EVALUATION OF ANTICANCER ACTIVITY (HCC) IN *NELUMBO NUCIFERA (NELUMBONACEAE)* FLOWER EXTRACT BY *INVITRO* SCREENING METHOD

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ABSTRACT

Nelumbo Nucifera, a revered traditional medicine has been scientifically investigated for its potential therapeutic application in treating Hepatocellular Carcinoma (HCC), a devastating form of liver cancer. A Comprehensive phytochemical analysis revealed the presence of an array of bioactive compounds, including alkaloids, flavonoids, polyphenols, tannins and terpenoids. The extract demonstrated an impressive spectrum of biological activities, encompassing enhanced cell viability, potent antiproliferative effects, robust antioxidant properties and significant anti-inflammatory activity. The antioxidant efficacy was meticulously evaluate using the DPPH (2,2 diphenyl-1-picrylhydrazyl) method, while the DLA (Dalton's Lymphoma assay) method was employed to investigate apoptotic cell death. Additionally, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cytotoxicity, proliferation, and viability of (Hepg2-cell line) cells. The extracts potential anti-HCC activity was rigorously analyzed using UV spectrophotometry (200-400nm) and quality control tests, including thin – layer chromatography (TLC). This study provides compelling statistical data supporting the therapeutic potential of Nelumbo Nucifera against HCC, underscoring its promise as a novel adjunctive treatment strategy for this debilitating disease.

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Introduction:

Nelumbo Nucifera, also known as the sacred lotus, is a plant that has been used in traditional medicine for centuries. Recent studies have shown that it possesses anticancer properties, making it a potential adjunct therapy for various type of cancer.

Nelumbo Nucifera contains several bioactive compounds, including the presence of Alkaloids such as Iso-quinoline and Aporphine alkaloids have been shown to inhibit cancer cell growth and induce apoptosis and the presence of Flavonoids such as Quercetin and Kaempferol have anti-oxidant and anti-inflammatory property which can help prevent cancer cell proliferation and the presence of Phenolic acids such as Ferulic acid and Sinapic acid have been shown to inhibit cancer cell growth and induce apoptosis.

Anti-Cancer mechanisms studies have demonstrated that *Nelumbo Nucifera* extracts and isolated compounds can inhibit cancer cell growth by inducing cell cycle arrest and apoptosis and prevent Angiogenesis by inhibiting the formation of new blood vessels that feed cancer

cells and enhance chemotherapy by increasing the sensitivity of cancer cells to chemotherapy. And also reduce inflammation by inhibiting pro inflammatory cytokine and enzymes.
Hepatocellular carcinoma (HCC) is a type of cancer that originates in the liver's hepatocytes, which are the main cell type of the liver. HCC is characterized by Uncontrolled cell growth, Invasion and Metastasis and Dysplasia and architectural distortion. Symptoms of Hepatocellular Carcinoma are Abdominal swelling and pain, Dark urine and Pale stools, Fatigue, Fever, Itching, Jaundice, Loss of appetite, Nausea and vomiting, Weight loss**.**
Chronic Hepatitis B, Chronic Hepatitis C, Liver Cirrhosis, Alcohol consumption, Smoking, Genetic disorders, Hepatitis D virus, Hepatitis E virus and Schistosomiasis are the common risk factors. Imaging tests (Ultra- sound CT-scan, MRI-scan), Biopsy-Tissue culture, Blood tests-CBC, LFT and Viral markers test are the diagnosis of Hepatocellular Carcinoma and the treatment carries Surgical resection, Liver transplantation, Loco-reginal therapies and Systemic therapies.

MATERIAL AND METHODS:

PLANT MATERIAL:

The flowers of Nelumbo Nucifera were collected from a local botanical garden. The collected flowers were authenticated by a Scientist "F" & Head of Office in Tamil Nadu Agricultural University which is located in Coimbatore and were washed thoroughly with distilled water to remove any dirt or impurities. The flowers were then air dried at room temperature.

Preparation of Plant Material

1. Collect fresh lotus flowers depending on the desired extract.

2. Clean the plant material thoroughly with distilled water.

3. Dry the plant material using a dryer or air dryer to remove excess moisture.

Solvent Selection and Extraction

1. Choose a suitable solvent, such as ethanol or methanol, depending on the desired compound to be extracted.

2.Weigh the flower.

3. Add the chosen solvent to the extraction vessel, ensuring the plant material is completely submerged

4. Grind the flower with suitable solvent of 1:3 ratio.

5. Seal the extraction vessel and allow the mixture to steep for a specified period (e.g., 24-48 hours).

6. Filter the resulting extract using a filter paper.

STATISTICAL ANALYSIS:

UV

The UV spectroscopy was performed using the sample at 200nm,300nm,400nm.

TLC

TLC was performed for the individual components such as

Alkaloids	Nuciferine:λmax=280-300nm(0.567)
Flavonoids	Quercetin: λmax=255-265nm (0.579) Kaempferol: λmax=230-255nm (0.568)
Phenolic Acids	FerulicAcid:λmax=280-300nm (0.5789) GallicAcid:λmax=210-220nm (0.567)

INVITRO CYTOTOXICITY SCREENING METHOD:

- DPPH Assay - to investigation of antioxidant property
- DLA Assay - to investigation of apoptotic cells
- MTT Assay - to investigation of cell viability, Proliferation and Cytotoxicity.

DPPH Assay

DPPH radical scavenging assay

Materials required

1. 2.2, -Di phenyl -1-pieryl Hydroxyl Hydrate

2. Methanol

3. Test material

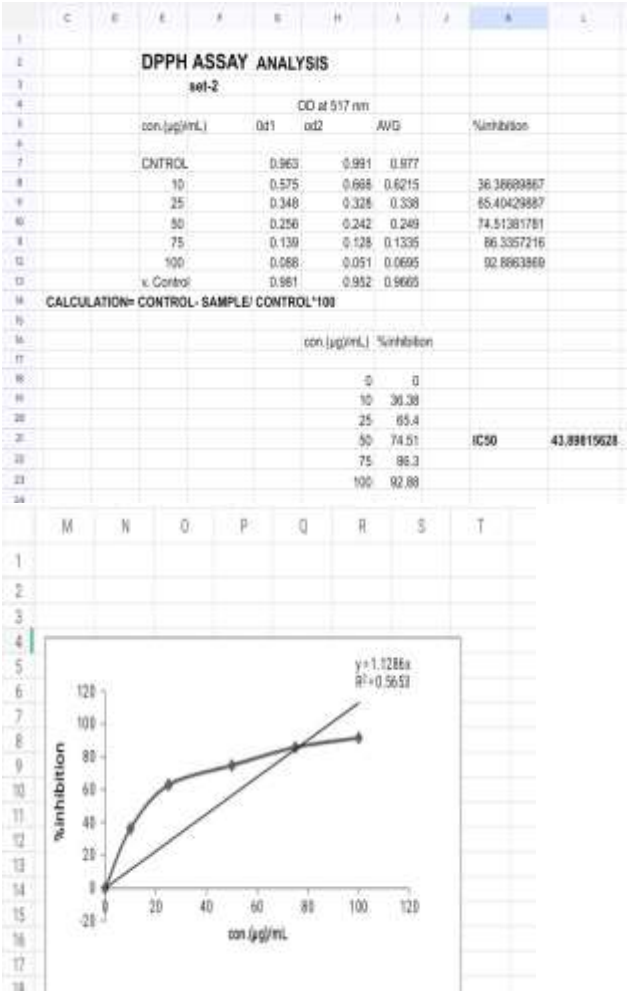
DPPH solution was prepared fresh (0.304 mM; 3 mg in 25 ml of Methanol). The freshly prepared DPPH reagent was kept in the dark bottle at room temperature for One to two hours to stabilize the reagent.

Procedure

About 187µl of DPPH and different concentrations (10-100µg/ml) of test samples were made up to 1ml with methanol (Final concentration of DPPH solution is 0.056 mM). The tubes were then kept in dark for 20 minutes at RT ant thereafter optical measurement was made at 517nm against methanol as blank. Percentage inhibition was calculated with respect to Reading of control tubes.

Calculation % of Inhibition:

(O.D of control – O.D of sample) / O.D of control x 100



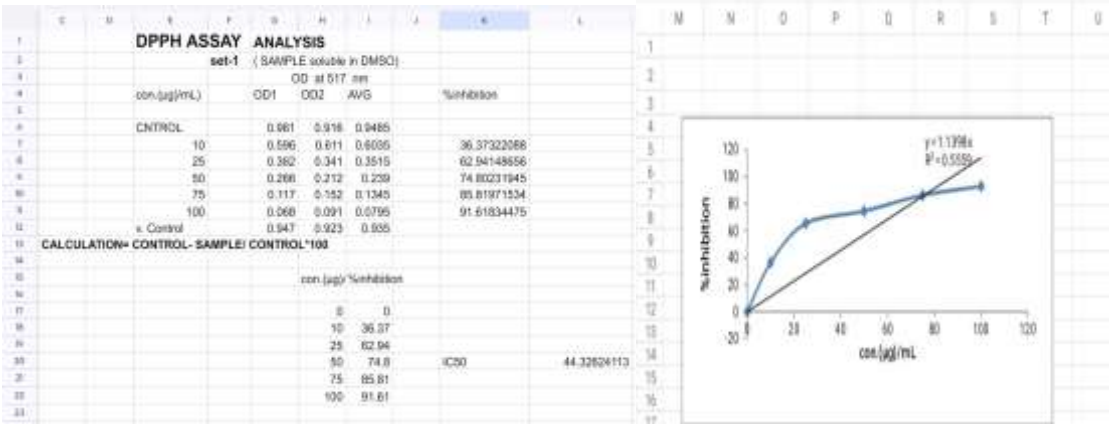
DPPH ASSAY ANALYSIS

set-2

con.(µg/mL)	OD at 517 nm od1	od2	AVG	%inhibition
CNTROL	0.963	0.991	0.977	
10	0.575	0.668	0.6215	36.38689867
25	0.348	0.328	0.338	65.40429887
50	0.256	0.242	0.249	74.51381781
75	0.139	0.128	0.1335	86.3357216
100	0.068	0.051	0.0695	92.8863869
v. Control	0.981	0.952	0.9665	

CALCULATION= CONTROL- SAMPLE/ CONTROL*100

con.(µg/mL)	%inhibition		
0	0		
10	36.38		
25	65.4		
50	74.51	IC50	43.89815628
75	86.3		
100	92.88		



DPPH ASSAY ANALYSIS

set-1 (SAMPLE soluble in DMSO)

con.(µg/mL)	OD1	OD2	AVG	%inhibition
CNTROL	0.961	0.916	0.9485	
10	0.596	0.611	0.6035	36.37322088
25	0.362	0.341	0.3515	62.94148656
50	0.266	0.212	0.239	74.80231945
75	0.117	0.152	0.1345	85.81971534
100	0.068	0.091	0.0795	91.61834475
c. Control	0.947	0.923	0.935	

CALCULATION= CONTROL- SAMPLE/ CONTROL*100

con.(µg)	%inhibition		
0	0		
10	36.37		
25	62.94		
50	74.8	IC50	44.32624113
75	85.81		
100	91.61		

DLA Assay:

The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS or normal cell line. Cell viability was determined by trypan blue exclusion method. Viable ceps suspension ($1x10^6$ cells in 0.1ml) was added to tubes containing various concentrations of the test compounds and the volume was made up to 1ml using phosphate buffered cell line (PBS). The control tube contained only cell suspension. These assay mixtures were incubated for 3 hours at 37^0C. Further cell suspension was mixed with 0.lml of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. In contrast to living cells, dead cells absorb the blue hue of trypan blue. Separate counts of labelled and unstained cells were made.

$$\% \text{ cytotoxicity} = \frac{\text{No. of dead cells}}{\text{No. of live cells} + \text{No. of dead cells}} \times 100$$

Drug concentration (µg/ml)	% Cell Death
12.5	3.57±0
25	3.57±0
50	3.78±0.4
100	7.09±0.7
150	10.5±1.9
200	14.8±1.8

	A	B	C	D	E	F	G	H
1	DLA CELL LINE							
2								
3	Drug con (µg/ml)							
4	200	1	2	3	4	AVG	SD	
5	Live cells	87	95	92	90			
6	Dead cells	18	14	15	16			
7	Total no. of cells	105	109	107	106			
8	% cytotoxicity	17.14285714	12.8440367	14.01869159	15.09433962	14.77498126	1.828594913	
9	150							
10	Live cells	99	102	104	102			
11	Dead cells	15	10	11	12			
12	Total no. of cells	114	112	115	114			
13	% cytotoxicity	13.15789474	8.928571429	9.565217391	10.52631579	10.54449984	1.861932893	
14	100							
15	Live cells	105	105	105	104			
16	Dead cells	9	7	8	8			
17	Total no. of cells	114	112	113	112			
18	% cytotoxicity	7.894736842	6.25	7.079646018	7.142857143	7.091810001	0.6723316055	
19	50							
20	Live cells	108	108	108	108			
21	Dead cells	5	4	4	4			
22	Total no. of cells	113	112	112	112			
23	% cytotoxicity	4.424778761	3.571428571	3.571428571	3.571428571	3.784766119	0.4286750948	
24	25							
25	Live cells	108	108	108	108			
26	Dead cells	4	4	4	4			
27	Total no. of cells	112	112	112	112			
28	% cytotoxicity	3.571428571	3.571428571	3.571428571	3.571428571	3.571428571	0	
29	12.5							
30	Live cells	108	108	108	108			
31	Dead cells	4	4	4	4			
32	Total no. of cells	112	112	112	112			
33	% cytotoxicity	3.571428571	3.571428571	3.571428571	3.571428571	3.571428571	0	
34								
35								

MTT Assay:

1. MTT reagent (the solution is filtered through a 0.2 µm filter and stored at 2–8°C for frequent use or frozen for extended periods)

2. DMSO

3. CO2 incubator

4. Micro Plate reader

5. Inverted microscope

6. Refrigerated centrifuge

Preparation of test solutions

This experiment was used to create repeated two-fold dilutions (6.25–100 µg) for the MTT assay.

Cell lines and culture medium

HepG2 cell line was procured from NCCS, stock cell was cultured in MEM medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO_2 at 37 ^{0}C until confluent.

Procedure

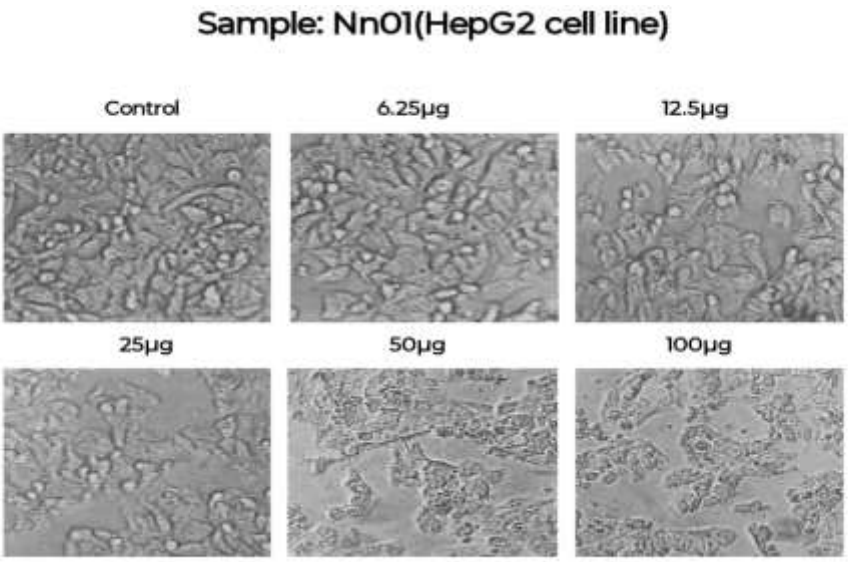
The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 105 cells/mL using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100 µL of the diluted cell suspension (1 x 104 cells/well) was added. When

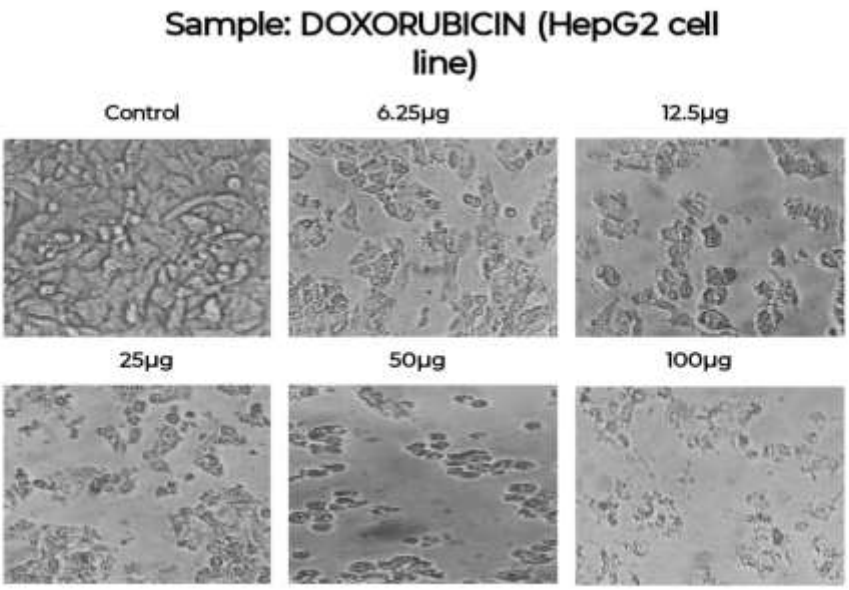
a partial monolayer had developed after 24 hours, the supernatant was removed, the monolayer was once again cleaned with medium, and 100 µL of test material at various concentrations was applied to the microtiter plates.. The plate was then incubated at 37^2C for 24 h in 5% CO_2 atmosphere. After incubation the test solutions in the wells were discarded and 20 µL of MTT (2 mg/1 mL of MTT in PBS) was added to each well. The plate was incubated for 4 h at 37oC in 5% CO_2 atmosphere. After removing the supernatant, 100 µL of DMSO was added, and the plate was gently shaken to dissolve the formazan that had formed. The absorbance was measured using a microplate reader set at 570 nm.

Percentage viability = Sample absorbance / Control absorbance x 100

MTT assay - HepG2 cell line

		OD at 570 nm			% viability					
Sample	Concentration	Singlet	Duplicate	Triplicate	Singlet	Duplicate	Triplicate	Mean	SD	IC50 (µg)
Control	0	1.378	1.361	1.353	100	100	100	**100**		
Nn101	**6.25**	1.292	1.307	1.311	93.7S907112	96.03232917	96.89578714	**95.56239581**	1.620300859	
	12.5	1.168	1.153	1.14	84.7605225	84.71711978	84.25720621	**84.57828282**	0.2789080661	
	25	0.826	0.809	0.81	59.94194485	59.44158707	59.86696231	**59.75016474**	0.2696531643	73.12
	50	0.763	0.741	0.735	55.3701016	54.44526084	54.32372506	**54.71302916**	0.5722769204	
	100	0.666	0.674	0.692	48.25834543	49.52240999	51.14560237	**49.64211926**	1.447346153	
DOXORUBICIN	**6.25**	0.706	0.721	0.714	51.16110305	52.97575312	52.77161863	**52.30282493**	0.9940142699	
	12.5	0.623	0.649	0.635	45.21044993	47.68552535	46.93274205	**46.60957244**	1.268790185	8.77
	25	0.519	0.536	0.52	37.66328012	39.38280676	38.4331116	**38.49306616**	0.8613297168	
	50	0.378	0.353	0.361	27.43105951	25.93681117	26.68144863	**26.68310644**	0.7471255486	
	100	0.206	0.228	0.214	14.94920174	16.75238795	15.81670362	**15.8394311**	0.9018079227	





RESULT:

1.DPPH

The sample showed dose depended DPPH scavenging efficacy with in the range of 10-100 µg/ml. The average IC50 values observed in two different assay is 42.5 µg/ml (excel file provided) this suggest the DPPH radical reduction potential of the sample supplied.

2.DLA

Using Dalton's lymphoma ascites cells (DLA), the test compound's short-term in vitro cytotoxicity was investigated. The test compound was dissolved in DMSO and concentration range between 200 µg/ml to 12.5 µg/ml was used for the study. Control tube contains 3 dead cells.

3.MTT

IC50 value of the given test sample Nn01 and DOXORUBICIN were found to be 73.12µg, and 8.77µg.

DISCUSSION:

The present study investigated the antioxidant, cytotoxic, and antigenotoxic potential of Nelumbo nucifera flower extract (Nn01). The results demonstrated that Nn01 possesses moderate antioxidant activity, as evidenced by its DPPH radical scavenging efficacy. The extract also exhibited cytotoxic activity against cancer cells, although its potency was lower compared to the positive control, Doxorubicin. Furthermore, the DLA assay results suggested that Nn01 may have a protective effect against DNA damage or genotoxicity. The findings of this study are consistent with previous reports on the pharmacological

activities of Nelumbo nucifera. The antioxidant and cytotoxic properties of Nn01 may be attributed to the presence of bioactive compounds such as flavonoids, phenolic acids, and alkaloids. These compounds may play a crucial role in protecting cells against oxidative stress, inflammation, and damage caused by free radicals. The results of this study have implications for the development of novel therapeutic agents against cancer and other diseases associated with oxidative stress and DNA damage. However, further studies are necessary to fully elucidate the mechanisms of action underlying the observed activities and to evaluate the in vivo efficacy and safety of Nn01. In conclusion, the present study demonstrates the potential of Nelumbo nucifera flower extract as a natural antioxidant, anticancer, and antigenotoxic agent. Further research is warranted to unlock its therapeutic potential and to explore its potential applications in medicine.

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