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Original Research Article

Nephroprotective Effects of *Spirulina platensis* on NRK-52E Cell Line: LC-HRMS and Docking Studies Targeting Epidermal Growth Factor Receptor

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ABSTRACT

Diabetic nephrotoxicity is a significant health concern that may lead to end-stage renal disease. This study explored the nephroprotective potential of *Spirulina platensis* extract fractions on NRK-52E (Normal Rat Kidney-52E) cell lines. *Spirulina platensis* underwent Soxhlet extraction using methanol as the solvent. The extracted material was then subjected to column chromatography for the purpose of separating the phytoconstituents. This process successfully yielded four distinct fractions: n-hexane, dichloromethane, ethyl acetate, and methanol. HR-LCMS (High-Resolution Liquid Chromatography Mass Spectrometry) analysis revealed a rich phytochemical profile (methanol: 79 compounds, ethyl acetate: 51, dichloromethane: 32, n-hexane: 19). Molecular docking (AutoDock tool) highlighted several compounds with high EGFR binding affinity, including Atalanine from the ethyl acetate fraction (score -11.9 kcal/mol), indicating potential EGFR (Epidermal Growth Factor Receptor) inhibition, a known factor in diabetic nephropathy. NRK-52E cells were treated with varying concentrations of these fractions, and % inhibition was assessed using the MTT assay. Fractions of methanol (10,40,100 µg/mL), ethyl acetate (10,40,100 µg/mL), and dichloromethane (40,100 µg/mL) demonstrated the most significant ($p<0.001$), while n-hexane (100 µg/mL) showed significant ($p<0.05$) protective effects on NRK-52E cells. Methanol fraction exhibited the strongest effect (% inhibition of 36.725 ± 6.54 at 100 µg/mL). Morphological evaluations of NRK-52E cells demonstrated a decreasing cell growth trend in response to the fractions of *Spirulina platensis*, listed in descending order of effect: methanol, ethyl acetate, dichloromethane, and *n*-hexane. The results indicate that *Spirulina platensis*, particularly the methanol fraction obtained through extraction, shows promise as a natural therapeutic for addressing diabetic nephrotoxicity.

Keywords*:Spirulina platensis*, Nephroprotective Effects, NRK-52E Cell Line, EGFR, HR-LCMS..

Introduction

Diabetes is a chronic metabolic disorder that affects millions of people worldwide and is associated with various complications, including nephrotoxicity. Nephrotoxicity is a major complication of diabetes and is characterized by progressive renal dysfunction, leading to end-stage renal disease if left untreated. [1] One potential strategy for preventing or treating diabetic nephrotoxicity is using natural compounds. [2] EGFR is a transmembrane receptor protein that is crucial in regulating cell proliferation, survival, and differentiation. Previous studies have shown that EGFR signaling is altered in diabetic nephropathy and may contribute to the development and progression of diabetic kidney disease (DKD). In DKD, hyperglycemia and other metabolic abnormalities can lead to oxidative stress, inflammation, and fibrosis in the kidney, resulting in progressive renal dysfunction. EGFR signaling is involved in these processes, and targeting EGFR has been proposed as a potential therapeutic strategy for DKD.[3, 4]

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Previous studies have indicated that algae such as Chlorella vulgaris, Ulva lactuca, and Porphyra yezoensis exhibit potential nephroprotective effects in the context of diabetic kidney disease. [5, 6, 7] These algae contain common metabolites such as phycocyanin, chlorophyll, carotenoids, vitamins, minerals, polysaccharides, and essential fatty acids. These metabolites, known for their antioxidant properties, have been suggested to play a role in attenuating oxidative stress and inflammation in the kidneys, thereby potentially contributing to the management of diabetic kidney disease. [8, 9, 10, 11]

Spirulina platensis is a blue-green alga shown in previous studies to have various potential health benefits, including antioxidant, anti-inflammatory, and anti-diabetic effects. [12]

NRK-52E cell lines have been widely used as a model system for studying renal epithelial cell biology and kidney disease. The study was aim to investigate the potential nephroprotective effects of *Spirulina platensis* on NRK-52E cell lines.[13,14]

To further investigate the potential molecular mechanisms underlying the effects of *Spirulina platensis* extract, LC-HRMS was used to identify the phytochemical compounds in the extracts and followed by docking studies using EGFR as a target protein to predict the potential binding interactions between the active compounds. Overall, this study will provide important insights into the potential nephroprotective effects of *Spirulina platensis* extract in diabetic nephrotoxicity and may help identify novel therapeutic targets and compounds for treating diabetic kidney disease.[15,16]

Material and Methods

Collection and authentication:
The algae, *Spirulina platensis*, belonging to the family Phormidiaceae, was collected on November 2018 from the Pravara Institute of Research and Education in Natural and Social Sciences (PIRENS), Krishi Vigyan Kendra (KVK), Bhableshwar, Tal-Rahata, Dist-Ahmednagar, Maharashtra (GPS location: 19.60972432135021, 74.50565297676332). After collection, the sample was authenticated by the Botanical Survey of India (BSI), Pune 411001. The authentication certificate (Ref No-BSI/WRC/100-1/DEN.CER/2018/101, Specimen No.204830) confirmed the sample to be *Spirulina platensis* (Nordstedt) Geitler 1925, belonging to the family Phormidiaceae.

Soxhlet extraction and fractionation:
The obtained *Spirulina platensis* samples were air-dried at room temperature and finely ground. A 20 g of the powdered samples underwent Soxhlet extraction with 250 mL of methanol as the solvent, lasting 24 hours. The extract was then filtered using Whatman filter paper (No.42). The solvent was evaporated under reduced pressure employing a rotary evaporator (1297, Dolphin, Mumbai, India), yielding the crude methanolic extract. The crude extract was subjected to column chromatography to segregate phytoconstituents from non-polar to polar. Elution was carried out using solvents n-hexane (Sigma Aldrich, analytical grade, anhydrous, 95%), dichloromethane (Sigma Aldrich, analytical grade, anhydrous, 99.8%), ethyl acetate (Sigma Aldrich, analytical grade, anhydrous, 99.8%), and methanol (Sigma Aldrich, analytical grade, anhydrous, 99.8%). Elution continued until no spots appeared on thin-layer chromatography for each solvent, signifying the successful separation of the compounds in the extract into their respective fractions. [17, 18]

Phytochemical analysis (HR-LCMS)
HR-LCMS was employed to identify and characterize the phytochemical constituents present in the *Spirulina platensis* extract fractions. The analysis used an Agilent HR-LCMS (model 6200 series TOF/6500 series Q-TOF B.09.00 (B9044.1 SP1), Agilent and California, U.S.) metabolite screening system with optimized chromatographic and mass spectrometric conditions.

Identification of Compounds by Matching with METLIN Database
After obtaining the HR-LCMS data in negative mode, the detected mass-to-charge (m/z) ratios of the phytochemical constituents in the *Spirulina platensis* extract fractions were compared to the METLIN Metabolite Database (https://metlin.scripps.edu). The search parameters were set within a predefined mass tolerance to account for potential mass deviations. Candidate compounds were further validated by comparing their retention times and fragmentation patterns with available reference standards. This approach enabled the comprehensive identification and characterization of the bioactive phytochemicals in the *Spirulina platensis* extracts. [19, 20, 21]

Selection of Target Receptor
In this study, the EGFR was selected as the target receptor for investigating nephrotoxic diabetic activity, given its significant role in the progression of diabetic nephropathy. The crystal structure of EGFR (PDB ID: 3W32) was utilized to gain insights into the receptor's activation and interaction with potential drug candidates. Through a rational drug design approach, novel compounds were identified and optimized, targeting EGFR to modulate its activity and prevent diabetic nephropathy progression. Molecular docking was employed to assess these compounds' binding affinity and stability with EGFR. This selection strategy enabled the identification of promising therapeutic agents for treating diabetic nephropathy. [22, 23]

Docking studies:
Molecular docking studies were conducted to evaluate the binding interactions between the identified phytochemical compounds and target proteins implicated in Nephroprotective Effects. The three-dimensional structures of the target proteins were retrieved from the Protein Data Bank (PDB). In contrast, the structures of the phytochemicals were drawn using ChemDraw software and optimized using Chem3D software (v.16). Docking simulations were performed using the AutoDock Vina software (Auto Dock Vina v.1.2.0) with default parameters. The generated docking poses were then analyzed using Discovery Studio Visualizer (v.4.5) to assess the binding affinities, conformations, and key interactions between the ligands (phytochemical compounds) and the target proteins. To further illustrate the binding interactions, two-dimensional interaction plots were generated using LigPlot++ software (v.2.2). [24, 25, 26]

Cell line
The NRK-52E cell line was used as an *in vitro* model in our study. The cells were cultured DMEM with high glucose (Cat No-11965-092), FBS (Gibco, Invitrogen) Cat No -10270106 Antibiotic – Antimycotic 100X solution (Thermo Fisher Scientific)-Cat No-15240062. The cells were maintained at 37°C in a 5% CO_2 incubator (Thermo Scientific BB150,Massachusetts, U.S.). Cells at the exponential stage were used for experimentation, and the medium was changed every 2-3 days.

In Vitro Nephroprotective effect: MTT Assay
Cells were incubated at 1×10^4 cells/mL in a culture medium for 24 hrs at 37°C and 5% CO_2. Cells were seeded at 1×10^4 cells/well in 100 µL culture medium into 96-well microplates (tissue culture grade). Fractions of n-hexane, dichloromethane, ethyl acetate, and methanol were added (10, 40, 100 µg/mL) to the wells along with IC_{50} of 5-FU to induce cytotoxicity. Control wells were incubated with 0.2% DMSO in PBS and the cell line. All samples were prepared in triplicate. Controls were maintained to determine the control cell survival and the percentage of live cells after culture. Cell cultures were incubated for 48 hrs at 37°C and 5% CO_2 in a CO_2 incubator (Thermo Scientific BB150, Massachusetts, U.S.). After incubation, the medium was completely removed, and 20 µL of MTT reagent (5 mg/mL in PBS) was added to each well. The cells were incubated for another 4 hrs at 37°C in the CO_2 incubator. Wells were observed for formazan crystal formation under a microscope (SGL-11 B- Digital Microscope, Miraj, India). Viable cells reduced the yellow MTT to dark-coloured formazan crystals. The medium was removed completely, and 200 µL of DMSO was added to each well to dissolve the formazan crystals. The plates were incubated at 37°C for 10 min while wrapped in aluminum foil to protect them from light. The absorbance of each sample was measured in triplicate using an ELISA microplate reader at a wavelength of 570 nm (Benesphera E21, India). The percent cytotoxicity (% inhibition) was calculated using equation number 1. [27, 28, 29]
Equation number 1:

$$\%\text{Cytotoxicity} = \frac{(\text{OD control sample} - \text{OD test sample})}{\text{OD control sample}} \text{x}100$$

Statistical analysis
The results of parametric data are expressed as mean±SD n=3 and were tested with two-way ANOVA followed by Dunnett's multiple comparisons test (GraphPad Prism 8. Ink) was performed. [30]

Result and Discussion

Phytochemical Identification and Molecular Docking
The HR-LCMS analysis of *Spirulina platensis* extract fractions demonstrated the presence of various phytochemical constituents within the different solvent fractions. The methanol fraction contained the highest number of compounds, with a total of 79 (Figure 1 and Table 1), followed by the ethyl acetate fraction, which displayed 51 compounds (Figure 2 and Table 2). The dichloromethane fraction came next, exhibiting 32 compounds (Figure 3 and Table 3), while the n-hexane fraction revealed the fewest, with 19 compounds (Figure 4 and Table 4). The identified compounds through HR-LCMS analysis were subjected to molecular docking studies using the target protein EGFR (PDB ID: 3W32). The binding affinities, conformations, and interactions between the identified compounds and the target protein were investigated, leading to the recognition of potential lead compounds exhibiting high binding affinities (Table 1 to 4). Three-dimensional (3D) and two-dimensional (2D) interactions of the compounds were reported, with docking scores exceeding -9.5 kcal/mol. (Table 5 and Figure 5 to 15). [31, 32, 33] In the methanol fraction, four compounds showed a docking score above -9.5 kcal/mol and

significant 2D and 3D interaction (Figure 5 to 8), out of which Corchoroside B, with a score of -10.8 kcal/mol, has the highest binding affinity (Table 1). Compound, (3b,5a,6b,22a,25R)- Furostane-22-methoxy3,6,26-triol 3-[glucosyl- (1->2)-[xylosyl-(1->3)]- glucosyl-(1->4)- galactoside] 26-glucoside belonging to class of steroidal glycoside. This class, steroidal glycoside, compounds has been reported to exhibit nephroprotective effects in rats. Chinenoside IV, a type of steroidal saponin, has shown great potential in various areas, including antifungal properties, cytotoxicity, anti-inflammatory effects, antithrombotic activity, and hypocholesterolemic effects. Nigericin has been reported to exhibit remarkable activity in effectively addressing factors such as glucose levels, dyslipidaemia, oxidative stress, and antioxidant enzyme activities. Nigericin was identified in both the methanol and ethyl acetate fractions (Table 1 and 2).[34,35,36]

Table 1: List of compounds identified in Methanol Fraction with molecular docking score

Sr.No.	Name	Retention time (minutes)	Docking Score (kcal/mol)
1	Thalassemine	2.787	-6.3
2	L-Arginine phosphate	2.821	-6
3	trans-S-(1-Propenyl)-L cysteine	3.314	-4.8
4	Isoamyl isothiocyanate	3.42	-4.6
5	S-Prenyl-L-cysteine	3.987	-5.6
6	Sch 59884	6.583	-8.4
7	Fluvoxamine	10.238	-7.3
8	(1xi,3S)-1,2,3,4-Tetrahydro-1-methyl beta-carboline-1,3-dicarboxylic acid	10.443	-7.7
9	Maculosin	11.377	-7.8
10	Methylthio 2- (propanoyloxy)propanoate	12.476	-5.4
11	Furegrelate	12.52	-8.5
12	Dihydro-2-methoxy-2- methyl-3(2H)- thiophenone	13.044	-5.3
13	Zanamivir	14.51	-6.9
14	3,3'-Dimethoxybenzidine	15.054	-8.7
15	(+/-)-Isobutyl 3- methylthiobutyrate	15.24	-4.7
16	(+)-2,7- Dideoxypancratistatin	15.385	-6.7
17	3,3'-Dimethylbenzidine	16.619	-8.6
18	Lycoricidine	18.033	-8.4
19	1,4-beta-D-Glucan	18.073	-7.2
20	Ptelatoside A	18.712	-8.2
21	Hydroxypropyl cellulose	18.723	-6.9
22	Methionyl butyrate	18.756	-6.1
23	4-Hydroxydiphenylamine	19.196	-7.6
24	7-(3-Methylbut-2-enyl)- L-tryptophan	19.516	-8.5
25	Nigericin	19.551	-9.8
26	Molinate	19.722	-5.8
27	Dubamine	19.782	-8.9
28	Ascorbigen	19.874	-8.4
29	Methyl-2-alpha-L-fucopyranosyl-beta-D-galactoside	20.097	-6.2
30	Tetrahydrofurfuryl	20.751	-7.5
31	PE(22:4(7Z,10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16 Z,19Z))	21.186	-6.9
32	Corchoroside B	21.735	-10.8
33	N-Acetyl-leucyl-leucine	21.737	-6.3
34	dolichyl D-xylosyl phosphates	22.487	-8.3
35	Cadiamine	23.85	-8.6
36	3-[4-Hydroxy-3-(3- methyl-2- butenyl)phenyl]-2- propenal	23.904	-7.8

37	4,4'-dihydroxy-3,5- dimethoxydihydrostilbene	25.883	-8.1
38	Oseltamivir	25.992	-6.6
39	Dihydroshikonofuran	26.809	-8.5
40	Vinaginsenoside R12	27.371	-8.6
41	(E,E)-Lansamide I	29.514	-8.8
42	Pisumoside B	29.965	-8.8
43	(3b,5a,6b,22a,25R)- Furostane-22-methoxy3,6,26-triol 3-[glucosyl- (1->2)-[xylosyl-(1->3)]- glucosyl-(1->4)- galactoside] 26-glucoside	31.231	-9.5
44	Geranylbenzoquinone	31.405	-8.3
45	Coumachlor	31.41	-7.4
46	(E,E)-1,6-bis(4- methoxyphenyl)-1,5- hexadiene	33.055	-7.5
47	Quillaic acid 3- [galactosyl-(1->2)- glucuronide]	36.436	-9
48	Purothionin AII	36.641	-7.9
49	TR-Saponin C	38.1	-7.8
50	Tragopogonsaponin G	38.134	-9.4
51	Ipecac (Emetamine)	39.811	-8.9
52	Tylosin	40.942	-7.6
53	N-Acetyl-leu-leu-tyr	41.002	-8.5
54	Ivermectin B1b	41.27	-9.4
55	Leu-leu-tyr	41.76	-8.3
56	Nebramycin factor 4	41.819	-7.4
57	Actinonin	42.287	-7.4
58	Glaucarubol 15-O-betaD-glucopyranoside	42.33	-8
59	Melilotoside D	42.424	-8.8
60	alpha-Amylcinnamyl isovalerate	42.863	-7.2
61	1-Methyl-2-nonyl-4(1H)- quinolinone	43.206	-7.8
62	Chinenoside IV	43.293	-10.2
63	Talinolol	43.786	-8.6
64	Belladonnine	44.026	-7.9
65	17-Methyl-18- norandrosta-4,13(17)- dien-3-one	45.228	-8.8
66	DG(22:6(4Z,7Z,10Z,13Z, 16Z,19Z)/16:0/0:0)	46.164	-8.4
67	3-Oxo-delta1-steroid	47.302	-6.7
68	Leucyl-leucyl-norleucine	47.534	-8.9
69	DG(15:0/22:6(4Z,7Z,10Z ,13Z,16Z,19Z)/0:0)	48.482	-8.1
70	3-(8,11,14- Pentadecatrienyl)phenol	48.613	-7.4
71	Perindopril	48.699	-7.5
72	Lucidenic acid L	49.834	-8.7
73	(x)-p-Menth-1-en-4-yl 5- isopropyl-2-methylphenyl ether	50.556	-6.1
74	RU 5135	50.56	-9.2
75	Lamtidine	51.011	-8.4
76	Ipecoside	51.57	-8.8
77	Citronellylbetasophoroside	51.826	-7.4
78	Cannabisativine	53.022	-8.1
79	Ginsenoside Rg5	57.32	-9.2

Table 2: List of compounds identified in Ethyl Acetate Fraction with molecular docking score

Sr.No	Name	Retention time (minutes)	Docking Score (kcal/mol)
1	3-(3'- Methylthio)propylmalic acid	2.514	-6.6
2	Lactucin	5.59	-8.3
3	Molinate	12.123	-5.7
4	Cerberoside	12.491	-8.4
5	(Ac)2-L-Lys-D-Ala-D-Ala	12.497	-6.9
6	N-Acetyl-D-galactosamine 1- phosphate	12.706	-6.2
7	Sanguisorbin E	13.233	-8.9
8	5,6-Dihydro-4-methoxy6-[2-(4- methoxyphenyl)ethyl]- 2H-pyran-2-one	13.515	-5
9	PE(20:3(5Z,8Z,11Z)/22:6 (4Z,7Z,10Z,13Z,16Z,19Z))	13.731	-6.8
10	Atalanine	13.742	-11.9
11	Haplopine	13.748	-6.9
12	Nigericin	13.803	-9.8
13	4-Ethyl-2-propylthiazole	13.932	-5.1
14	4-Oxoglutaramate	14.316	-5.4
15	7-(3-Methylbut-2-enyl)- L-tryptophan	14.322	-8.4
16	Sinalexin	14.329	-6.2
17	Hexanethioic acid S-propyl est	14.536	-5.2
18	L-Thyronine	14.94	-8.3
19	N-Acetyl-leucyl-leucine	15.228	-6.4
20	4,4'-dihydroxy-3,5- dimethoxydihydrostilbene	16.533	-7.1
21	cis- and trans-LMercapto-p-menthan-3- one	16.944	-7.7
22	Lansiumamide A	17.308	-8.3
23	4-tert-Butylphenyl salicylate	18.288	-8.6
24	Vinaginsenoside R12	18.512	-8.6
25	[10]-Dehydroshogaol	18.632	-8.1
26	(E,E)-Lansamide I	18.68	-8
27	Alfentanil	18.772	-8.4
28	17-Hydroxyprogesterone	19.513	-8.5
29	Muricoreacin	19.519	-7.1
30	Lemobiline	19.749	-8.3
31	Lamtidine	19.749	-7.1
32	Dehydroaporheine	19.859	-9.3
33	N(6)-(Octanoyl)lysine	19.907	-6.4
34	alpha-Amylcinnamyl isovalerate	20.371	-7.1
35	Oseltamivir	20.472	-6.6
36	Linalyl benzoate	20.778	-7.2
37	Linalyl phenylacetate	22.028	-7.9
38	4-Nerolidylcatechol	22.349	-8.4
39	17-Methyl-18- norandrosta-4,13(17)- dien-3-one	23.574	-8.8
40	(Z,Z)-2,9,16- Heptadecatriene-4,6- diyn-8-ol	23.696	-6.9
41	Arachidonyl Trifluoromethyl Ketone	25.02	-8
42	4-Acetyl-6-tert-butyl-1,1- dimethyl indane	25.534	-7.7
43	Talinolol	26.733	-8.7
44	2-Undecyl-4(1H)- quinolinone	27.033	-8
45	Trandolapril	27.185	-8.2
46	DG(20:4(8Z,11Z,14Z,17Z)/22:6(4Z,7Z,10Z,13Z,16 Z,19Z)/0:0)	27.211	-7.9

47	Ramipril	28.313	-7.9
48	2-Octaprenyl-6-methoxy-1,4-benzoquinon	28.503	-6.3
49	3-Oxo-delta1-steroid	28.542	-8.9
50	3-(8,11,14- Pentadecatrienyl)phenol	29.165	-7.6
51	DG(20:2(11Z,14Z)/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	29.257	-7.8

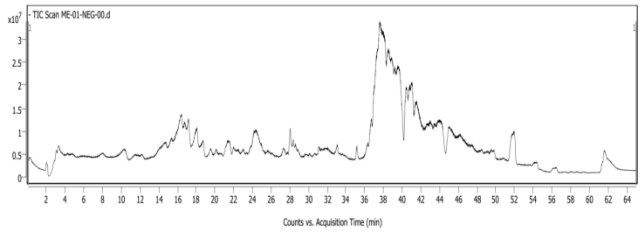


Figure 1: HR-LCMS profile of the methanol fraction from *Spirulina platensis*

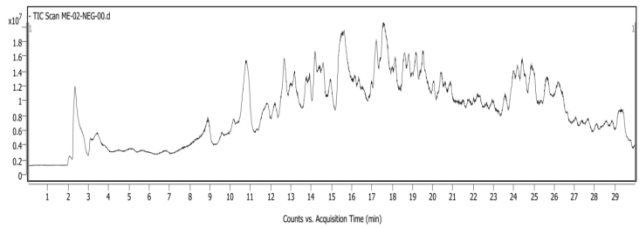


Figure 2: HR-LCMS profile of the ethyl acetate fraction from *Spirulina platensis.*

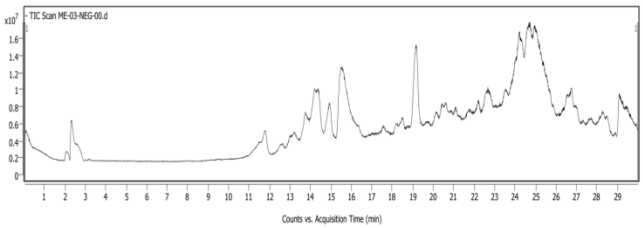


Figure 3: HR-LCMS profile of the dichloromethane fraction from *Spirulina platensis*.

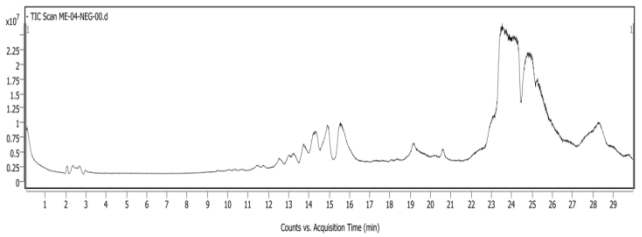


Figure 4: HR-LCMS profile of the n-hexane fraction from *Spirulina platensis.*

In the ethyl acetate fraction, two compounds showed a docking score above -9.5 kcal/mol and significant 2D and 3D interaction (Figure 8 to 9), out of which Atalanine showed the highest docking score of -11.9 kcal/mol from all four fractions (Table 2). The highest binding affinity compound Atalanine interacts with active amino acids, including ASP837, ASP855, UNL1, ARG841, ALA722, LEU844, PRO877, LYS745, and LEU718. These interactions involve different types of bonds, including electrostatic, hydrogen, and hydrophobic (Table 5 & Figure 9). Previous studies show that Atalanine contributes to antidopaminergic and antiadrenergic activities.[37]

In the dichloromethane fraction, four compounds showed a docking score above -9.5 kcal/mol and significant 2D and 3D interaction (Figure 10 to 13), out of which Withaperuvin H, with a score of -10.5 kcal/mol, has the highest binding affinity (Table 3). Licorice saponin B2 has demonstrated anti-diabetic potential. Withaperuvin H is classified as a withanolide compound, which is known for its reported anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective effects.[38, 39]

In the n-Hexane fraction, two compounds showed a docking score above -9.5 kcal/mol and significant 2D and 3D interaction (Figure 14 to 15), of which 6-alpha-Fluoro-17-beta-hydroxyandrost-4-en-3- one acetate with a score of -10.1 kcal/mol has a highest binding affinity (Table 4). Sanguisorbin E is classified as a tannin compound, which has been reported to possess the ability to scavenge free radicals.[40]

Evaluation of Nephroprotective Effect of Spirulina platensis Fractions and 5-FU on NRK-52E Cells

The potential nephroprotective effects of the fractions obtained from *Spirulina platensis* and 5-FU were evaluated in NRK-52E cells through the utilization of the MTT assay, as presented in Table 6. IC_{50} value of 5-FU was found to be 32.07 µg/mL. The nephroprotective effects by MTT assay revealed that methanol fractions (10, 40, 100 µg/mL), ethyl acetate fractions (10, 40, 100 µg/mL), and dichloromethane fractions (40, 100 µg/mL) showed the most significant protective effects ($p<0.001$) on NRK-52E cells. The n-hexane fraction at a 100 µg/mL concentration also showed significant ($p<0.05$) protective effects. However, the dichloromethane fraction at 10 µg/mL and n-hexane fractions at 10 and 40 µg/mL concentrations demonstrated non-significant ($p>0.05$) effects. The methanol fraction exhibited the most pronounced protective effect at all tested concentrations. The highest % inhibition was observed at 100 µg/mL (36.725 ± 6.54), indicating its relatively lower cytotoxicity than other fractions and 5-FU. This was followed by the ethyl acetate fraction and dichloromethane fractions, which showed a significant inhibition rate at all concentrations compared to the 5-FU control (Table 6). But the fraction of n-hexane did not reach the same level of inhibition as other fractions.

Assessment of Cell Viability of NRK-52E Cells after MTT Assay

The cell viability was photographed and evaluated (Figure 16). The results revealed that the Methanol, Ethyl Acetate, Dichloromethane, and n-Hexane fraction of *Spirulina platensis* had shown decreasing order of cell growth. In contrast, the normal cells treated with 5-FU were severely affected and resulted in a decrease in cell growth. The increase in the number of phytochemicals in each fraction directly correlated with the enhanced nephroprotective effects.

Conclusion

The research focused on evaluating the nephroprotective capabilities of *Spirulina platensis* fractions against diabetic nephrotoxicity using the NRK-52E cell line. Four fractions were obtained and analyzed using HR-LCMS, revealing multiple phytochemical constituents. Molecular docking studies indicated potential EGFR inhibition by compounds in the methanol, ethyl acetate and dichloromethane fractions. The MTT assay showed concentration-dependent nephroprotective effects, with the methanol and ethyl acetate fractions exhibiting the highest protection. These findings highlight the potential of these fractions as therapeutic agents for diabetic nephropathy, warranting further research for confirmation and understanding of their mechanism of action.

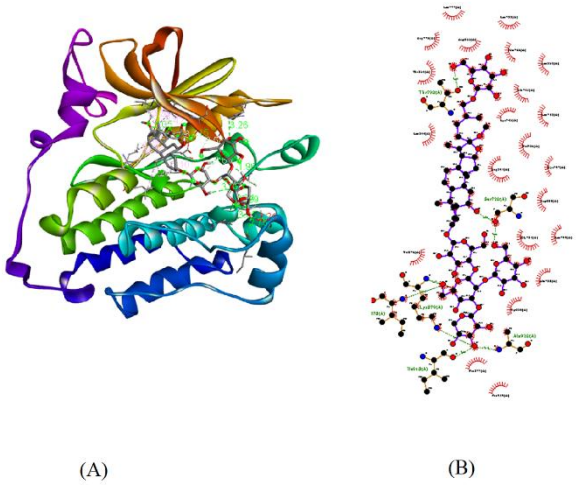
Table 3: List of compounds identified in Dichloromethane Fraction with molecular docking score

Sr.No.	Name of compound	Retention time (minutes)	Docking Score (kcal/mol)
1	Isofraxidin	2.297	-6.4
2	(Ac)2-L-Lys-D-Ala-D-Ala	12.509	-7.4
3	Licoricesaponin B2	13.768	-9.6
4	4-Oxoglutaramate	14.269	-5.4
5	N-trans-p-Coumaroyloctopamine	16.966	-9
6	5-Methylbarbiturate	17.015	-5.9
7	Koeniginequinone B	17.031	-8
8	Skimmianine	17.382	-6.7
9	[10]-Dehydroshogaol	17.596	-8.1
10	Arborinine	18.125	-8.1
11	Quinacridone	18.284	-10.2
12	Elaterinide	18.728	-9.7
13	Dehydroxymethylflazine	19.153	-9.4
14	Lamtidine	19.748	-8.3
15	Juzirine	20.156	-8
16	7- (Methylthio)heptanenitrile	20.235	-4.8
17	alpha-Amylcinnamyl isovalerate	20.386	-7.2
18	4-Nerolidylcatechol	20.512	-8.3
19	(E,E)-1,6-bis(4- methoxyphenyl)-1,5- hexadiene	20.591	-7.5
20	Tetrahydrogestrinone	21.206	-8.7
21	a-Tetrasaccharide	22.572	-7.1
22	S-Prenyl-L-cysteine	22.688	-6.9
23	7,8-Dihydrovomifoliol 9- [rhamnosyl-(1->6)- glucoside]	22.718	-7.6
24	3'-N-Debenzoyltaxol	23.101	-7.9
25	Tributyl phosphate	24.734	-5.1
26	6-[2,3-Dihydroxy-1- (hydroxymethyl) propyl]- 1,2-dihydro-7-hydroxy-9- methoxycyclopenta[c][1]benzopy ran-3,4-dione	24.961	-8.3
27	Withaperuvin H	25.127	-10.5
28	LysoPE(0:0/18:2(9Z,12Z))	25.41	-6.5
29	Hydroxyhomodestruxin B	25.41	-6.9
30	Prosulfocarb	27.031	-6.6
31	(4beta,5beta,6beta,14be ta,15alpha,20S,22R) -5,6- Epoxy-4,14,15- trihydroxy-1-oxowitha2,24-dienolide	28.314	-9.4
32	Norpropoxyphene	29.163	-6.8

Table 4: List of compounds identified in n-Hexane Fraction with molecular docking score

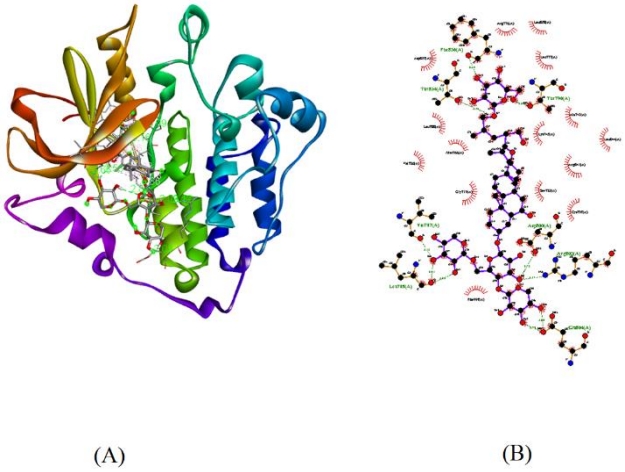
Sr.No.	Name of compound	Retention time (minutes)	Docking Score (kcal/mol)
1	L-Arginine phosphate	2.26	-5.9
2	Thalassemine	2.319	-6.3
3	Isofraxidin	2.454	-6.4
4	Maculosin	8.963	-8.8
5	1,2-Benzisothiazol-3(2H)-one	9.547	-6.3
6	3-Carboxy-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-1- propanoic acid	9.601	-7.7

7	AminoDAHP	10.64	-5.7
8	1-(9H-Pyrido[3,4-b]indol1-yl)-1,4-butanediol	11.735	-7.4
9	1-O-[2-(L-Cysteinamido)-2-deoxy-alpha-D-glucopyranosyl]-1D-myo-inositol	11.773	-7.4
10	Lycoricidine	12.317	-8.4
11	2-Oxo-8- methylthiooctanoic acid	13.337	-5.2
12	Sanguisorbin E	13.76	-9.6
13	[10]-Dehydroshogaol	17.624	-8.3
14	Lycaconitine	18.062	-8
15	Linalool oxide D 3- [apiosyl-(1->6)- glucoside]	20.101	-8.1
16	Ustiloxin D	20.629	-6.4
17	Purothionin AII	23.295	-8.2
18	Septentriodine	23.847	-8
19	6alpha-Fluoro-17betahydroxyandrost-4-en-3-one acetate	27.918	-10.1



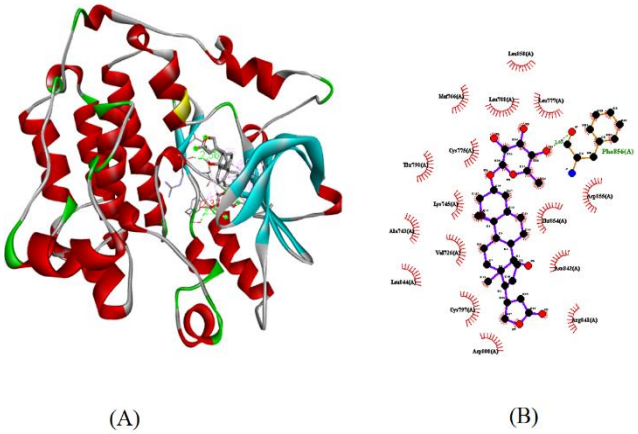
(A) (B)

Figure 5: The 2D (A) and 3D (B) interaction of compound (3b,5a,6b,22a,25R)- Furostane-22-methoxy3,6,26-triol 3-[glucosyl- (1->2)-[xylosyl-(1->3)]- glucosyl-(1->4)-galactoside] 26-glucoside with target protein EGFR (PDB ID: 3W32)



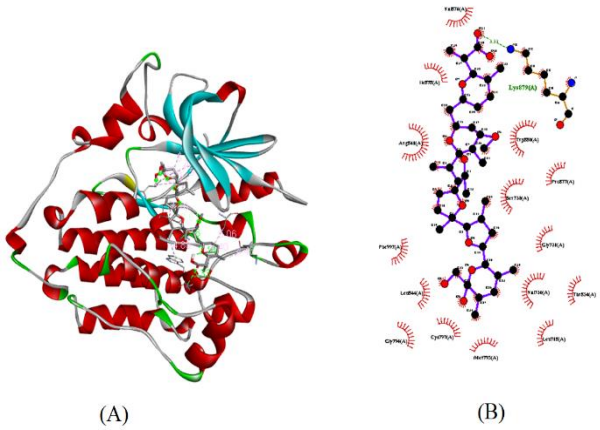
(A) (B)

Figure 6: The 2D (A) and 3D (B) interaction of compound Chinenoside IV with target protein EGFR (PDB ID: 3W32)



(A) (B)

Figure 7: The 2D (A) and 3D (B) interaction of compound Corchoroside B with target protein EGFR (PDB ID: 3W32



(A) (B)

Figure 8: The 2D (A) and 3D (B) interaction of compound Nigericin with target protein EGFR (PDB ID: 3W32).

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 5: Summary of molecular docking interactions between selected compounds that shows the highest binding affinity and their respective active amino acids, bond types, and docking scores

Compound Name	Active Amino acid	Bond Type	Docking Score
(3b,5a,6b,22a,25R)- Furostane-22-methoxy3,6,26-triol 3-[glucosyl- (1->2)-[xylosyl-(1->3)]- glucosyl-(1->4)- galactoside] 26-glucoside	ARG748,THR790,ILE878,LYS879,LYS879 SER720,UNL1:O27,UNL1:O23, ILE918,UNL1,UNL1,GLY721,UNL1, ASP855,VAL876,VAL726 ,ALA743, ACYS797 ,ARG841 ,LEU799,LEU718, CYS797,LEU844,LEU844,VAL726.	Hydrogen Bond, Hydrophobic Bond	-9.5
Chinenoside IV	THR790,ARG803,THR854,PHE856 VAL717,LEU718,UNL1,GLU804, ASP800,UNL1,THR854,THR854, VAL726 ,VAL726 ,ALA743 ,ALA743, CYS797 ,LEU844,CYS797,LEU844, LEU844,LYS745,LEU788.	Hydrogen Bond, Hydrophobic Bond	-10.2
Corchoroside B	PHE856,ARG841,VAL726 ,VAL726, VAL726 ,VAL726 ,ALA743 ,LYS745 , LYS745 ,LEU844,CYS775,PHE856.	Hydrogen Bond, Hydrophobic Bond	-10.8
Nigericin	ARG841,LYS879,UNL1,ARG841, ALA722 ,VAL726 ,VAL726 ,ALA743 , ARG841 ,LEU844 ,PRO877 ,LEU844, LEU844,LYS875,TRP880.	Hydrogen Bond, Hydrophobic Bond	-9.8
Atalanine	ASP837,ASP855,UNL1,ASP855, UNL1 ,ARG841,ALA722,LEU844, ALA722 ,PRO877,LYS745,LEU718.	Electrostatic, Hydrogen Bond, Hydrophobic Bond	-11.9
Elaterinide	LYS745,THR854,ASP855,PHE856 ASP800,LEU718,CYS797 ,LEU844 LEU777,LEU788	Hydrogen Bond, Hydrophobic Bond	-9.7
Licoricesaponin B2	ASP994,SER991, ARG841 ,ASN842, ILE878, UNL1,UNL1,PRO877 LYS879 ,LEU799,ARG841,LYS879	Hydrogen Bond, Hydrophobic Bond	-9.6
Quinacridone	ASP855,THR854,LEU788,MET766 VAL726,ALA743,LYS745, LEU777 LEU858,LEU718,VAL726,ALA743 LEU844	Electrostatic, Hydrogen Bond, Hydrophobic Bond, Others	-10.2
Withaperuvin H	GLY719,THR854,ASN842,VAL726 , ALA743 ,ARG841 ,VAL726,LYS745, LEU844,VAL726,LYS745,LEU788, LEU858,LEU777.	Hydrogen Bond, Hydrophobic Bond,	-10.5
6alpha-Fluoro-17betahydroxyandrost-4-en-3- one acetate	CYS797,THR854,ASP855,THR854, VAL726 ,VAL726 ,VAL726 ,ALA743, CYS797,THR854,ASP855,THR854, VAL726	Hydrogen Bond, Hydrophobic Bond,	-10.1
Sanguisorbin E	ALA722,ARG841,ARG841,THR854 GLY721,VAL726 ,LEU718, VAL726	Hydrogen Bond, Hydrophobic Bond,	-9.6

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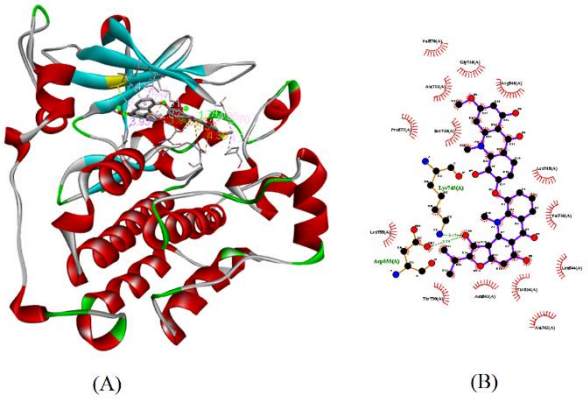
Table 6: Results of Nephroprotective Effect of *Spirulina platensis* Fractions and 5-FU on NRK-52E Cells

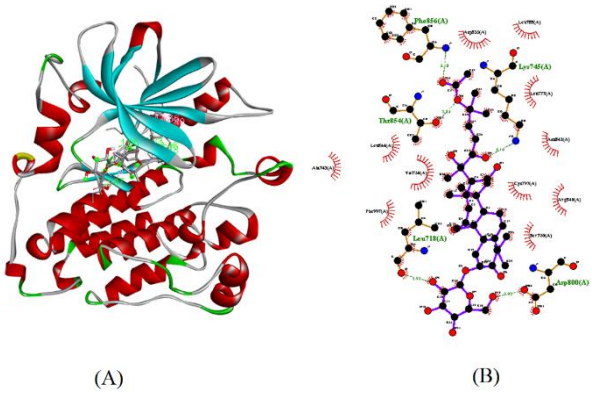
Concentration (µg/mL)	% Inhibition				
	5-FU	*Spirulina platensis* Fractions			
		n-Hexane	Dichloromethane	Ethyl Acetate	Methanol
10 µg/mL	71.9 ± 2.95	80.20 ± 2.95^{ns}	64.58 ± 2.48^{ns}	53.54 ± 3.11***	46.76 ± 2.03***
40 µg/mL	80.34 ± 3.1	75.14 ± 3.1^{ns}	58.905 ± 3.31***	51.715 ± 3.72***	43.805 ± 3.59***
100 µg/mL	84.42 ± 4.86	71.44 ± 4.86*	58.79 ± 4.87***	47.255 ± 3.69***	36.725 ± 6.54***

Data are presented as mean ± S.D.; n = 3. Two-way ANOVA with Dunnett's multiple comparisons test was performed; the p-value was <0.0001, considered extremely significant. Significance levels in comparison with 5-FU: ***p < 0.001; **p < 0.01; *p < 0.05; ns (non significant) p > 0.05.



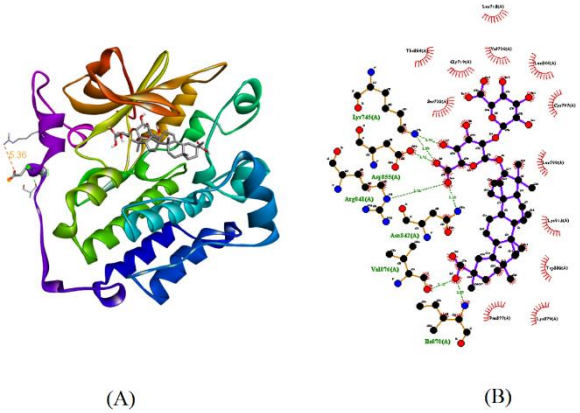
(A) (B)

Figure 9: The 2D (A) and 3D (B) interaction of compound Atalanine with target protein EGFR (PDB ID: 3W32)



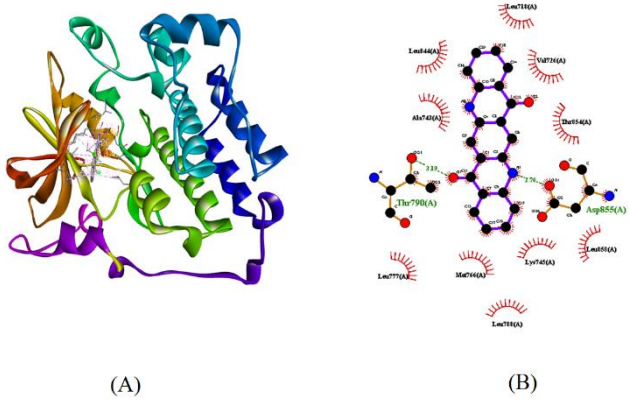
(A) (B)

Figure 10: The 2D (A) and 3D (B) interaction of compound Elaterinide with target protein EGFR (PDB ID: 3W32)



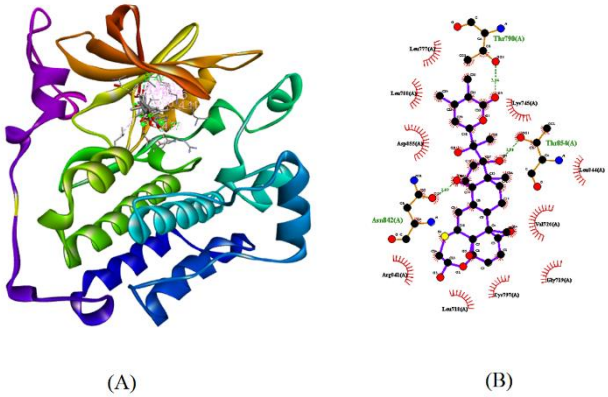
(A) (B)

Figure 11: The 2D (A) and 3D (B) interaction of compound Licoricesaponin B2with target protein EGFR (PDB ID: 3W32)



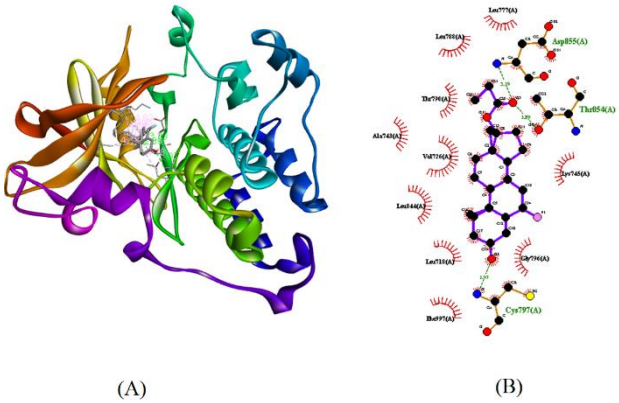
(A) (B)

Figure 12: The 2D (A) and 3D (B) interaction of compound Quinacridone with target protein EGFR (PDB ID: 3W32)



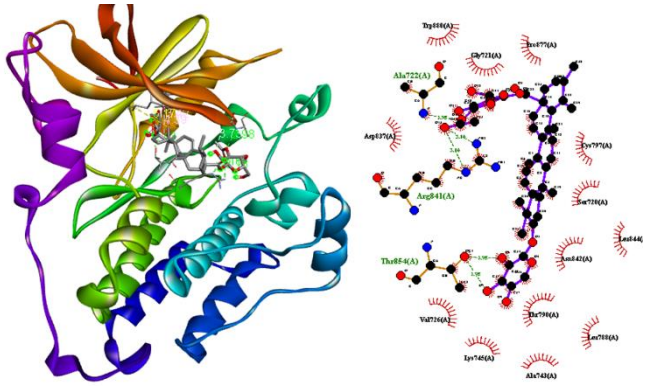
(A) (B)

Figure 13: The 2D (A) and 3D (B) interaction of compound Withaperuvin H with target protein EGFR (PDB ID: 3W32)



(A) (B)

Figure 14: The 2D (A) and 3D (B) interaction of compound 6alpha-Fluoro-17betahydroxyandrost-4-en-3-one acetate with target protein EGFR (PDB ID: 3W32)



(A) (B)

Figure 15: The 2D (A) and 3D (B) interaction of compound Sanguisorbin E with target protein EGFR (PDB ID: 3W32)

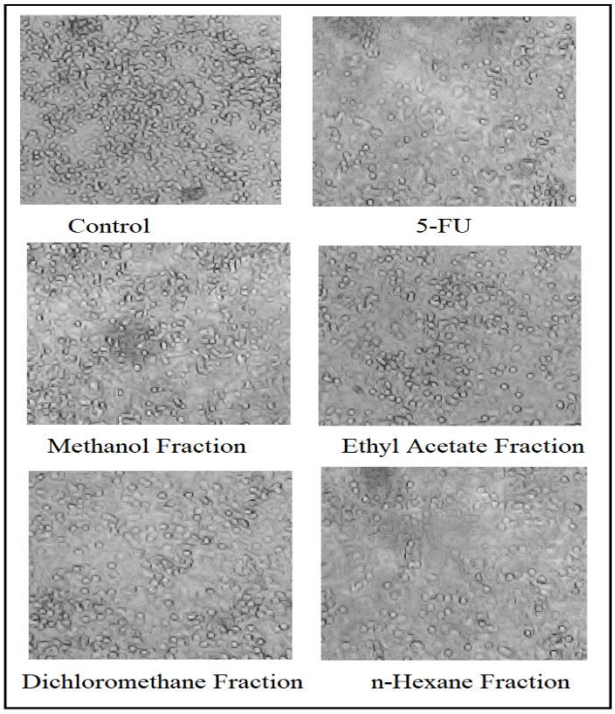


Figure 16: Cell viability of NRK-52E Cells after MTT Assay

Conflict of Interest

The authors declare no conflict of interest.

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