethanol. In total, 416 fractions were collected and subjected to thin layer chromatography. Each fraction was tested for RF calculation and similar RF values were combined and further eluted by column chromatography with dichloromethane and Ethyl Acetate[14-16]. Twenty-two fractions were collected and tested by thin layer chromatography to obtain a pure bioactive compound of 45 mg. The compound was further confirmed through ferric chloride and sulphuric acid tests. The identified flavonoids were pale white in color and subjected to spectral analysis.

### Characterisation studies of isolated flavanone Ultra violet-Visible and Fourier Transform Infrared Spectroscopy studies

The isolated flavanone sample was exposed to UV and FTIR spectral analysis to determine the characteristic peaks of specific functional groups occurring in the compound. The sample was examined using a Perkin Elmer UV-Visible spectrometer model Lambda 15 at a wavelength range of 200 to 800nm, and an FTIR spectrometer Brucker Germany model with KBr pellet at a range of 400 to 4000 $cm^{-1}$ with 4 $cm^{-1}$ resolution scale. The spectral analysis provided information about the chemical bonds and functional groups in the isolated compound[14,17].

### HPLC-Purity Test

The level of purity of the flavanone sample was determined using the Shimadzu HPLC (High

Performance Liquid Chromatography), which was equipped with a UV-Vis detector and C18 HPLC auto sampler. The analysis adapted a solvent system of HPLC-grade methanol and water in a ratio of 60:40v/v for the mobile phase, with at a volume range of 20 µL and a flow rate was maintained as 1.0 mL/min at a wavelength of 260nm[18].

### NMR

The nature of Carbon and hydrogen atoms present in the flavanone was studied by $^{1}H$ and $^{13}C$ NMR conducted using a Bruker NMR (Nuclear magnetic resonance spectroscopy) instrument. The Avance III 400 MHz spectrophotometer was employed for the analysis, with samples recorded at 400 MHz for proton NMR study and 100 MHz for carbon NMR study using DMSO ($\delta$ = 39.50ppm) as a solvent[14,16]. The studies were conducted at 310K, and the data obtained from $^{1}H$ and $^{13}C$ NMR will aid in validating the structure of the isolated compound.

### High resolution mass spectrometry

The isolated sample was subjected to High-resolution mass spectrometry (HRMS) using (Waters, USA), model XEVO-G2-XS-QTOF, undertaken in the Cortecs C18 column, 90 Å, 1.6µm, 2.1mm 150mm. The high-resolution electrospray ionization spectrometry positive mode was adopted for the current study[19]. The fragmentation and other data will aid in determining the molecular composition of the isolated compound.

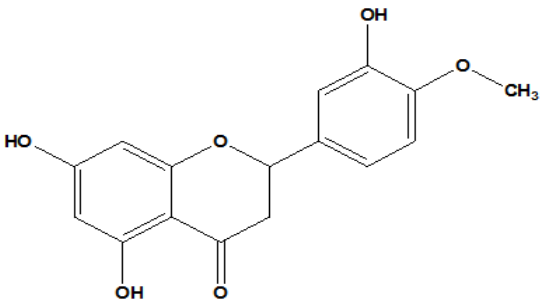


**Fig. 3. Structure of isolated flavanone-Hesperetin**

### MTT assay

A MCF-7 cell line was employed in the study and cultured using "Dulbecco's Modified Eagle Medium (DMEM)" supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin, and 100 µg/mL streptomycin. After trypsinization, the cells were plated into tissue culture plates. The culture plates with FBS and antibiotic solution were incubated at 37°C for 24 to 48 hours[10,20]. Following this, sterile phosphate buffer saline was used to wash the wells, which were subsequently treated with the test compound and synthetic drug (2DG) at equal concentrations. The system was then incubated for one day, after which MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)at the volume of 10 µL of 5 mg/mL) was added, and kept for 2 to 4 h of incubation process[21,22]. The wells were then washed with PBS, and DMSO was added. This step will dissolve the formazan crystals. The resulting absorbance was evaluated at 570nm using a Microplate reader and the cell viability and $IC_{50}$ values were calculated (Model: Thermo Fisher Scientific, Graph Pad Prism 6.0 software USA)[3,23].

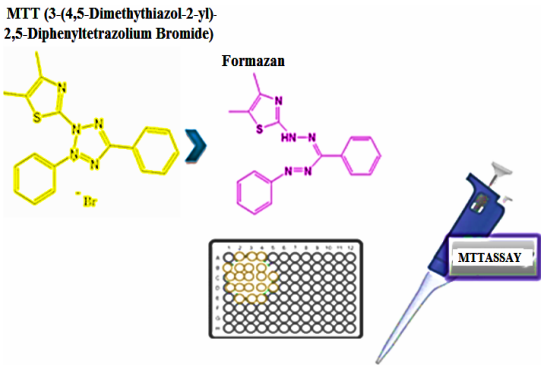


**Fig. 4. Schematic diagram of anticancer activity MTT assay**

## RESULT AND DISCUSSION

The characterization studies and literature review of the isolated compound from citrus confirmed that it belongs to the flavanone class, specifically hesperetin. The spectral data of hesperetin aligns with the previously reported data, and the preliminary ferric chloride test yielded a bluish-violet color, indicating that the compound belongs to the phenolic group[24,25]. Further confirmation through spectral analysis provided additional validation of the compound. The UV-Vis spectrum of isolated hesperetin is shown in Fig. 6. The absorption peaks of hesperetin were found to be 231 and 288nm. These absorption ranges support that the basic flavonoid structure consisting of one benzoyl

group gives a peak near 300nm, and the cinnamyl group in the range of 250nm[15]. The $\pi-\pi^*$ transition of the benzoyl group with hydroxyl group may be responsible for the peak at 288nm and the minimal absorption peak at 337nm. Thus, the UV-Vis spectroscopy results validate the structure of isolated flavanone.

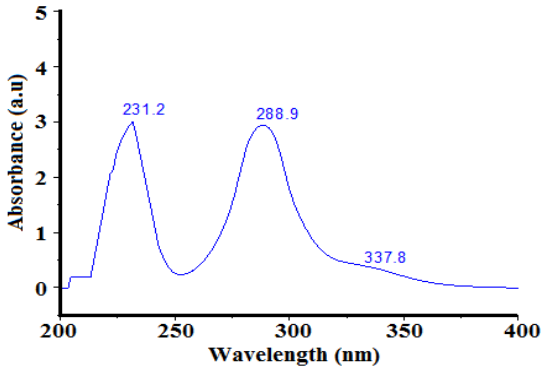


**Fig. 5. UV-Vis spectrum of isolated hesperetin**

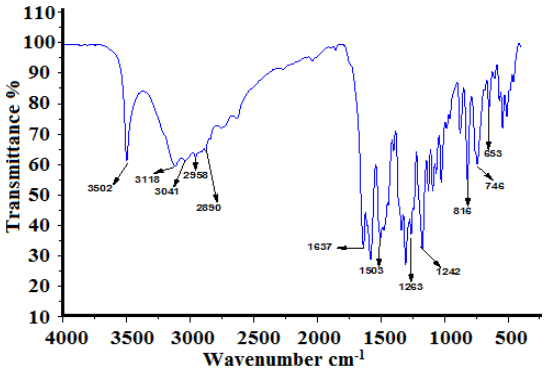


**Fig. 6. FTIR spectrum of isolated hesperetin**

The FTIR image of hesperetin (Fig. 6.) showed 1637 $cm^{-1}$ band indicates carbonyl group (C=O), -OH stretching vibration at 3502 $cm^{-1}$. The bands at 3118 and 3041 $cm^{-1}$ may correspond to the aromatic C-H group, the bands at 2890 & 2957 $cm^{-1}$ may be due to the C-H group(aliphatic)[14,26]. The aromatic ether group may be found at a range of 1263 $cm^{-1}$, and C-C may be found at 1242 $cm^{-1}$, which became recognized for hesperetin. Similar FT-IR ranges have been previously described by other researchers[27,28].

The isolated flavanone's retention time was 4.57 min, as determined by HPLC chromatography[18]. The area of the obtained peak was used to calculate the purity level of the isolated compound, which was found to be 95.69%. The sample had an absorbance range of 254nm.

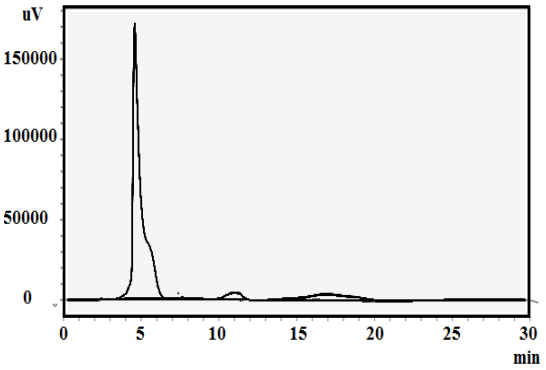


**Fig. 7. HPLC chromatogram of isolated hesperetin**

The nuclear magnetic resonance (NMR) spectrum of isolated flavonoid exhibits signals at specific proton positions, as follows:δ(ppm) at 2.741 (1H, dd; J=2.8&2.4 Hz) represents a doublet of doublets, attributed to splitting, with a downfield shift; δ : 3.231 (1H, q) represents a proton (M) in a three-neighboring proton environment, with an upfield shift; δ : 3.781 (3H, s) represents methyl protons in the upfield region due to benzene resonance; δ : 5.423 (dd, J=3.1, 17.1 Hz); δ : 12.124 (1H, s); δ : 10.762 (1H, br, s); and δ : 9.062 (1H, s) represent protons in the hydroxyl group with a singlet peak[14,19,29].

The $^{13}C$ NMR spectrum of isolated flavonoid also reveals signals at specific carbon positions, including: δ(ppm) at 56.19 ($-OCH_3$), 42.53 (3-C), and 78.68 (2-C), which correspond to aliphatic carbon atoms in the compound. Additionally, signals at δ : 146.97(3'-C), 163.27(5-C), and 167.12(7-C) indicate carbon atoms with direct hydroxyl group attachments[14]. The carbon atoms at δ : 163.94 (9-C) and 196.65(4-C) correspond to carboxyl group carbons, with downfield shifts. Other carbon signals in the spectrum include δ : 95.46(8-C), 96.27(6-C), 102.29 (10-C), 112.54(5'-C), 114.55 (2'-C), 118.12(6'-C), 131.66(1'-C), and 148.37 (4'-C)[17,25]. The mass spectrum data indicates that the principal parent molecular ion with a molecular weight of 301.143 is present at m/z = 301.143. The characterization evidence suggests that the isolated flavonoid is likely to be hesperetin, with a molecular weight of 302.27 g/mol and a molecular formula of $C_{16}H_{14}O_6$. The structure of the isolated flavonoid is depicted in Figure 3.

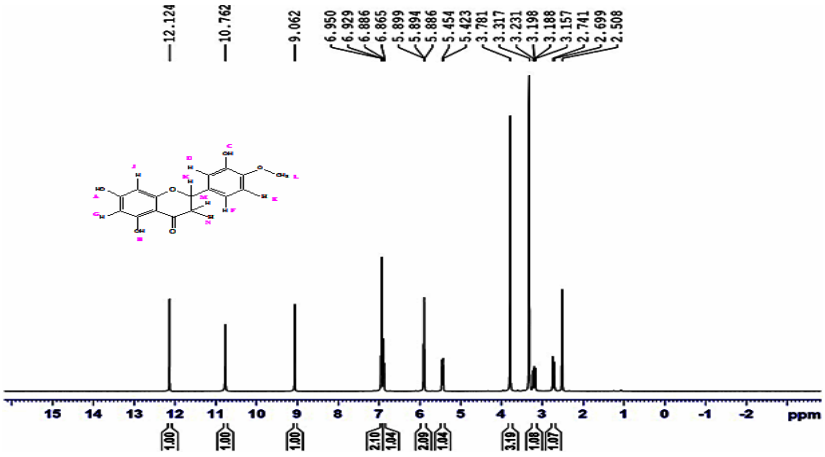


**Fig. 8. $^{1}$H NMR spectrum of isolated hesperetin**

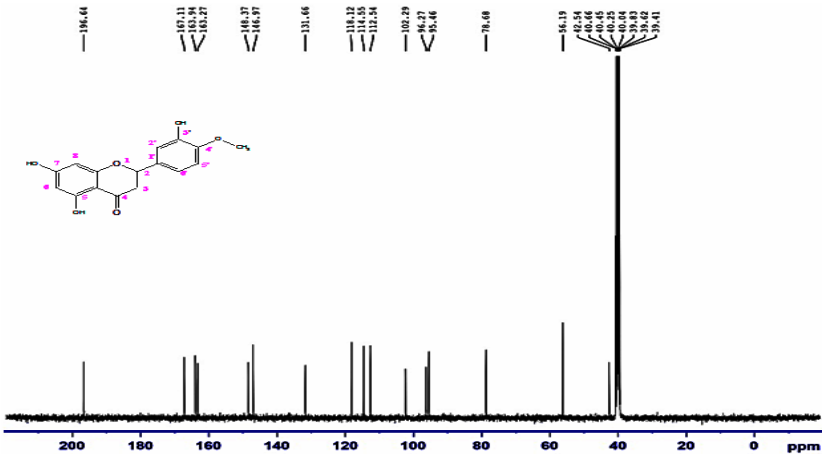


**Fig. 9. $^{13}$C NMR spectrum of isolated hesperetin**

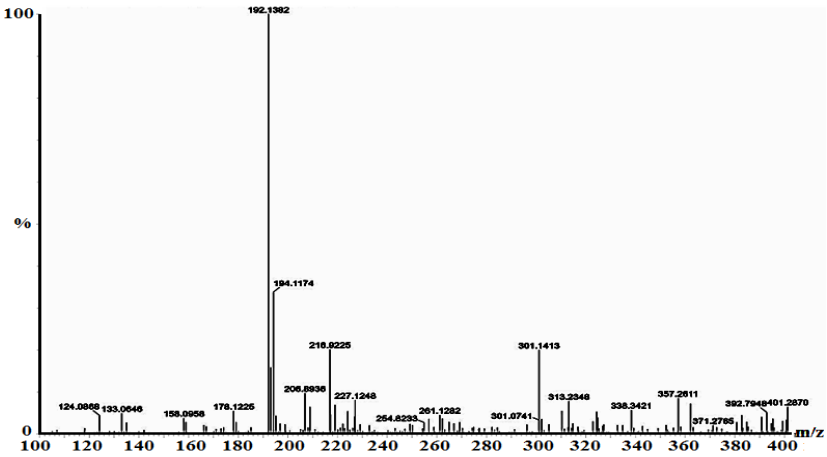


**Fig. 10. HRMS spectrum of isolated hesperetin**

The isolated flavonoid hesperetin is commonly found in citrus plants and was identified in *Citrus medica* leaves in this study for the first time. Hesperetin belongs to the flavanone category, which has a documented history of possessing numerous pharmacological benefits[30,31]. The isolated flavanone and synthetic drug were evaluated using the MTT assay for breast cancer cell lines.

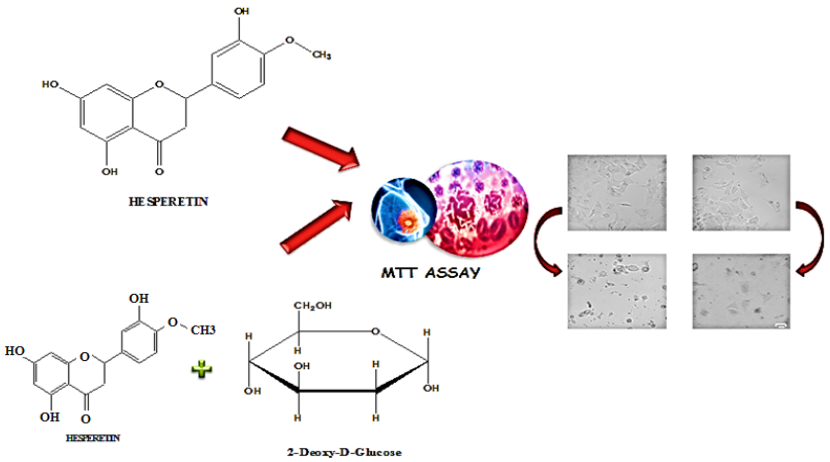


**Fig. 11. Schematic diagram of combination study of Hesperetin and 2DG on breast cancer cell**

The isolated flavanone and H2DG were examined for their cytotoxic potential and both samples demonstrated promising outcomes. The $IC_{50}$ values for hesperetin and the combination of 2DG with hesperetin were 57.10 and 34.09 µg/mL, respectively. The anticancer activity of hesperetin was surprisingly high and may prove to be an effective treatment for cancer cells. Additionally, the combination of hesperetin and 2DG resulted in a 23% increase in effectiveness, making it a potentially promising combination therapy for cancer treatment. It is worth noting that 2DG has fewer side effects and a simpler mechanism of action on cancer cells, making this combination of therapies a viable option for further research.

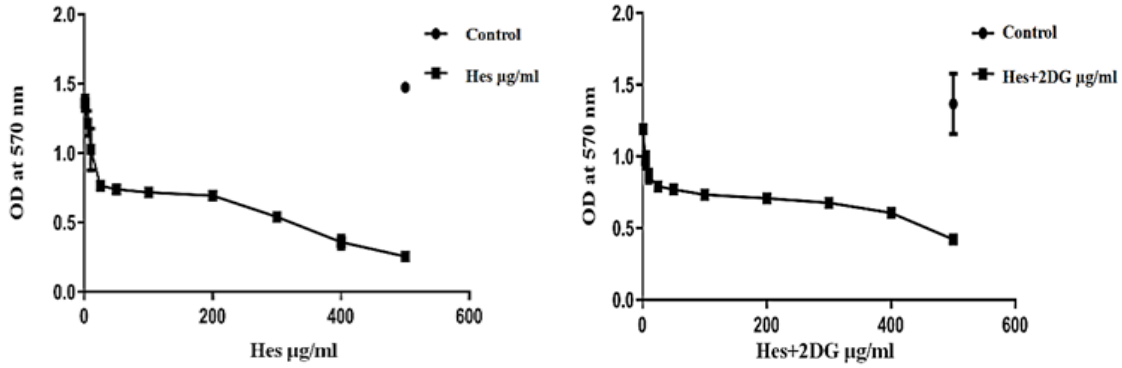


**Fig. 12. OD values obtained from MTT assay of isolated hesperetin and combination of hesperetin and 2DG in various concentration**

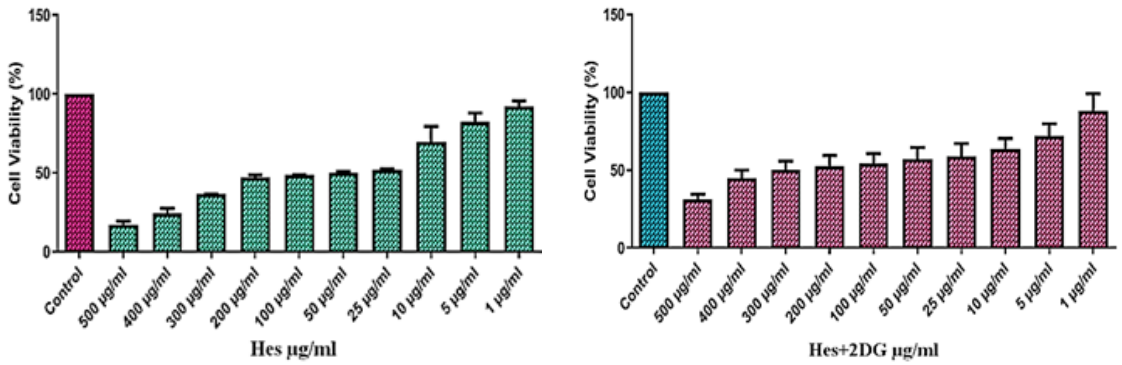


**Fig. 13. Cell viability result of isolated hesperetin and combination of hesperetin and 2DG in various concentration**

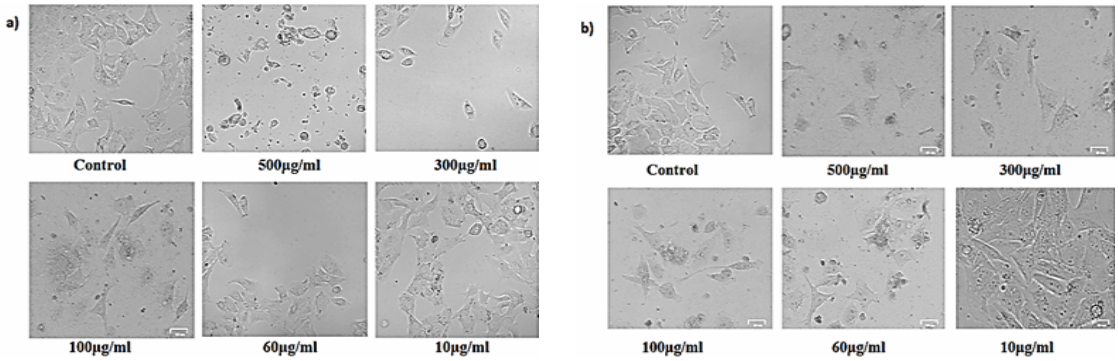


**Fig. 14. Morphological changes of MCF-7 cells tested with isolated hesperetin(a) and combination of hesperetin along with 2DG(b)**

## DISCUSSION

Citrus plants have been widely cultivated worldwide and are highly valued for their numerous health benefits and pharmacological activities. Numerous literatures recorded that phenolic compounds present in citrus plants exhibits significant biological importance[20,41-43]. However, there is still a need for further research to establish scientific evidence for some of these bioactive compounds[32-34]. Hesperetin, a pale white compound, was isolated from *Citrus medica* leaf extract using column and thin layer chromatography. The compound was eluted in repeated fractions of dichloromethane and ethyl acetate. Given the insufficient effects of current cancer medications, there is a strong rationale for finding combination drugs with plant-based sources[35]. Several bioactive compounds have been identified and studied for their various cancer activities. Among them, Hesperetin and other related compounds have shown promising results in cancer activity[44,45]. To improve the efficacy of these bioactive compounds and cancer cells, better linkages and combination drugs are needed. Additionally, drugs used for cancer treatment should have minimal side effects and be safe for prolonged usage[46]. This presents a good reason for combining natural product-based drugs with uncomplicated synthetic drugs for cancer treatment, especially for breast cancer treatment[8,35]. According to literature reports, long-term usage of metformin can lead to adverse effects in cancer patients[8,47]. The use of aspirin, for instance, may exacerbate bleeding in certain cases. Cancer prevention research suggests that increased glycolysis can increase the risk of cancer cell growth[36-39]. Therefore, targeting glycolysis may be an effective solution. In light of these findings, a synthetic analogue of glucose, 2DG, could be a more suitable option for study in combination with plant-based hesperetin. This study demonstrates the potential use of a combination of hesperetin and 2DG as an anticancer therapy.

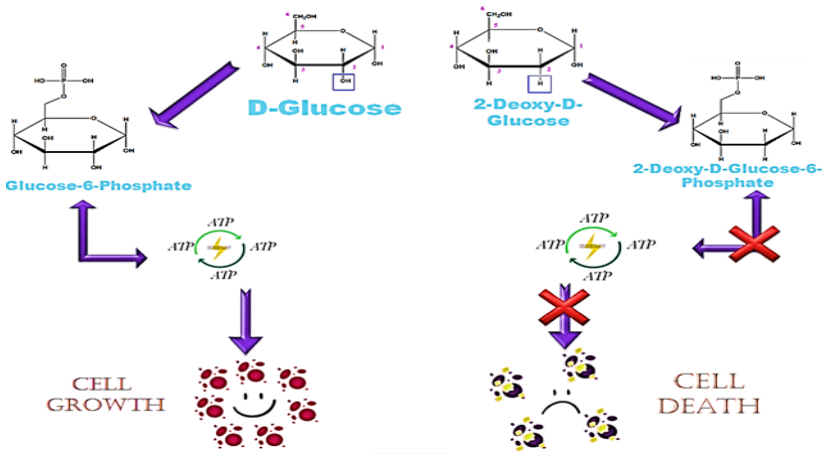


**Fig. 15. Schematic diagram of mechanism behind 2DG on cancer cells**

The implementation of this modified glucose molecule leads to its entry into cancer cells, subsequently inhibiting the glycolysis cycle, which generates adenosine triphosphate (ATP) for cellular energy[48-50]. As ATP is absent, the cells are unable to access the energy required for growth, ultimately leading to cell death at some point[11,37]. Since cancer cells demand more energy than normal cells, they consume greater amounts of the modified glucose, ultimately failing to proliferate[51-53]. Consequently, the outcomes indicate that combining a synthetic drug with a natural flavonoid enhances the effect on cancer cells.

## CONCLUSION

The isolated compound from leaf samples of *Citrus medica* has demonstrated promising anticancer properties, which may prove to be valuable in the field of anticancer drug discovery. Although the effect was enhanced when combined with 2DG, further studies are necessary to assess the potential of synergy between hesperetin and 2DG for various cancer treatment. The findings suggest a potential synergistic effect between plant-based products and synthetic drugs on breast cancer activity, which could be beneficial for future research in this area. This information may also aid in understanding the underlying mechanisms involved in the synergistic effect. The expansion of these studies to include various cancer cell lines offers a thorough examination of the outcomes of this novel combination of therapy.

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**Conflict of interest**

The authors have no conflicts of interest.

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