RESEARCH PAPER

Crystal Engineering of Daidzein: Development, Characterization, and In Vitro Cytotoxicity Against Human Breast Cancer Cells (MCF-7)

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Abstract

Breast cancer is the most common cancer and the leading cause of cancer death in women globally. Even though lots of chemotherapeutic agents are available, however, their clinical application is commonly associated with severe side-effects and development of drug resistance. Daidzein is a naturally occurring soy isoflavone with great antioxidant, anti-inflammatory and anticancer effects. However, limited aqueous solubility and poor oral bioavailability hinder its therapeutic application. The objective of the present work was to improve the physicochemical properties and anticancer potential of daidzein through cocrystallization with Isonicotinamide. The daidzein–Isonicotinamide cocrystal was prepared (1:1 molar ratio) via solvent evaporation method. The cocrystal was characterized by FTIR, PXRD, DSC, TGA, SEM and SCXRD. All characterization results confirmed the successful formation of a novel crystalline phase. The cytotoxic potential of the prepared cocrystal was evaluated by MTT assay against MCF-7 human breast cancer cell line using doxorubicin as a standard reference drug. Analysis through nonlinear regression demonstrated concentration-dependent inhibition of the viability of cells, with an IC_{50} value of 714.9 ± 0.15 µM for the daidzein cocrystal, whereas doxorubicin showed an IC_{50} value of 9.539 ± 0.16 µg/mL. The dose-response curves displayed excellent fitting to the Hill equation according to the high R^2 values (cocrystal = 0.926, doxorubicin = 0.902). The cocrystal was less cytotoxic than doxorubicin but had good antiproliferative activity and the advantage of solubility and bioavailability. The outcome of the research has shown that the crystal engineering strategy can be used effectively to augment the pharmaceutical performance of daidzein. The study also supports further preclinical evaluation of the developed cocrystal for breast cancer therapy.

Keywords: Daidzein, Pharmaceutical Cocrystal, Crystal Engineering, Isonicotinamide, Breast Cancer, MCF-7, MTT Assay, Cytotoxicity, IC_{50}, Bioavailability.

How to cite this article: Kaur P, Pandey M, Singh B. Crystal Engineering of Daidzein: Development, Characterization, and In Vitro Cytotoxicity Against Human Breast Cancer Cells (MCF-7). Int J Drug Deliv Technol. 2026;16(71s): 1716-1732. DOI: 10.25258/ijddt.16.71s.200

1.Introduction:

Daidzein is a naturally occurring soy isoflavone (4′,7-dihydroxyisoflavone) which has antioxidant, anti-inflammatory, anti-proliferative and pro-apoptotic properties that have garnered much interest as an anticancer agent [1] Multiple in vitro and in vivo studies showed daidzein inhibits the growth of several cancer cells, especially breast cancer cells. Daidzein induces cell cycle arrest and apoptosis while inhibiting angiogenesis. Moreover, it modulates various signaling pathways like PI3K/Akt, MAPK, NF-kB, and estrogen receptor-mediated pathways. Poor aqueous solubility, low dissolution rate and poor oral bioavailability limit the clinical application of daidzein. [2] This will result in an inadequate therapeutic concentration at the site of action. Pharmaceutical cocrystallization has evolved as an effective crystal engineering tool aimed at addressing these biopharmaceutical insufficiencies without altering the molecular framework of the active pharmaceutical ingredient. The daidzein–Isonicotinamide cocrystal demonstrates the largest improvement in pharmacokinetic performance with significantly higher Cmax and increased dissolution when compared with pure daidzein. The improved physicochemical properties of the daidzein cocrystals should lead to better anticancer efficacy due to enhanced cellular uptake and availability of the drug inside the cell. When the bioavailability of anticancer drugs improves, they can better inhibit cancer cell proliferation, induce apoptosis and suppress tumor progression.[3]

Daidzein's ability to increase sensitivity of breast cancer cells to chemotherapeutic agents, reversing multidrug resistance, and inducing apoptosis via modulation of estrogen receptor signalling and inhibition of breast cancer resistance protein (BCRP) has been documented by earlier studies.[4] Thus, this can be explored for use in breast cancer treatment. To enhance the therapeutic performance of the natural anticancer molecule daidzein, the development of its cocrystals is a potential strategy. Testing the cytotoxicity of daidzein cocrystals on human breast cancer cell lines, MCF-7 could provide useful information related to the link between improved pharmaceutical properties and enhanced biological activity. The findings suggest that daidzein cocrystals may serve as an effective breast cancer oral formulation, indicating the need for further preclinical and clinical studies.

MCF-7 Cell Line Description In 1970, a 69-year-old female patient with metastatic breast cancer who experienced pleural effusion underwent a biopsy. [5] The pleural effusion harbors tumor cells, which were then isolated into the MCF-7 line. Furthermore, estrogen receptor-positive cells are suitable for hormone treatment, making them appropriate for clinical prognosis.[6] Considered the most widely used cell line and reliable for breast cancer (BC) research, it displays stable growth characteristics and is responsive to hormones. zileuton supplier. [7,8] The first established human breast adenocarcinoma cell line, MCF-7, has increased the estrogen receptor. All Culture of the Cells Mixed with Dulbecco's Modified Eagles Medium F12 (DMEM-F12) in answer to Emma Koonse.[9] MCF-7 cells have an epithelial-type morphology and express estrogen receptors (ER+) and progesterone receptors (PR+) but have low or negligible HER2 expression. So, they are classified as a luminal subtype of breast cancer. Due to their hormone-dependent growth and relatively weak invasive potential, MCF-7 cells are extensively used for studies on breast cancer, anticancer agents, estrogen signaling and assessment of the cytotoxicity of new drug delivery systems and formulations. MCF-7 cells are an experimental standard cell line for screening compounds for hormone responsive breast cancer as they are reproducible, easy to culture, and clinically relevant. [10]

2. Experimental Details

2.1 Material method

Solvent Evaporation Method of Preparation of Daidzein-Isonicotinamide Cocrystal. A 1:1 molar mixture of daidzein and Isonicotinamide cocrystal was prepared through solvent evaporation. In a minimum volume, a measured quantity of daidzein and isonicotinamide was dissolved in ethanol or methanol at room temperature under continuous magnetic stirring until a clear homogeneous solution was obtained. [11]The resulting solution was stirred for complete interaction of the drug with the coformer for 30–60 minutes. The solvent was then allowed to evaporate slowly at ambient conditions or in a hot air oven at 40-45 °C until completely dry. The crystalline solid obtained was collected and ground gently using a mortar and pestle, then passed through a #60 sieve and stored in a desiccator for further characterisation. [12]

2.2 Identification and Characterizations

2.2.1 *Powder X – ray diffraction (PXRD)*

The PXRD patterns were examined using Rigaku Miniflex 600, Rigaku Corporation with Cu Kα. radiation (1.54060 Å).[13]

2.2.2 DSC – differential scanning calorimetry.

All samples were analyzed using a Shimadzu DSC 60 Plus (Asia Pacific, Private Limited). Limited A sample weighing 5 mg was sealed in aluminium pans and scanned at a rampling rate of 20°C per minute under a nitrogen atmosphere. The origin 8.5 software was used to collect the data. The range of temperature in DSC was 0°C to 350°C.[14]

2.2.3 *Fourier transform – infra red spectroscopy (FTIR)*

The KBr diffuse-reflectance mode (sample concentration 2mg) used on a spectrum FTIR 4X spectrometer Jasco was built in India. Data analyses were carried out using 8.5 origin software and the range of 4000-400 cm-1. Fourier transform infrared spectrometer in chemometrics A spectrum FT/IR-4X spectrometer (Jasco, India) was employed in the KBr diffuse reflectance mode (sample concentration 2mg) over a range of 4000-400 cm-1). Furthermore, data analysis utilized the Origin 8.5 software.[15,16]

2.2.4 *Scanning Electron Microscopic (SEM)*

Examination of daidzein, Isonicotinamide and daidzein cocrystal through scanning electron microcopy was carried out at International University of Business Agriculture and Technology (IUBAT) using ZEISS EVO 18 Research, USA.[17]

3 Result

3.1 Absorption Maxima

The UV–Visible spectrum of pure daidzein shows a characteristic absorption maximum at 256 nm that confirms the integrity of its conjugated isoflavone chromophore.

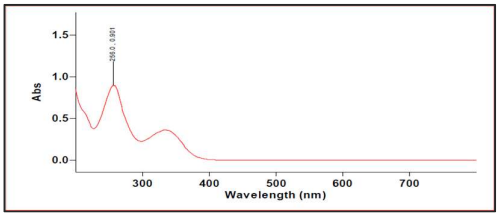


Figure 1

Absence of absorption in the visible region indicates that compound is UV active but not visible-active The λ max observed was taken as the analytical wavelength for preparation of the calibration curve, drug content assay, dissolution studies and other quantitative evaluations. The UV–Visible spectrum of pure daidzein exhibited a characteristic absorption maximum at 256 nm which confirms its integrity of the conjugated isoflavone chromophore. The compound does not absorb light in the visible region where it is likely to be UV-active. The λ max observed was taken as the analytical wavelength for the preparation of calibration curves, drug content assay, dissolution studies, and other quantifications.[18]

3.2 Powder X – ray diffraction (PXRD)

The PXRD pattern of pure daidzein (Figure a) revealed a large number of sharp and intensified diffraction peaks in the 2θ range of 5-50°, indicating it is highly crystalline. Reflections characteristic of quartz were detected at around 10.3° and at about 15.8°, 16.8°, 24.2°, 25.0°, 26.0°, 27.5°. 28.6°. The PXRD for pure Isonicotinamide (Figure b) also showed sharp crystalline reflections with diffraction peaks at □17.8º, □20.7º, □23.3º, □25.8º, □26.5º, and □30.8º assignable to crystalline Isonicotinamide. The PXRD pattern of the daidzein–Isonicotinamide cocrystal shows an obvious difference from daidzein (Figure c). The essential peaks of pure daidzein and pure Isonicotinamide were severely weakened or missing, which led to the appearance of new diffraction reflections at approximately 16.0ø, 17.0ø, 22.8ø, 24.5ø, 25.3ø, and 27.8ø. The strongest diffraction peak was observed around an angle of approximately 22.8° (2θ), which is not the major reflection for either of the parent materials. The emergence of novel reflections and changes in the intensity of parent peaks signify the formations of a new crystalline phase rather than the formation of a simple physical mixture. Changes in structure happen because the molecule forms intermolecular hydrogen bonds between the hydroxyl functionalities of daidzein and the amide/pyridine of Isonicotinamide. This allows rearranging into a new crystal lattice. Further, the cocrystal's crystalline diffraction pattern, which lacks diffuse halo, indicates that this cocrystal is non-amorphous in nature.[19]

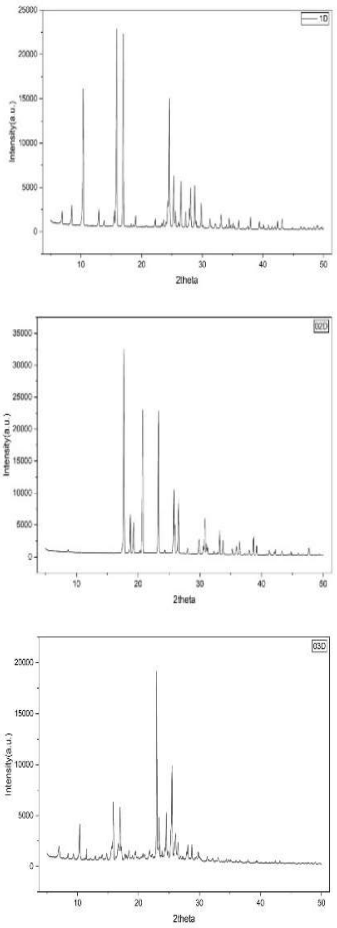


Figure 2a Figure 2b Figure 2c

3.3 DSC – differential scanning calorimetry

The thermal behavior of the cocrystals DIS constructed by solvent evaporating technique was studied. Daizein and Isonicotinamide peaks at 336 °C The melting point of a cocrystal is when the individual components in pure form lose structural order to form an alternate molecule arrangement. Furthermore, melting point studies aid in characterizing substances. Two materials produce a unique cocrystal that is different from each pure component. The DSC thermogram of pure daidzein shows single sharp endothermic peaks falling in the range of 336–339 °C indicating melting of crystalline daidzein. There was appearance of sharp and vigorous endothermic peak indicating highly crystalline and pure nature. No polymorph transformation or impurity-related phenomenon occur before the melting process. Evidence from thermogravimetric analysis daidzein, being a heat-stable compound, shows no exothermic and endothermic transitions below 300 °C. After

melting, the thermogram shows the beginning of thermal decomposition which is common for the flavonoid compounds at a higher temperature.[20]

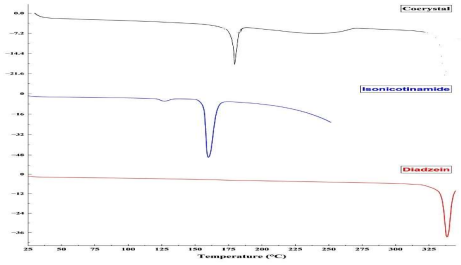
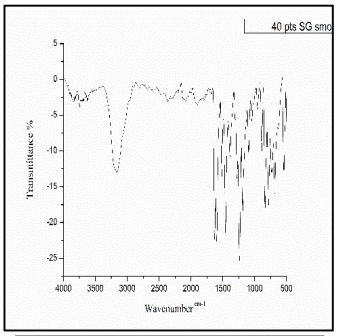
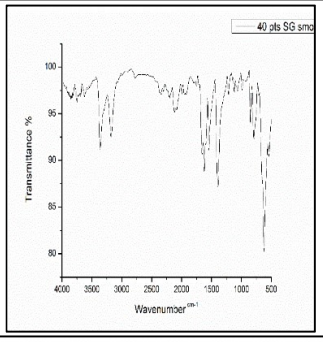


Figure 3

3.4*Fourier transform – infra red spectroscopy (FTIR)*

FTIR characterization of different cocrystals is based upon the shift in position and intensity of characteristic peaks. The FTIR spectrum of DAID exhibited numerous sharp, distinct peaks at 3218.12 cm_1 associated with OH stretch, 2833.30 cm_1(CH Stretch), 1630.68 cm_1 (C¼O Stretch), 1598.50 cm_1(C¼C vibration). The alterations in. The various cocrystals were observed to have bands assigned to OH and C¼O deformation in DAID (Fig. 4). The approach to produce a successful DIS requires careful consideration of various factors. In particular, the chosen instrument must meet at least three levels of design criteria: a demanding mission profile, a successful testing history, and near failure-free operation. Successful DIS instruments will often be challenging and/or costly to achieve.[21]





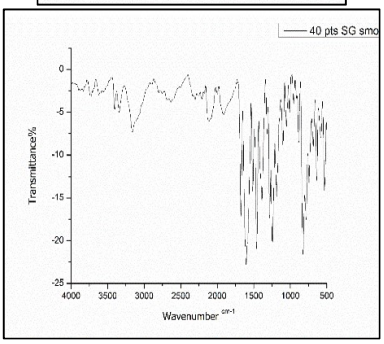
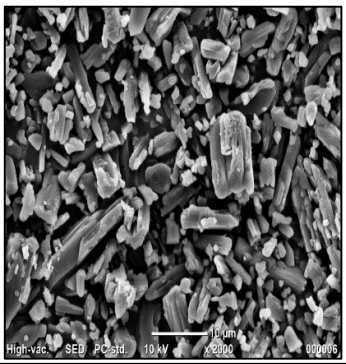


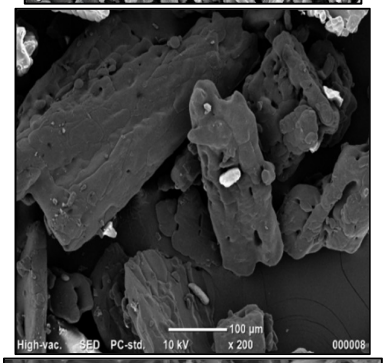
Figure 4a Figure 4b Figure 4c

3.5 SEM

Scanning Electron Microscopic (SEM)

Micrograph of Daidzein – Isonicotinamide co-crystal (×2000) is shown. The morphological analysis suggests the presence of crystallites in the form of rods and plates with well-defined crystal faces and smooth surfaces. The particles are uniformly distributed with slight agglomeration and are significantly different in crystal size. Crystals have a long and sharp edge which is indicative that crystals have grown in a controlled manner during the process of the solvent evaporation. Hence, the dry crystals are a crystalline co-crystal phase.





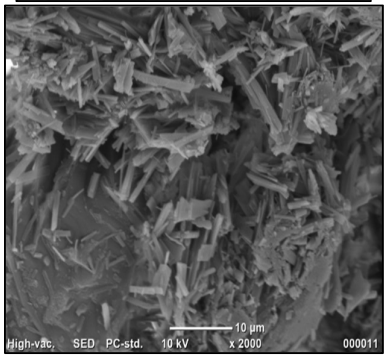


Figure: (5a) Figure: (5b) Figure: (5c)

Figure 5a displays the SEM micrograph of Daidzein–Isonicotinamide co-crystal at ×200, which has been evaluated for overall morphology of bulk particle. The sample consists of large compact agglomerates whose lethargy is more or less smooth. The presence of smaller crystal particles attached to larger aggregates shows the possible growth via particle association. We believe that the small particle size and compact morphology represent the feature morphology of the co-crystal provided as well as the crystalline phase of the solvent evaporation method.[22,23]

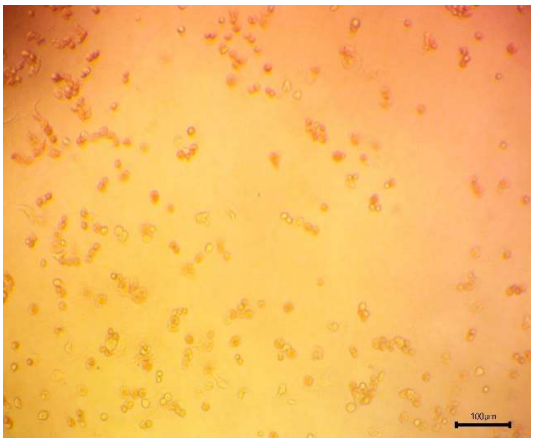
The figure 5b is of the Daidzein-Isonicotinamide co-crystal at × 500 magnification. Clusters made up of many rod and needle-shaped crystallites of various sizes are observed equally distributed on the particle's surface. The closely defined crystals form a three-dimensional framework that is interconnected and irregular. The particles aggregated possibly due to crystal formation and particles association during solvent evaporation. The co-crystal of Daidzein–Isonicotinamide has a unique crystal structure and well-shaped crystals, which facilitates the formation of Daidzein–Isonicotinamide co-crystal.[24]

The SEM micrograph of Daidzein-Isonicotinamide (figure 5c) represents co-crystal at ×2000. Most of the particles were mainly rod and plate like in shape, having well-defined crystal faces with sharp edges will reveal their crystalline nature. The crystals are agglomerated to smaller extents and densely packed. Few smaller crystals are sticking to a larger one. The uniformity of the crystal habit and the orderly surface morphology of the synthesized co-crystals suggest controlled crystallization during solvent evaporation, leading to the successful growth of Daidzein−Isonicotinamide co-crystal.[25]

3.6 MTT Assay

Cytotoxicity of the provided samples on MCF-7 (Procured from NCCS Pune- Passage No.- 31, RRID- CVCL_0031) cell line was determined by MTT Assay. [26] The cells (10000 cells/well) were cultured in 96 well plate and incubated (Air-Jacketed CO_2 incubator, Heal Force- HF90) for 24 h in DMEM medium (Dulbecco's Modified Eagle Medium-AT149-1L- HIMEDIA) supplemented with 10% FBS (Fetal Bovine Serum - HIMEDIA-RM 10432) and 1% antibiotic solution (Penicillin-Streptomycin-Sigma-Aldrich P0781) at 37°C with 5% CO_2. Next day cells were treated from different concentrations of the samples (concentration as per mentioned in excel sheet).[27,28] Stock solution of samples was prepared in ethanol and further diluted to get different concentrations in incomplete Cell culture Medium (Without FBS).[29,30] Cells without treatment were considered as Control and cells without MTT were considered as Blank. After incubation for 24 hours, MTT Solution (5 mg/ml) was added to cell culture and further incubated (Air-jacketed CO2 incubator-Heal force-HF90) for 2 h. [31,32] At the end of the experiment, culture supernatant was removed,[33] and cell layer matrix was dissolved in 100 µl ethanol and read in an Elisa plate reader (iMark, Bio-Rad, USA) at 540 nm. IC_{50} was calculated by using software Graph Pad Prism 6.[34,35] Images were captured under inverted microscope (Olympus ek2) using Camera (AmScope digital camera 10 MP Aptima CMOS).

50% inhibitory concentration (IC_{50}) was presented as Mean ± SEM (Standard Error of Mean). [36,37]



3.7 Result:

3.7.1 MTT Assay to Determine Effect of Doxorubicin on MCF-7 Cells.

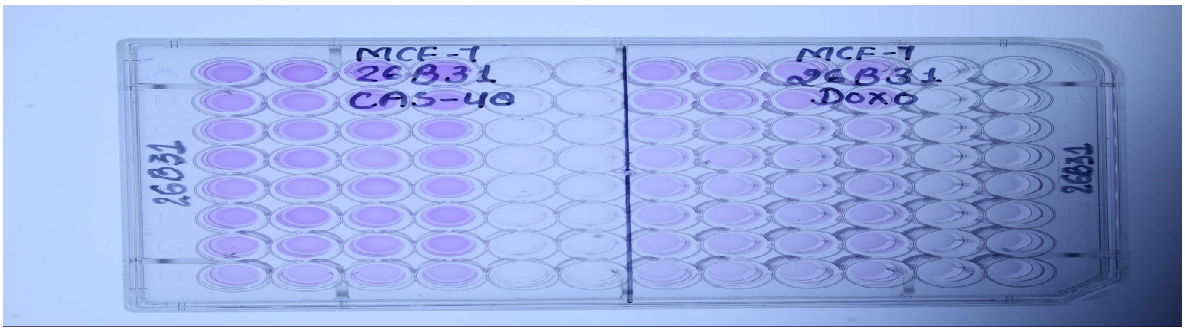


Fig 6:Pure**Plate image of Standard (Doxorubicin) and sample (Daidzein Cocrystsal)**

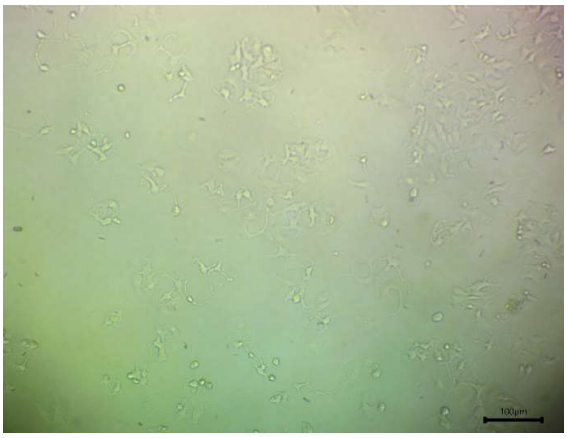


Figure7:Doxorubicin

Figure 8: Daidzein Cocrystal

Sample Conc.	Test Replicates			
	1	2	3	4
0	0.291	0.292	0.296	0.293
1	0.229	0.235	0.227	0.23
10	0.169	0.159	0.169	0.173
50	0.161	0.149	0.159	0.162
100	0.155	0.147	0.142	0.156
125	0.147	0.146	0.148	0.155
250	0.146	0.132	0.146	0.155
500	0.135	0.118	0.119	0.128
Control		0.249		

Calculation Table for Normal Doxorubicine

Total Volume of Reaction Mixture			100	µl
Volume of Treatment Compound			100	µl
Volume to be dispensed for Treatment			5	µl
	Working Concentration (µg/ml)	**Stock (mg/ml)**	**Prev Stock (µl)**	**Solvent (µl)**
1	0	0	0	100
2	1	0.02	10	90
3	10	0.2	20	80
4	50	1	50	50
5	100	2	80	20
6	125	2.5	50	50
7	250	5	50	50
8	500	10	100	0
	Stock Conc.	10	mg/ml	

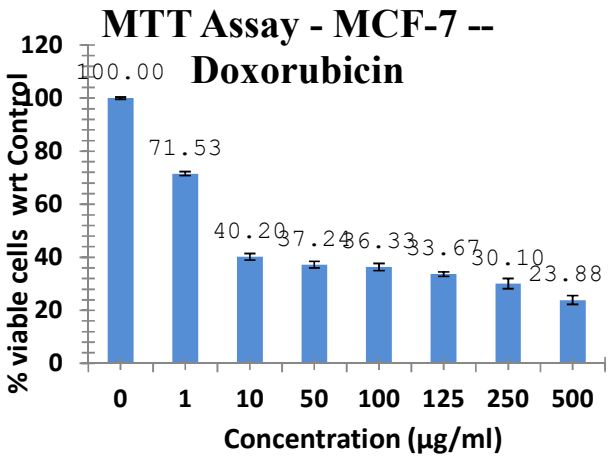
Final Values for Analysis

Stats

Sample Conc.	Mean	SD	SEM	N
0	100	0.88173	0.44087	4
1	71.5306	1.38915	0.69458	4
10	40.2041	2.43762	1.21881	4
50	37.2449	2.43477	1.21738	4
100	36.3265	2.72788	1.36394	4
125	33.6735	1.66632	0.83316	4
250	30.102	3.87755	1.93878	4
500	23.8776	3.28227	1.64113	4

Corrected Values

1	2	3	4
0.243	0.244	0.248	0.245
0.174	0.18	0.172	0.175
0.1	0.09	0.1	0.104
0.0945	0.0825	0.0925	0.0955
0.094	0.086	0.081	0.095
0.0805	0.0795	0.0815	0.0885
0.075	0.061	0.075	0.084
0.0685	0.0515	0.0525	0.0615



Abbreviations:
Set 1= Control (No treatment)
Set 2= Control Blank
Set 3= Treatment
Set 4= Treatment Blank
SD= Standard Deviation
SEM = Standard Error of Mean
N - Number of Test Replicates
Corrected Value (Blank Corrected value) = Absorbance (Test) - Absorbance (Average of Blank)
Final Replicate value = calculation of % inhibition with respect to control
Absorbance (Test)=Blank Corrected value
Absorbance (Control) = Average of Blank corrected value at no Treatment (Concentration "0")
Mean= Average of Final Replicate values
Control = Cell without Treatment i.e. at "0' Concentration

$$\%\ Viable\ cells\ wrt\ Control\ =\ \frac{Absorbance\ (Test)}{Absorbance\ (Control)}\ x100$$

3.7.2 Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0	4	400	100	0.7774 54
1	4	286.12 24	71.530 61	1.9297 51
10	4	160.81 63	40.204 08	5.9419 69
50	4	148.97 96	37.244 9	5.9280 86
100	4	145.30 61	36.326 53	7.4413 44
125	4	134.69 39	33.673 47	2.7766 21
250	4	120.40 82	30.102 04	15.035 4
500	4	95.510 2	23.877 55	10.773 29

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18650.6	7	2664.3	421.21	1.58E-23	2.4226
Within Groups	151.817	24	6.3254			
Total	18802.41	31				

In MTT Assay, the cytotoxic effect of Doxorubicin was assessed in MCF-7 cell line. Cells were treated with doxorubicin (0-500 µg/mL) and cell viability was measured in relation to the untreated control group. We found the untreated control grew normally and was 100% viable. Doxorubicin's Effects on the Viability of MCF-7 Cancer Cells. At 1 µg/mL cell viability decrease to 71.53 ± 1.39% indicates start of toxicity.

The viability dropped significantly when exposed to 10 µg/mL (40.20 ± 2.44%) indicating strong antiproliferative activity. After increasing the concentration even further, cell viability was reported to decrease and at which we got the values – 50, 100, 125, 250, and 500 µg/mL – 37.24 ± 2.43%, 36.33 ± 2.73%, 33.67 ± 1.67%, 30.10 ± 3.88%, and 23.88 ± 3.28%. The findings of the study show that doxorubicin is an effective MCF-7 cell proliferation inhibitor, with the maximum concentration yielding maximum cytotoxicity. Statistical Analysis of Difference Among Treatment Groups was analysed by one way analysis of variance.

The further analysis showed that F-value was 421.21 or much greater than table F (2.42) Additionally, it had a very low P value (1.58x10-23). P-value was <0.0001 meaning statistically significant difference between the cell viability of different treatment groups. Based on this evidence, the decrease in cell viability is caused by doxorubicin concentrations and not a mere coincidence.

The power to kill cancerous cells has been evaluated using the MTT assay. Doxorubicin killed MCF-7 breast cancer cells efficiently, downregulating their functions. As the drug concentration increased, a drop in the viability of the cell occurred, which shows the sensitivity of the assay to detect the growth-inhibitory effects of the compound under study. The result with doxorubicin (which is a positive control) shows the efficacy of the assay for in vitro anticancer studies. The results may provide a benchmark for future biological studies for comparative cytotoxic efficacies of different cocrystal formulations like Daidzein–Isonicotinamide.

3.8 MTT Assay to Determine Effect of Daidzein (CAS-48) cocrystal on MCF-7 Cells.

Test Name	MTT Assay - MCF-7
Sample ID	CAS-48
Graph Title	MTT Assay – MCF-7 –CAS-48
X Title	Concentration (µM)
Y Title	% viable cells wrt Control

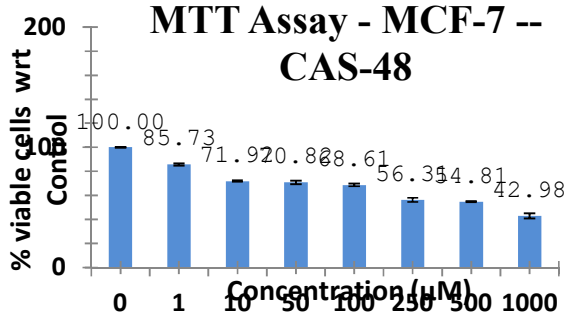
Average Values	
Blank	0
Control	0.317

3.8.1 Calculation Table for Sample (Daidzein Cocrystal)

Total Volume of Reaction Mixture			100	**µl**
Volume of Treatment Compound			100	**µl**
Volume to be dispensed for Treatment			5	**µl**
	Working Concentration (µg/ml)	**Stock (mg/ml)**	**Prev Stock (µl)**	**Solvent (µl)**
1	0	0	0	100
2	1	0.02	10	90
3	10	0.2	20	80
4	50	1	50	50
5	100	2	40	60
6	250	5	50	50
7	500	10	50	50
8	1000	20	40	60
	Stock Conc.	50		

Sample Conc.	Test Replicates				Blank	
	1	2	3	4	1	2
0	0.376	0.374	0.37	0.376	0.059	0.055
1	0.32	0.329	0.323	0.333	0.056	0.053
10	0.29	0.295	0.288	0.287	0.065	0.059
50	0.287	0.273	0.273	0.293	0.06	0.054
100	0.272	0.271	0.262	0.279	0.054	0.053
250	0.23	0.246	0.244	0.224	0.058	0.057
500	0.229	0.234	0.231	0.235	0.057	0.06
1000	0.197	0.189	0.17	0.199	0.056	0.049

Corrected Values			
1	2	3	4
0.319	0.317	0.313	0.319
0.2655	0.2745	0.2685	0.2785
0.228	0.233	0.226	0.225
0.23	0.216	0.216	0.236
0.2185	0.2175	0.2085	0.2255
0.1725	0.1885	0.1865	0.1665
0.1705	0.1755	0.1725	0.1765
0.1445	0.1365	0.1175	0.1465



Final Values for Analysis

Stats				
Sample Conc.	Mean	SD	SEM	N
0	100	0.89225	0.44612	4
1	85.7256	1.84617	0.92308	4
10	71.9243	1.12272	0.56136	4
50	70.8202	3.19117	1.59558	4
100	68.612	2.20068	1.10034	4
250	56.3091	3.378	1.689	4
500	54.8107	0.8687	0.43435	4
1000	42.9811	4.17212	2.08606	4

Abbreviations:

Set 1= Control (No treatment)

Set 2= Control Blank

Set 3= Treatment

Set 4= Treatment Blank

SD= Standard Deviation

SEM = Standard Error of Mean

N - Number of Test Replicates

Corrected Value (Blank Corrected value) = Absorbance (Test) - Absorbance (Average of Blank)

Final Replicate value = calculation of % inhibition with respect to control

Absorbance (Test)=Blank Corrected value

Absorbance (Control) = Average of Blank corrected value at no Treatment (Concentration "0")

Mean= Average of Final Replicate values

Control = Cell without Treatment i.e. at "0' Concentration

$$\%\ Viable\ cells\ wrt\ Control\ = \frac{Absorbance\ (Test)}{Absorbance\ (Control)}\ x100$$

3.8.2Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
				0.796
		4	1	1
		0	0	0
0	4	0	0	7
		3	8	3
		4	5	.
		2	.	4
		.	7	0
1	4	9	2	8
			0	5
			2	5
			2	5
			2	7
			8	1
			7	.
			.	9
			6	2
			9	4
1			7	2
0	4	2	9	3
		2	7	1
		8	0	0
		3	.	.
		.	8	1
		2	2	8
		8	0	3
5		0	1	5
0	4	8	9	4
		2	6	4
		7	8	.
		4	.	8
		.	6	4
		4	1	2
1		4	1	9
0		7	9	8
0	4	9	9	4
		2	5	1
		2	6	1
		5	.	.
		.	3	4
		2	0	1
2		3	9	0
5		6	1	8
0	4	6	5	7
		2	5	0
		1	4	.
		9	.	7
		.	8	5
		2	1	4
5		4	0	6
0		2	7	4
0	4	9	3	3
		1	4	1
		7	2	7
		1	.	.
		.	9	4
1		9	8	0
0		2	1	6
0		4	0	5
0	4	3	7	5

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	91		13	20	6.	2.
	68.		09	09.	23	42
	21		7	29	E-20	62
	4	7	45	33		9
Within Groups	150		6.			
	.19		257			
	06	24	94			
Total	931					
	8.					
	405	31				

The in vitro cytotoxic effect of Daidzein–Isonicotinamide cocrystal (CAS-48) against human breast adenocarcinoma (MCF-7) cell line was performed in MTT assay. The viability of cells was tested with varying concentrations (1-1000 µg/mL) of cocrystal and expressed as percentage untreated control. The untreated control shows all cells to have 100% viability indicating they are metabolically active and viable.

The cocrystal of daidzein decreased A549 cells' cell viability in a dose-dependent manner. At concentrations of 1,10,50,100,250,500,1000 µg/mL, the average cell viability was measured as 85.72 ± 1.86%, 71.92 ± 1.10%, 70.82 ± 3.08%, 68.61 ± 2.20%, 56.31 ± 3.33%, 54.81 ± 0.84% and 42.98 ± 4.31% respectively.

As a result, the percentage growth inhibition of the plants was increased from 14.28% at 1 µg/mL to 57.02% at 1000 µg/mL. As the concentration increased, the cell viability decreased which shows the dose-dependent cytotoxicity of the cocrystal. All treatment groups exhibited low standard deviation values, which indicated good reproducibility of the experimental data in all treatment groups. Recording as high as 1000 µg/mL doses indicated an inhibitory effect on the cellular metabolism, with a 43% viability loss exhibited.

The Daidzein–Isonicotinamide cocrystal has effective inhibition of MCF-7 cells growth. It is implied that improved wants physicochemical properties which will subsequently increase aqueous solubility & dissolution rate the well-known advantage of pharmaceutical cocrystal formation, and thus it may increase cytotoxic activity.

By enhancing dissolution of Daidzein, it is expected that Daidzein will be available in a higher concentration inside the cancer cell leading to greater uptake and more robust activity compared to poorly soluble parent drug. The compound Daidzein can also induce apoptosis, cause cell cycle arrest and modulate multiple signalling pathways involved in breast cancer progression.

The improved delivery characteristics of the cocrystal, therefore, could explain the concentration-dependent growth inhibitory effect on MCF-7 cells. According to MTT assay data, Daidzein–Isonicotinamide cocrystal's in vitro anticancer activity was significant against MCF-7 breast cancer cell line. Evidence that cocrystal reduced cell viability in a concentration dependent manner shows that cocrystal could be a better formulation of Daidzein for breast cancer activity. These promising findings warrant further mechanistic studies and in vivo experiments to evaluate its clinical potential.

3.9 IC_{50} Analysis of Doxorubicin and Daidzein Cocrystal Against MCF–7 Cells.

Half-maximal inhibitory concentration (IC_{50}) is a pharmacological parameter that describes the concentration of a test compound that causes a 50% inhibition of cell viability or biological activity in a

defined experimental system. In the field of anticancer research, the IC_{50} is a crucial measure of the cytotoxicity of a compound to cancer cell lines. The cell viability MTT, SRB, Alamar Blue assay-based dose-response curves often determine effective dose of anti-cancer agents. A lower IC_{50} value indicates a greater cytotoxic effect and anticancer potency, whereas a higher IC_{50} value indicates lower inhibition. Consequently, IC_{50} is frequently utilized for evaluating the therapeutic potential of pure drugs, co-crystals, and new drug formulations in in vitro cancer studies.

log(inhibitor) vs. normalized response -- Variable slope	**Doxorubicin**	**CAS-48**
Best-fit values		
LogIC50	0.9795	2.854
HillSlope	-0.2893	-0.2819
IC50	**9.539±0.16**	**714.9±0.15**
Std. Error		
LogIC50	0.1679	0.1525
HillSlope	0.04662	0.04252
95% CI (asymptotic)		
LogIC50	0.5478 to 1.411	2.462 to 3.246
HillSlope	-0.4091 to -0.1694	-0.3912 to -0.1726
IC50	3.530 to 25.77	289.8 to 1764
Goodness of Fit		
Degrees of Freedom	5	5
R squared	0.9021	0.926
Sum of Squares	137.7	87.84
Sy.x	5.247	4.191
Number of points		
# of X values	7	7
# Y values analyzed	7	7

The MTT assay was employed to determine the cytotoxic potential of the standard drug Doxorubicin as well as Daidzein–Isonicotinamide cocrystal (CAS-48) against MCF-7 human

breast cancer cell line. We used nonlinear regression analysis (log inhibitor vs normalized response variable slope) to evaluate the dose-response data and derive the half-maximal inhibitory concentration (IC_{50}). The $LogIC_{50}$ of Doxorubicin was 0.9795 which is equivalent to an IC_{50} of 9.539 ± 0.16 µg/mL (95% CI: 3.530–25.77 µg/mL). The Hill slope observed was −0.2893 indicating a concentration-dependent cell viability loss. This indicates that the model selected properly describes the experimental data with an R^2 value of 0.9021. In comparison, the Daidzein cocrystal (CAS-48) had a $LogIC_{50}$ value of 2.854, which corresponds to an IC_{50} of 714.9 ± 0.15 µM (95% CI: 289.8–1764 µM). The slope (-0.2819) of hill is comparable to that of Doxorubicin, indicating concentration-dependent inhibition, however, at much higher concentrations. The fitted dose–response curve was highly reliable (R^2 = 0.9260). The goodness-of-fit is excellent. Doxorubicin has well-known effective cytotoxic activity on MCF-7 breast cancer cells due to low IC_{50} value. The Daidzein cocrystal had an antiproliferative

activity of moderate measure, it only reach 50% at high concentration. Despite this, the cytotoxic effect obtained shows that the activity of Daidzein is maintained in the formulation of cocrystal which could also lead to an enhancement in properties such as aqueous solubility, dissolution rate and bioavailability as compared to pure drug. The enhanced physicochemical parameters may improve the therapeutic potential of daidzein which showed higher IC_{50}. The good values of R^2 (>0.90) for both samples show they are in good agreement with the experimentally observed data, and nonlinear regression model thus confirming the IC_{50} determination made is robust.

Conclusion:

The MTT assay demonstrated that both Doxorubicin and the Daidzein–Isonicotinamide cocrystal inhibited the proliferation of MCF-7 breast cancer cells in a concentration-dependent manner. Doxorubicin exhibited superior cytotoxic activity with an IC_{50} value of 9.539 ± 0.16 µg/mL, whereas the Daidzein cocrystal showed an IC_{50} of 714.9 ± 0.15 µM, confirming moderate but significant antiproliferative activity. The high coefficient of determination (R^2 > 0.90) for both dose-response curves demonstrates excellent model fitting and reliable IC_{50} estimation. Although the cocrystal was less potent than the standard chemotherapeutic agent, it retained the anticancer activity of Daidzein while offering the potential advantages of enhanced solubility and bioavailability through cocrystal formation. These findings support the further investigation of the Daidzein–Isonicotinamide cocrystal in advanced preclinical studies, including apoptosis assays, cell-cycle analysis, mechanistic investigations, and in vivo breast cancer models, to establish its therapeutic potential as a safer phytopharmaceutical candidate for breast cancer management.

Acknowledgement I acknowledge the guidance provided by Dr Manju Pandey and Dr Brijesh Singh for conducting this study, I also want to thanks Shree Ramswaroop Memorial University, Barabanki, Lucknow, Deva Road India , and Ashoka Institute of Technology and Management ,Varanasi for providing suitable environment for smooth conduction of study.

Area of conflict Author has no area of conflict in this study.

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