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Invitro And Insilico Study With GC-MS Profiling for Assessment of Antioxidant and Anticancer Activity of The Methanolic Extracts of Annona Squamosa L. Leaves

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ABSTRACT

Annona Squamosa L, (sugar apple or custard apple) belongs to tiny plant- family Annonaceae is a tropical plant species, known for its sweet edible fruits and is a reach source of secondary metabolites, (Quercetin, kaempferol, Catechin, Epicatechin, Epigallocatechin gallate etc) .In this study we have performed the phytochemical screening, Total phenolic, flavonoid and terpenoid contents Antioxidant activity, anti-cancer and cytotoxic activity of methanolic leaf extracts from *Annona Squamosa* L.. The free radical scavenging activities were investigated through H_2O_2 assay. To evaluate the anticancer and cytotoxic activity MTT Assay was performed. In this study we have done GC-MS and LC-MS to find out the name and % abundance of flavonoid of interest and from the result we have find out the presences of following flavonoid compounds those are most useful to treat different type of cancer and Endometriosis for its angiogenesis and antifibrotic properties. (1) Kaempferol, (2) Epigallocatechin (EGC), (3) Quercetin and (4) Epigallocatechin – 3 – Gallate (EGCG). Molecular docking method was employed to find out the anticancer activity of the phytoconstituents. Ethnopharmacological study (the study of interrelation between humans and plants including plants used as food, medicine and in other human applications) revels the information of different plant part and their useful preparation method. At the same time in vitro and in vivo studies and clinical tests will provide the proof of evidence that will support the ethnopharmacological report.

Keywords: *A. Squamosa Polyphenols, MTT Assay, Antioxidant, Cytotoxic, Anti-cancer. Ethnopharmacological study. Molecular docking.*

1. INTRODUCTION

Nowadays cancer is one of the leading causes of death throughout the world. Several cytotoxic, immunotherapeutic and chemotherapeutic agents are used to treat the cancer. These drugs are more expensive and associated with Sevier side effects. To avoid these side effects continuous search is going on for an ideal treatment to beat the cancer with minimal side effects and in cost effective way. The *Annona Squamosa* L leaf extracts contain some flavonoids, terpenoids and polyphenolic compounds like kaempferol, Quarcetin etc. These compounds have some medicinal merits as well as some toxicological effects [1], Traditional and folk medicine has utilized the fruit pericarp and leaf extract of Annonaceae family to cure of variety of tumours and malignancies [1,2] Chemotherapeutics like Vinca Alkaloids and Taxol are obtained from medicinal plant sources, used as anti-cancer drugs widely. Vincristine, Vinblastine, Vindesine etc. are used clinically in the treatment of haematological and lymphatic neoplasms. Similarly In vivo studies of the crude extracts and isolated flavonoid from *A. Squamosa* L and *A. Muricata* L have shown anti-stress, anti-inflammatory, anti-oxidant and anti-tumoral activity [3,6] In addition, crude extracts and isolated polyphenols compounds form A. Squamosa and A. Muricata produces neurotoxic effect in vivo and in vitro [4]. Therefore, further investigation is required for these crude extracts and isolated polyphenols, to identify the magnitude of toxicity, mechanism of Pharmacological action, safety profile, optimal and lethal doges form and side effects. Ethnobotanical study [5]. The main aim to use these kinds of anti-cancer drugs from medicinal plant sources is to provide additive effect to the conventional anti-cancer drugs (Ex. Platinum based chemotherapeutics-Cisplatin, Oxaliplatin, Carboplatin etc.) and to decrease the side effects of chemotherapy and radio therapy. In this regard polyphenols

are used to treat various pathological conditions, as anti-inflammatory, anti-collagenase, anti-oxidant and anticancer etc. [7, 8]. Recently scientist is very interested with polyphenols due to its anti-proliferative effects. In this study we have find out the flavonoids present in *A. Squamosa* L methanolic leaf extract, estimate the phenolic and terpenoids content present, quantify the antioxidant properties (scavenging effects for free radicals) [9,10] and compare the cytotoxic activity [11] with the standard drug paclitaxel by using MTT assay technique of mcf-7 cell line.

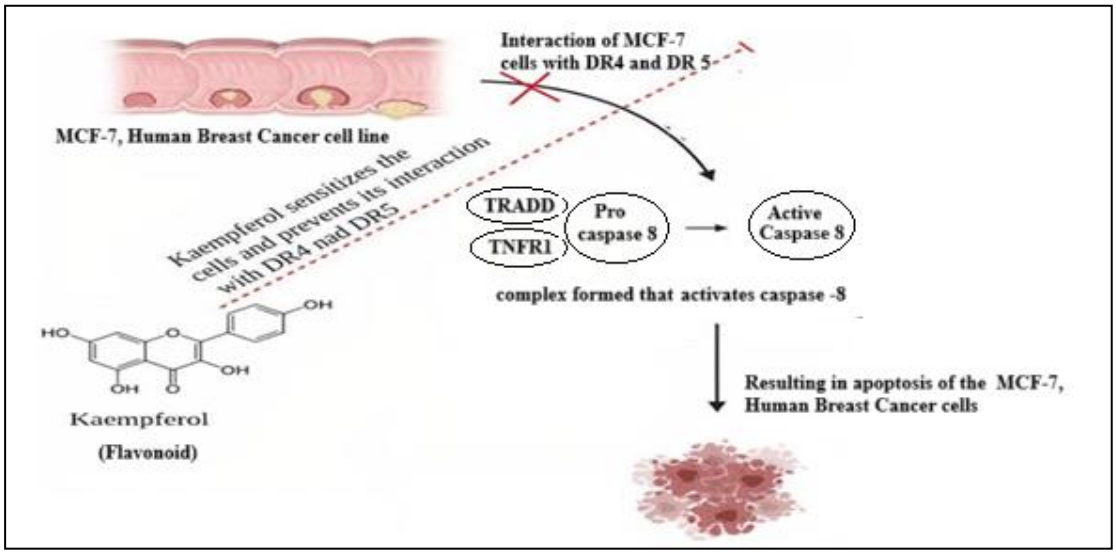


Fig 1: Graphical Abstract

2. MATERIAL AND METHODS

Materials

The extraction procedure made use of methanol (Merck, India), ethanol (Loba Chemie Pvt. Ltd, India), and chloroform (Loba Chemie Pvt. Ltd, India). The phenolic content of the extract was estimated using gallic acid (Loba Chemie Pvt. Ltd, India), the Folin-Ciocalteu Reagent (Loba Chemie Pvt. Ltd, India), and hydrogen peroxide (Merck, India) was employed for the peroxide method of antioxidant activity.

Preparation of extract

The *A. Squamosa* L plant sample, which consisted of leaves, was gathered from local sources, washed with running tap water. The Herbarium identification and authentication was performed through Shibpur Botanical Garden, Howrah, Kolkata. It was then air dried for 7 days in the shade at room temperature, grounded into a fine powder with an electric grinder, and then stored in an airtight container for future use. **For extraction**, the powdered sample was combined with solvents in a 4:1 methanol to water ratio for 24 hours. The substance was then filtered using Whatman No. 1 filter paper, and the filtrate was combined with (2–3) drops of 2M HCl before being combined with an equivalent volume of chloroform. The dried residue was obtained by taking the lower organic layer after it had formed, separated, and then the solvent was evaporated. The organic layer by mechanism is supposed to have flavonoids/phenolic/terpenoids fraction of the extract which then was mixed with distilled water and 1% w/v of Tween 80 solution for the further antibacterial and antioxidant activity [12].

Preliminary phytochemical screening

The extracts were subjected to preliminary phytochemical screening for the detection of various phytoconstituents using standard procedures:

Test for Alkaloids

Around 5 mg of the extract was mixed in 5 ml of distilled water and 2 ml of HCl was included until total precipitation occurred. The filtrate was examined for the nearness of alkaloids as follows.

a) Dragandroff's test: 1ml of Dragandroff's reagent was included to 2 ml of the filtrate along the side of the test tube. Arrangement of a reddish-brown precipitate demonstrated the nearness of alkaloid.

b) Wagner's test: Two drops of Wagner's reagent were included to 1 ml of the test sample along the side of the test tube. The arrangement of yellow or brown precipitate affirmed the nearness of alkaloid.

Test for Flavonoids

Little amount of each extract was warmed with 10 ml of ethyl acetate in boiling water for 3 minutes. The blend was filtered and filtrates are utilized for the taking after tests.

a) Ammonium test: The filtrate was agitated with dilute ammonia solution (1 ml, 1% v/v). The layers were permitted to isolate. A yellow colour watched at ammonia layer shown the nearness of flavonoid.

b) Alkaline reagent test: The extract (2 ml) was attended with few drops of 20% w/v NaOH solution. Arrangement of strongly yellow colour, which turned colourless on the inclusion of diluted HCl demonstrated the nearness of flavonoids.

c) Shinoda test: A number of magnesium turnings and 5 drops of concentrated Hcl were included drop by drop to 1ml of test solutions. A crimson red colour showed up after few minutes, affirmed the nearness of flavonoid.

Test for Phytosterols/ Terpenoids

Liebermann-Burchard's test: 2 mg of the extract was mixed in 2 ml of acetic acid anhydride, warmed to boiling, cooled and after that 1 ml of concentrated H_2SO_4 was included along the side of test tube. A brown ring arrangement at the intersection affirmed the test for the nearness of phytosterols.

Test for Tannins

Ferric chloride test: A couple of drops of 5% w/v $FeCl_3$ were included in to the test extract (2 ml). Presences of bluish black colour showed the nearness of hydrolysable tannin.

Test for Glycosides

Felling test: A number of drops H_2SO_4 was included to the test arrangement (2 ml) and after that 5% NaOH was included for neutralization. At last, Fehling's arrangements A and B were included to the over blend. The above test produces a red colour to demonstrate the positive result.

Test for Phenols

Extracts were attended with 3-4 drops of 10% w/v $FeCl_3$ solution. Appearance of greenish black colour shows the nearness of phenol.

Test for Triterpenoids

Salkowski test: Dry extract (2 mg) was blended with chloroform (1 ml) and a number of drops of Conc. H_2SO_4 were included along the side of the test tube. A red-brown colour shaped at the interface shown the nearness of triterpenoid**.**

Estimation of the Total Phenolic Content

The total phenolic content (TPC) of *A. Squamosa* leaves extracts was estimated by spectrophotometric methods by using Folin-Ciocalteu reagent. A series of gallic acid standard solutions of 10, 20, 30, 40, 50 µg/ml were prepared in test tubes and 1 mL of Folin-Ciocalteu reagent was added to each tube and mixed well. After exactly 5 minutes, 1 mL of 7% sodium carbonate solution was added to each tube or and mixed thoroughly. The solutions were allowed to stand for 2 hours at room temperature, protecting them from light. The absorbance of each solution was measured at 765 nm using UV spectroscopy. A standard curve was prepared by plotting the concentration of gallic acid (µg/mL) on and the corresponding absorbance values on the x-axis and y-axis.

Sample, 100 mg, was weighed and transferred into a test tube. A suitable solvent ethanol or water was added to the test tube to extract the phenolic compounds. The contents were thoroughly mixed and allowed the extraction to proceed for 30 minutes with occasional shaking. The sample extract was centrifuged at a suitable speed and duration to remove any insoluble particles. The supernatant extract was transferred to a new test tube, which was used for analysis. The absorbance of this solution was measured by UV spectroscopy. Calculate the total percentage phenolic content of the sample using the equation obtained from the standard curve. The value obtained of the total flavonoid content were expressed as milligrams of gallic acid equivalent per 100 mg of dry mass [13].

Estimation of the Total Flavonoid Content

Aluminium chloride colorimetric assay was used to measure the total flavonoid content of the extract. A 10 ml volumetric flask keeping 4 ml of distilled deionized water was filled with a portion (1 ml) of extracts or a standard solution of (+)-quercetin (20, 40, 60, 80, 100 µg/ml). 0.3 ml of 5% $NaNO_2$ was put in to the flask. 0.3 millilitre of 10% $AlCl_3$ was added after 5 minutes. Following addition of 2 millilitre of 1M NaOH at the sixth minute, the volume was raised to 10 millilitres using deionized water. The solution was thoroughly mixed, and a Shimadzu UV-Visible spectrophotometer was used to measure the absorbance against a prepared reagent blank at 510 nm with Shimadzu UV-Visible spectrophotometer. The value obtained of the total flavonoid content were expressed as milligrams of (±) quercetin equivalent (QE) per 100 mg of dry mass [14].

Estimation of total terpenoids Content (TTC)

200 µl of extract solutions in methanol (0.1 mg/ml) was first mixed with 1 ml of perchloric acid and 300 µl vanillin/glacial acetic acid (5% w/v) solution. 5 ml of glacial acetic acid was then added to it and the absorbance was measured at 548 nm with a Shimadzu UV-Visible spectrophotometer. Ursolic acid at concentrations (20, 40, 60, 80, 100 µg/ml) were used to generate the standard calibration curve [15].

Evaluation of Anti-oxidant activity

The antioxidant activity of *A. Squamosa* leaves extracts was measured in vitro on the basis of the scavenging activity of hydrogen peroxide In a test tube, a fixed volume of 2.5 ml of 1 mM hydrogen peroxide was added and to another test tube a fixed volume of sample dilution 0.5 ml containing the hydrogen peroxide solution. A control tube was included with only the hydrogen peroxide solution without the sample to measure the background reaction. All the reaction mixtures were incubated at a specific temperature for 30 minutes to allow the reaction to proceed. After the incubation period, a suitable stop solution was added to terminate the reaction. Commonly used stop solutions include sulphuric acid, sodium hydroxide, or catalase enzyme. The absorbance of each reaction mixture was measured using a spectrophotometer at 230 nm that corresponds to the absorption peak of hydrogen peroxide. Subtracted the absorbance of the control from the absorbance of each sample to obtain the net absorbance. Ascorbic acid was used as standard compound.

$$\text{Antioxidant activity (\%)} = \frac{A_0 - A_1}{A_0} x100$$

Where A_0 is the absorbance of the plant extract and A_1 is the absorbance of the plant-peroxide sample [16].

Gas Chromatography coupled with Mass Spectrometry

GC-MS analysis was carried out on GC-MS-QP2010 Shimadzu system consisting of a mass spectrometer device coupled to a gas chromatograph using the following parameters: column VF-5MS fussed silica capillary column (30.0m x 0.25mm x 0.25µm, made of 5% phenyl/95% dimethylpolysiloxane), running in electron impact mode at 70eV; helium (99.999%) was utilized as the carrier gas at a steady flow of 1 ml/min, and an injection volume of 0.5µl was used (split ratio of 10:1), with an injector temperature of 2400C and an ion-source temperature of 2000C. The oven temperature was set to rise by 10 0C/min from 70 0C (isothermal for 3 minutes) to 240 0C, with a 9-minute isothermal at 280 0C at the conclusion. Mass spectra were obtained at 70 eV, with pieces ranging from 40 to 440 Da with a scan interval of 0.5 seconds. The GC runs for 40 minutes in total. The compounds have identified according to the comparison of their mass spectra with the reference mass spectra of several libraries like Wiley library, NIST and previously published data from various literature.

***Insilico* Study for Assessment of Cytotoxic Activity**

In silico Study

Molecular docking method was employed to find out the anticancer activity of the phytoconstituents. The active phytoconstituents of the extracts we taken as the ligands and the proteins on the cancer cells were designated as the receptors. A successful inhibition of those receptor proteins by the phytoconstituents was assumed as a prediction of anticancer activity.

Protein preparation

As per the reported mechanism of action of the selected phytoconstituents (ligand), receptor selected was BCL-2 [17, 18] target protein as it plays important role in the life cycle of cancer cells. It was obtained from the RCSB PDB database for docking study. The protein was then processed by eliminating water molecules, internal ligands, removing superfluous chains or heteroatoms, introducing polar hydrogen charges in Discovery Studio Visualizer. After that it was opened in PyRx (Algorithm is same as Auto Dock Vina) and was converted into PDBqt format. Ultimately, the ligand was then placed in the centre of the grid box and the docking process was performed in the presence of previously prepared ligand molecule. Receptors were validated with Discovery Studio Visualizer and Procheck web portal.

Ligand preparation

The bioactive ligand molecules - Quercetin and Kaempferol were selected on the basis of their docking scores with the specific receptor and downloaded from the PubChem directory as 3D Standard Data Format (3D SDF). OPEN BABEL interface was used to translate the ligand from 3D SDF files to Protein Data Bank (PDB) format. These ligand molecules were independently uploaded into the AutoDock Tools (PyRx) during ligand preparation. It was prepared by minimizing the energy of the structure and also by converting it to PDBqt format for the evaluation of the binding affinity (docking score) of the receptor-ligand complex.

Visualization of the Structure

After the completion of the docking process in the PyRx software, best fit of the ligand structure to the receptor surface was identified on the basis of the docking score of less than −7 and RMSD value of 0. The ligand was then placed in the prepared protein structure placed in Discovery Studio Visualizer and the interaction parameters (hydrogen bond formation with

residues, distance, donor-acceptor properties, etc.) were examined and all the non-bond parameters were recorded. The 3-dimensional and 2-dimensional structures of the complex was noted.

Insilico study was carried out with the reported flavonoid / polyphenolic part (ligand) of the plant extracts against the cancer cells (protein / receptor or specific enzyme) used in the *in vitro* study with the help of BIOVIA DISCOVERY STUDIO and AUTODOCK VINA (PyRx) software for the evaluation of specific parameters which was needed for the completion of the study.

Table 1: *In silico* study of Quercetin and Kaempferol obtained from *A. squamosa* L

Reported isolated compound (Ligand)	Activity	Mechanism of action	Protein/ Receptor name	Protein/Receptor specification (PDB ID)
Quercetin	Anticancer	BCL-2 is a protein that helps cancer cells survive by preventing apoptosis (programmed cell death). By binding to BCL-2, Quercetin allows apoptosis to occur, leading to the death of cancer cells.	BCL-2 [17]	6O0K
		Same as 6O0K	BCL-2[17]	4AQ3
Kaempferol	Anticancer	BCL-2 is a protein that helps cancer cells survive by preventing apoptosis (programmed cell death). By binding to BCL-2, Kaempferol allows apoptosis to occur, leading to the death of cancer cells.	BCL-2[18]	2O22
Kaempferol	Anticancer	BCL-2 is a protein that helps cancer cells survive by preventing apoptosis (programmed cell death). By binding to BCL-2, Kaempferol allows apoptosis to occur, leading to the death of cancer cells.	BCL-2[18]	2O22
		Same as 2O22	BCL-2[18]	1G5M

***In vitro* cytotoxicity evaluation of *A. Squamosa* leaf extract against -MCF-7 Cell Line by MTT Assay [19]**

The MTT Assay was used to assess the leaf extracts' cytotoxic effects on the MCF-7 cell line, which was purchased from NCCS Pune. A 96-well plate containing 10,000 cells per well was incubated for 24 hours at 37°C with 5% CO2 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) using an Air-Jackedeted CO2 incubator (Heal Force-HF90). The following day, cells were exposed to varying extract concentrations. To obtain varying quantities in incomplete cell culture medium (without FBS), stock solutions of the materials were made in DMSO and then further diluted. Cells without MTT were referred to as Blank, and cells without treatment were referred to as Control. Following a 24-hour incubation period, the cell culture was supplemented with MTT Solution (5 mg/ml) and incubated for an additional two hours using an air-jacketed CO2 incubator (Heal Force HF90). After removing the culture supernatant at the end of the experiment, the cell layer matrix was dissolved in 100 µl of Dimethyl Sulfoxide (DMSO) and measured at 540 nm using an Elisa plate reader (iMark, Bio-Rad, USA). Graph Pad Prism 6 was used to determine the IC50. Images were taken with an AmScope digital camera (10 MP Aptima CMOS) under an inverted microscope (Olympus ek2). The findings were displayed as Mean ± Standard Error of Mean, or SEM. Mean ± SEM (Standard Error of Mean) was used to represent the 50% inhibitory concentration (IC50).

3. RESULTS AND DISCUSSIONS

Phytochemical Screening

Table 2: Phytochemical screening of *Annona squamosa* L leaves extract

Terpenoids	Alkaloids Test		Glycoside	Flavonoids		Steroid	Phenol	Tannin
Salkowski test	Dragendorff test	Wagner test	Fehling test	Shinoda test	Alkaline reagent	Salkowski test	10% Ferric Chloride test	5% Ferric Chloride test
–	+	+	+	+	+	–	+	+

Table 3: Phytochemical screening of *Ocimum sanctum* leave extract taken as positive control

Terpenoids	Alkaloids Test		Glycoside	Flavonoids		Steroid	Phenol	Tannin
Salkowski Test	Dragendorff test	Wagner test	Fehling Test	Shinoda Test	Alkaline Reagent	Salkowski Test	10% FeCl3 Test	5% FeCl3 Test
+	+	+	+	+	+	+	+	+

Ocimum sanctum was chosen as the positive control plant for the validation of the reagents used in phytochemical screening.

Quantitative Estimation of Phenolic, Flavonoid, Terpenoid Contents and Antioxidant activity in *A. squamosa* L leaf extract

Table 4: Quantitative Estimation of Phenolic, Flavonoid and Terpenoid Contents

Extract	Total Phenolic content (eqv. to 100mg of gallic acid)	Total Flavonoid content (eqv. to 100mg of Quarcetin)	Total Terpenoid content (eqv. to 100mg of Ursolic acid)
1	**30.44 µg/ml**	**27.26 µg/ml**	**36.61 µg/ml**

Quantitative Estimation of Antioxidant activity

% Scavenged due to extract solution = $\frac{A_0 - A_1}{A_0} x100$

A_0 (absorbance of control) = the absorbance of H_2O_2 sample = 0.823

A_1 (absorbance of sample) = absorbance of mixture of plant sample and peroxide = 0.452

Percent Scavenged = $\frac{0.823 - 0.452}{0.823} x100$ = 45.07 (Moderate)

Similarly for the Ascorbic acid (Standard drug) it was found to be 46.9%.

The above result indicates that the final extract residue contains primarily the flavonoids/phenolic groups and terpenoids which was one of the experimental objectives. Which was further screened through by GC-MS & LC-MS study of the plant extract. The results are as follows-

GC-MS Profile of A. Squamosa L leaf extract

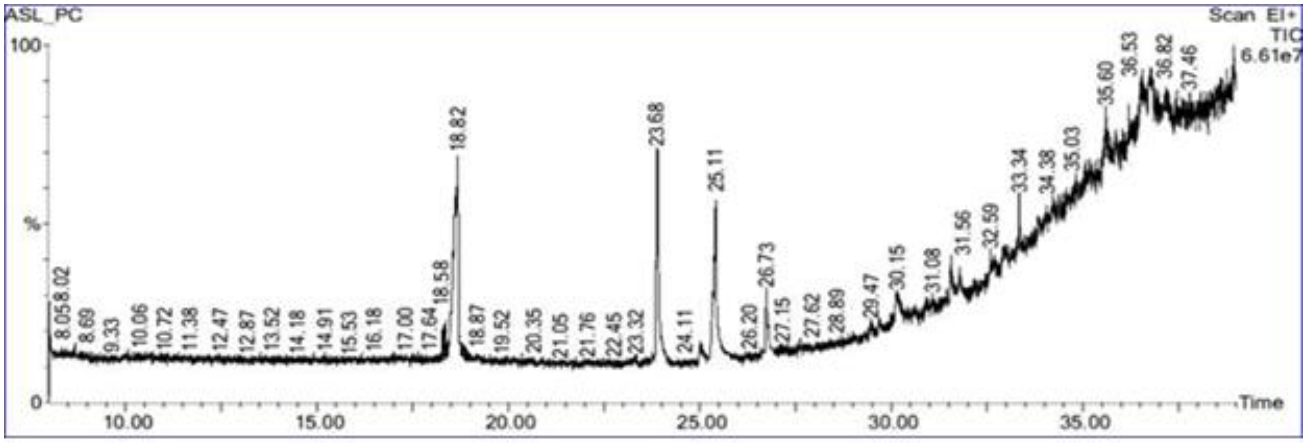


Figure 2: GC-MS of methanolic leaf extract of *A. Squamosa* L

Identification of components by GC-MS

The National Institute of Standards and Technology's (NIST) database, which contains over 62,000 patterns, was used to interpret the GC-MS mass spectrum. The unknown component's mass spectrum was contrasted with the known components' spectrums, which were kept in the NIST library at an interval of 0.5 seconds with pieces ranging from 40 to 440 Da. The relevant flavonoids and phenolic molecules are shortlisted (Table 4) based on retention time (RT) and Area%.

Table 5: Identified compounds in *A. squamosa* leaf extract from GC-MS spectrum using NIST database.

SL No	RT	Area %	Type of Compound	IUPAC Name of The Identified Compound	Compound Name
1	8.023	1.033	Low to medium polarity compounds or flavonoid glycosides	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one.	Quercetin
2	8.203	0.962	Methoxylated flavones or low MW flavonoid derivatives	2-phenyl-4H-chromen-4-one	Apigenin dimethyl ether, trimethoxy flavone
3	8.354	1.697	Flavonoid (aglycone)/Semi-volatile compounds	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one	Luteolin
4	8.464	1.938	Flavonoid aglycones or methylated derivatives	3',4',5,7-tetrahydroxy-3-methoxyflavone	Isorhamnetin
5	8.689	3.139	Aglycones or low-polarity glycosides	2-phenyl-4H-chromen-4-one	Trimethoxy flavone.
6	9.139	0.472	Not explicitly named in the row	Not Identified	Not Identified
7	9.239	0.860	Not explicitly named in the row	Not Identified	Not Identified
8	9.454	1.758	Flavonoid (aglycone)/Semi-volatile compounds	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one	luteolin
9	9.629	1.218	Small polar molecules/ alkylphenols	Benzenecarboxylic acid	Benzoic acids
10	9.709	1.135	Small polar molecules/ alkylphenols	Benzenecarboxylic acid	Benzoic acids
11	10.064	1.667	Small polar molecules/ alkylphenols	Benzenecarboxylic acid	Benzoic acids
12	10.209	0.998	Terpenoids, (monoterpenes, sesquiterpenes)	(1R,4R,6R,10S)-9-Methylene-4,12,12-trimethyl-5-oxatricyclo [8.2.0.04,6]	caryophyllene oxide

				dodecane	
13	10.389	1.119	Phenolic acid derivative, Phenolic but not a flavonoid	Benzene propanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	Benzenepropanoic acid
14	10.534	2.060	Low to medium polarity compounds or flavonoid glycosides	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one	Quercetin trimethyl ether
15	10.719	0.336	A phenolic antioxidant.	Benzene propanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	Benzenepropanoic acid
16	10.900	1.302	Sesquiterpene oxide, Terpenoid	(1R,4R,6R,10S)-9-Methylene-4,12,12-trimethyl-5-oxatricyclo [8.2.0.04,6] dodecane	Caryophyllene oxide
17	10.965	0.924	Not explicitly named in the row	Not Identified	Not Identified
18	11.130	0.614	Diterpene alcohol -PHYTOL-TERPINOIDS	2E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol.	Phytol
19	11.380	1.967	Diterpene alcohol -PHYTOL-TERPINOIDS	2E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol.	Phytol
20	11.505	0.881	Not explicitly named in the row	Not Identified	Not Identified
21	11.690	0.940	Not explicitly named in the row	Not Identified	Not Identified
22	11.875	1.422	A phenolic antioxidant	(2E)-3-(4-hydroxy-3-methoxyphenyl) prop-2-enoic acid	Ferulic acid
23	12.055	0.296	Not explicitly named in the row	Not Identified	Not Identified
24	12.180	1.063	Fatty acid methyl esters	Methyl hexadec-9-enoat	Hexadecenoic acid, methyl ester
25	12.360	0.921	Not explicitly named in the row	Not Identified	Not Identified
26	12.465	0.674	Not explicitly named in the row	Not Identified	Not Identified
27	12.585	0.397	Not explicitly named in the row	Not Identified	Not Identified
28	12.650	0.418	Not explicitly named in the row	Not Identified	Not Identified
29	12.695	0.473	Not explicitly named in the row	Not Identified	Not Identified
30	12.870	0.853	Fatty acid methyl esters	5,6,7-trimethoxyflavone or 3',4',5'-trimethoxyflavone	Trimethoxy flavones
31	12.950	0.461	Not explicitly named in the row	Not Identified	Not Identified
32	13.050	0.292	Not explicitly named in the row	Not Identified	Not Identified
33	13.120	0.955	Fatty acid methyl ester	(9Z)-hexadec-9-enoic acid	Palmitoleic acid
34	13.290	0.357	Fatty acid methyl esters	5,6,7-trimethoxyflavone or 3',4',5'-trimethoxyflavone	Trimethoxy flavones
35	13.391	0.584	Fatty acid methyl esters	5,6,7-trimethoxyflavone or 3',4',5'-trimethoxyflavone	Trimethoxy flavones
36	13.521	0.392	Not explicitly named in the row	Not Identified	Not Identified
37	13.651	0.386	Not explicitly named in the row	Not Identified	Not Identified
38	13.681	0.595	Flavonoid (aglycone)	5,7-dihydroxy-2-phenyl-4H-chromen-4-one	Chrysin

39	13.831	0.297	Not explicitly named in the row	Not Identified	Not Identified
40	13.911	0.277	Not explicitly named in the row	Not Identified	Not Identified
41	13.981	0.505	Not explicitly named in the row	Not Identified	Not Identified
42	14.146	0.338	Not explicitly named in the row	Not Identified	Not Identified
43	14.191	0.290	Not explicitly named in the row	Not Identified	Not Identified
44	14.241	0.645	Not explicitly named in the row	Not Identified	Not Identified
45	14.371	0.263	Not explicitly named in the row	Not Identified	Not Identified
46	14.531	0.797	Not explicitly named in the row	Not Identified	Not Identified
47	14.706	0.276	Not explicitly named in the row	Not Identified	Not Identified
48	14.911	1.033	Flavonoid (aglycone)	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one	Lueolin / Apigenin
49	15.146	0.249	Not explicitly named in the row	Not Identified	Not Identified
50	15.396	0.322	Not explicitly named in the row	Not Identified	Not Identified
51	15.531	0.468	Not explicitly named in the row	Not Identified	Not Identified
52	15.666	0.252	Not explicitly named in the row	Not Identified	Not Identified
53	15.836	0.455	Not explicitly named in the row	Not Identified	Not Identified
54	15.977	0.652	Not explicitly named in the row	Not Identified	Not Identified
55	16.112	0.236	Not explicitly named in the row	Not Identified	Not Identified
56	16.252	0.255	Not explicitly named in the row	Not Identified	Not Identified
57	16.362	0.545	Not explicitly named in the row	Not Identified	Not Identified
58	16.642	0.242	Not explicitly named in the row	Not Identified	Not Identified
59	16.752	0.459	Not explicitly named in the row	Not Identified	Not Identified
60	16.837	0.228	Not explicitly named in the row	Not Identified	Not Identified
61	16.927	0.245	Not explicitly named in the row	Not Identified	Not Identified
62	17.062	0.233	Not explicitly named in the row	Not Identified	Not Identified
63	17.137	0.476	Not explicitly named in the row	Not Identified	Not Identified
64	17.337	0.495	Not explicitly named in the row	Not Identified	Not Identified
65	17.557	0.251	Not explicitly named in the row	Not Identified	Not Identified
66	17.637	0.305	Not explicitly named in the row	Not Identified	Not Identified
67	17.882	0.288	Not explicitly named in the row	Not Identified	Not Identified
68	17.962	0.272	Not explicitly named in the row	Not Identified	Not Identified
69	18.147	0.476	Not explicitly named in the row	Not Identified	Not Identified
70	18.407	1.229	methoxylated or glycosylated flavonoid	3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4H-1-benzopyran-4-one	Isorhamnetin. / Methyl Quercetin
71	18.583	1.488	Medium-to-high molecular weight, less volatile compounds.	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-methoxychromen-4-one	Quercetin-3-methyl ether.

			Glycosylated/methylated flavonoid		
72	18.828	9.067	Methoxylated or glycosylated flavone/flavanol.	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one.	Quercetin
73	18.918	0.255	Large phenolic glycoside	2-phenyl-4H-chromen-4-one	Flavone
74	19.293	0.249	Not explicitly named in the row	Not Identified	Not Identified
75	23.685	4.895	Flavonoid compound or Methoxylated flavone	3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one	Kaempferol

Table 6: Compound Class Presence Summary

Compound Class	RT Range	Presence	Supporting Evidence
Flavonoids	13.5 – 23.5 min	Strong	Multiple peaks in this range; supported by earlier confirmed flavone, isorhamnetin etc.
Terpenoids	10.5 – 13.5 min	Strong	Peaks align with phytol and caryophyllene oxide RTs
Phenolics	8.5 – 10.5 min	Moderate	Consistent with methoxyphenol-type peaks
Other Compounds	30 – 39 min	Present	Likely plant waxes, fatty acids, sterols; common in late RTs

Estimated Relative Abundance (Based on TIC)

Flavonoids – ~30–35% (multiple moderate-to-high peaks in 13–23 min window)

Terpenoids – ~15–20% (moderate peaks in 10–13 min range)

Phenolics – ~5–10% (smaller early peaks)

Other lipophilic compounds – ~30% (strong peaks after 30 min)

Conclusion

Based on the chromatographic profile:

Annona squamosa leaf extract shows **strong presence of**

Flavonoids (e.g. flavone, isorhamnetin, quercetin),

Terpenoids (e.g. phytol, caryophyllene oxide), and

Phenolics (e.g. alkylphenols).

N**on-polar plant constituents** like sterols, waxes, or fatty acids.

Table 7: Important Compounds identified in methanolic and chloroform extract of A. Squamosa leaves by positive mode of analysis

Name	Retention time (min.)	Molecular formula	Molcular mass (g/mol)	Classification	Area%
Quercetin	8.023	$C_{27}H_{30}O_{15}$	594.1587	Flavonoid	1.033
Quercetin	18.828	$C_{27}H_{30}O_{15}$	594.1587	Flavonoid	9.067
Luteolin / Apigenin	14.911	$C_{15}H_{10}O_6$	286.24	Flavonoid	1.033
Benzenepropanoic acid	10.389	$C_{10}H_{13}N_5O_4$	267.0967	Phenolic	1.119

Flavone	18.918	$C_{15}H_{10}O_2$	222.293	Phenolic	0.255
Trimethoxy flavone	8.689	$C_{18}H_{16}O_5$	312.31	Flavonoid aglycones	3.139
Phytol	11.380	$C_{20}H_{40}O$	296.53	Terpenoids	1.967
Ferulic acid	11.875	$C_{10}H_{10}O_4$	194.18	A phenolic antioxidant	1.422
Quercetin trimethyl ether	10.534	$C_{18}H_{16}O_7$	344.32	flavonoid glycosides	2.060
Kaempferol	23.685	$C_{15}H_{10}O_6$	286.23	Flavonoid compound or Methoxylated flavone	4.895

Ligand: Quercetin

PDB ID: 6O0K

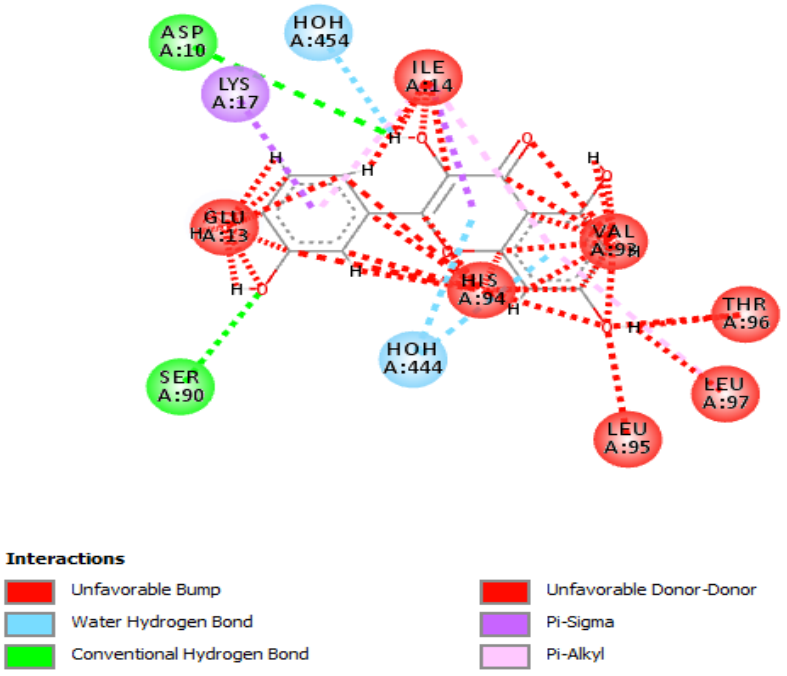


Figure 3: 2D diagram of Quercetin- 6O0K complex best fit interaction

Hydrogen-bond interacting active binding site residues: SER 90, ASP 10

PDB ID: 4AQ3

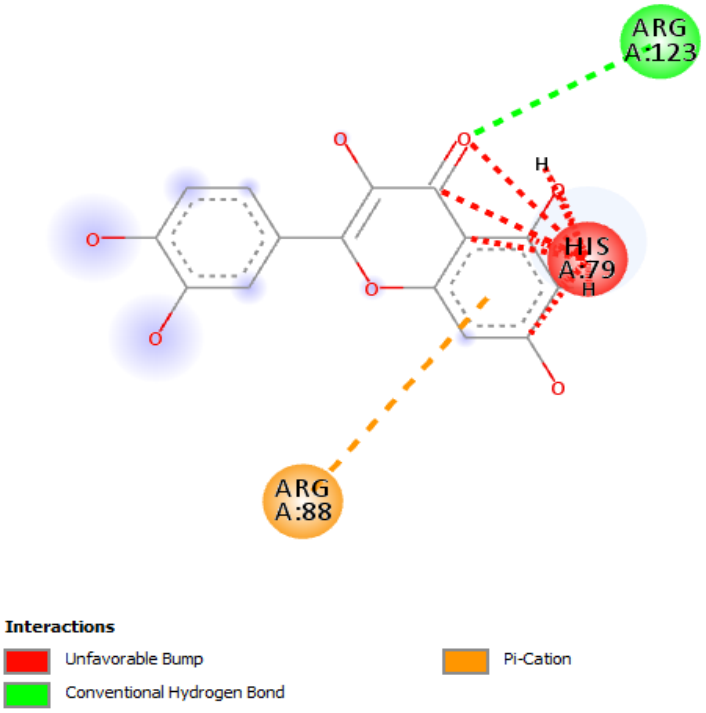


Figure 4: 2D diagram of Quercetin- 4AQ3 complex best fit interaction

Hydrogen-bond interacting active binding site residues: ARG 123

LIGAND: KAEMPFEROL

PDB ID: 2O22

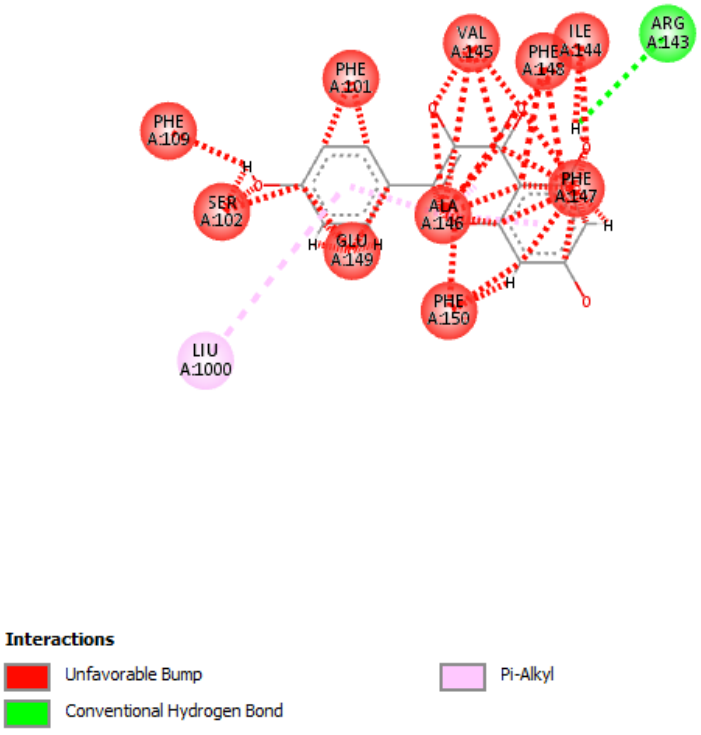


Figure 5: 2D diagram of Kaempferol- 2O22 complex best fit interaction

Hydrogen-bond interacting active binding site residues: ARG 123

PDB ID: 1G5M

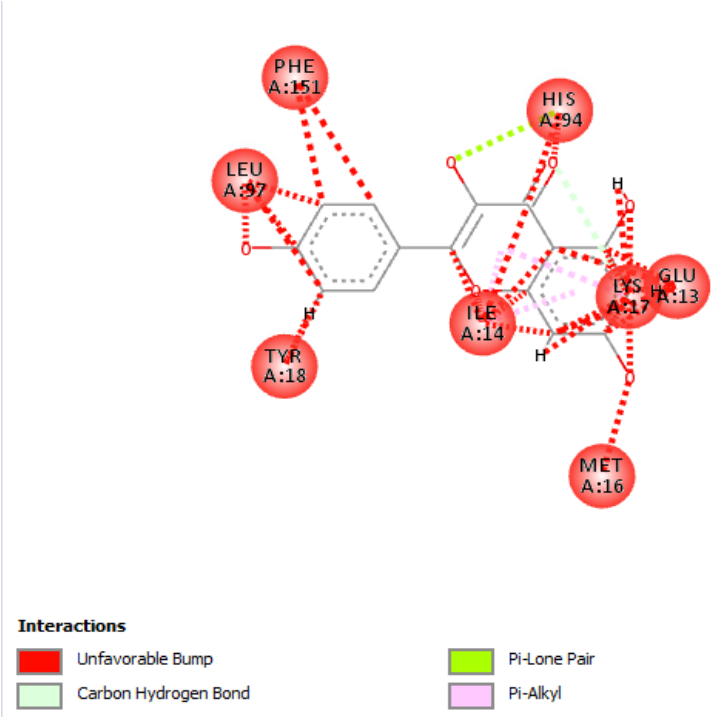


Figure 6: 2D diagram of Kaempferol- 1G5M complex best fit interaction

Table 8: Summary of Docking Study

Ligand	Receptor	Protein/ Receptor name	Docking Score (Best Fit)	RMSD Value
Reported Test Compound (Quercetin)	6O0K	BCL-2	-7.7	0
Standard Compound (Paclitaxel)			-7.3	0
Reported Test Compound (Quercetin)	4AQ3	BCL-2	-7.5	0
Standard Compound (Paclitaxel)			-7.2	0
Reported Test Compound (Kaempferol)	2O22	BCL-2	-7.3	0
Standard Compound (Paclitaxel)			-7.2	0
Reported Test Compound (Kaempferol)	1G5M	BCL-2	-7.1	0
Standard Compound (Paclitaxel)			-7	0

According to the above data, the plant extract claimed phytoconstituents like **Quercetin** and Kaempferol demonstrated more *in silico* anticancer efficacy against BCL-2 than the standard drug Paclitaxel and out of the both ligands Quercetin complex with receptor shows best docking score of the studies.

So, Quercetin was selected for the ADME analysis.

ADME analysis [20]

Quercetin

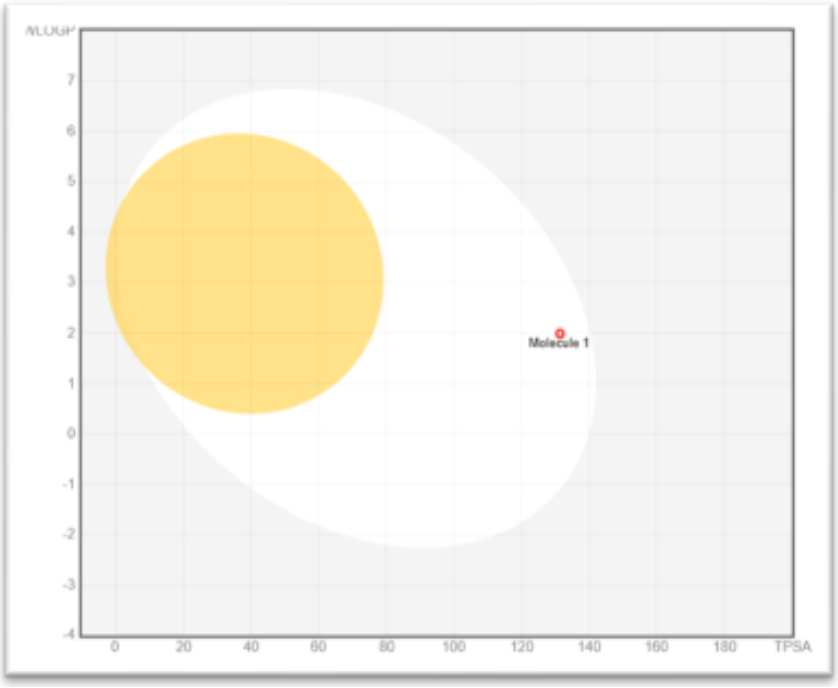


Fig 7: Boiled Egg model of Quercetin

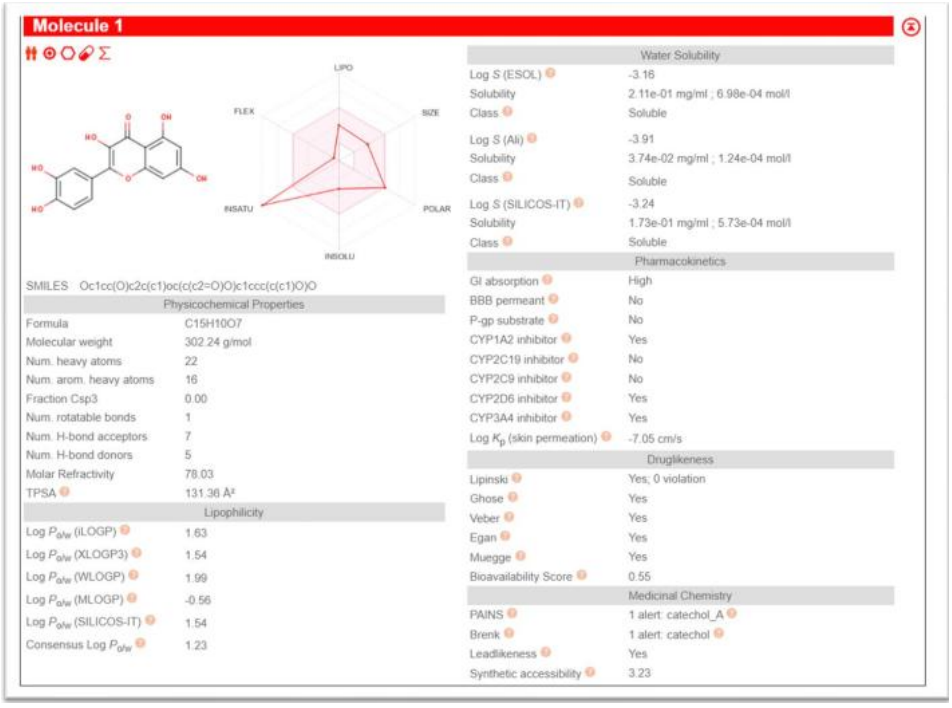


Fig 8: Structural details and physicochemical parameters of Quercetin

From the above figure it can be seen that Quercetin can cross the GI membrane & it also having good bio-availability score of 0.55 and it seemed to be obey Lipinski rules also.

***Protein structure validation* [21, 22]**

PDB ID: 6O0K

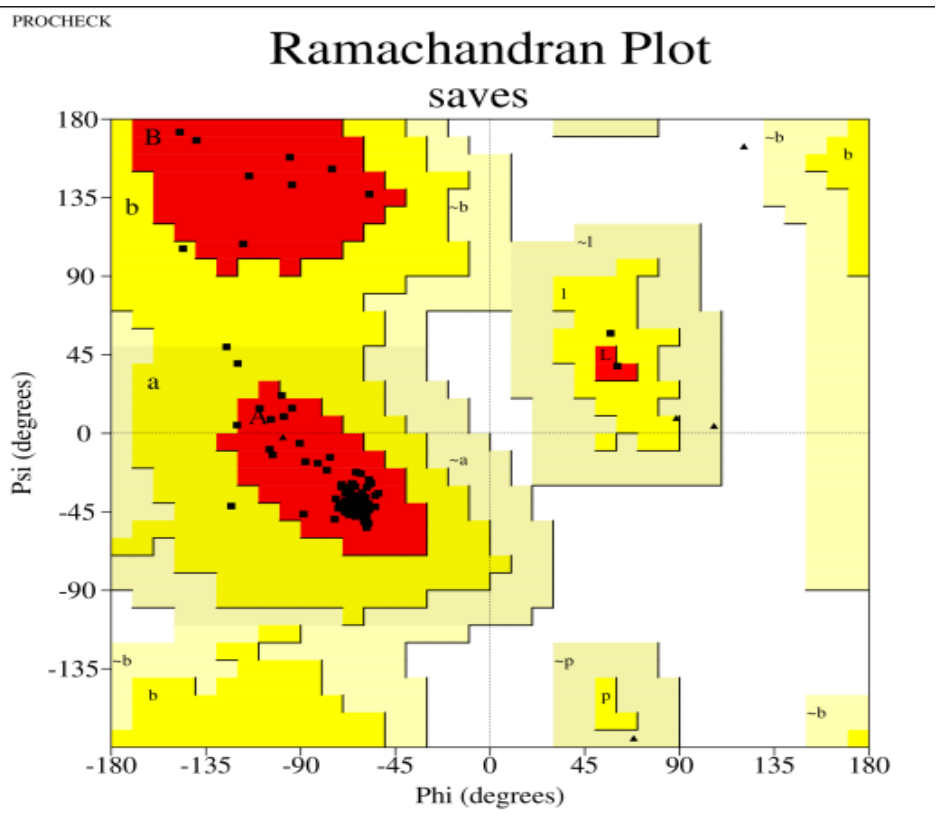


Fig 9: Ramachandran plot statistics

Plot statistics

Residues in most favoured regions [A.B.L]	118	94.4%
Residues in additional allowed regions [a.b.l.p]	7	5.6%
Residues in generously allowed regions [-a-b-l-p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	125	100.0%
Number of end-residues (excl. Gly and Pro)	3	
Number of glycine residues (shown as triangles)	11	
Number of Proline residues	2	
Total number of residues	141	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Since the most liked area of the plot included more than 90% (94.4%) of the amino acid residue, receptor structure of **6O0K** was validated and the model was approved as it was selected based on the best docking score observed when docked with quercetin.

***Simulation Study* [23]**

Receptor PDB ID: 6O0K

β-factor/ Mobility

The main-chain deformability is a measure of the capability of a given molecule to deform at each of its residues during thermal oscillation with it's stability profile.

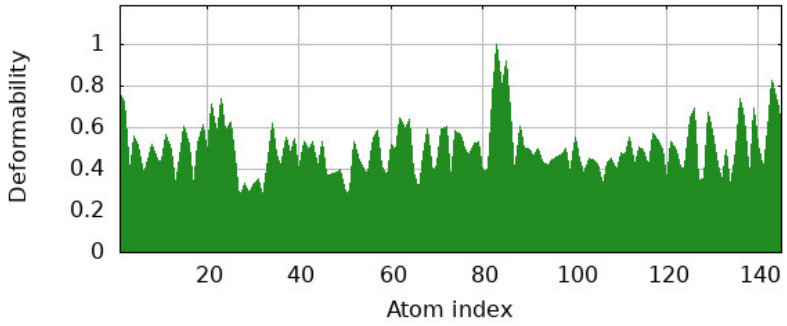


Fig10: B-factor/ Mobility

Above figure indicates the number of amino acid residue present in the selected chain of the receptor.

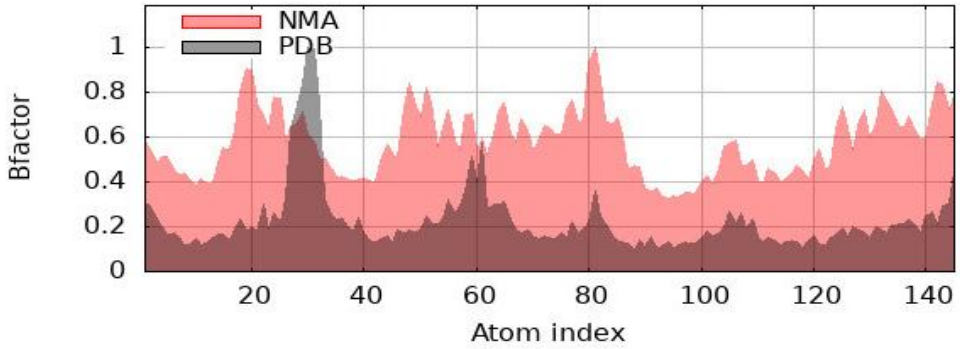


Fig 11: B-factor/ Mobility

Above figure shows the difference between PDB and NMA analysis. In general, experimental B-factors and NMA predicted mobilities are very similar.

NMA: Normal mode analysis

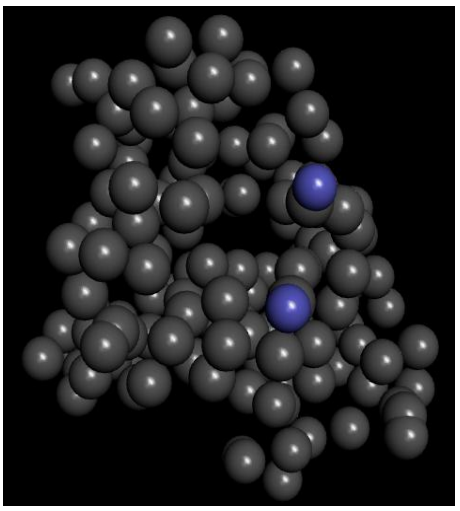


Fig 12: Deformability c-alpha chain of 6O0K

Eigenvalues

The eigenvalue associated to each normal mode represents the motion stiffness. Its value is directly related to the energy required to deform the structure. The lower the eigenvalue, the easier the deformation.

5.

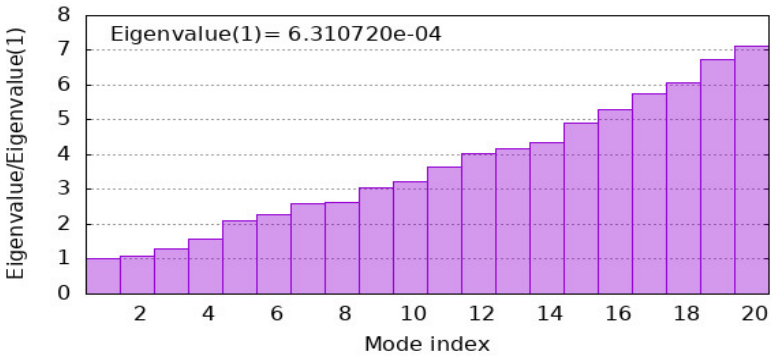


Fig 13: Eigenvalues of 6O0K

This figure represents the required energy, which is required for the deformation of the residue. Lower the value represents as the lower the energy, mean's that's easier for the deformation. This Eigen values plot evidences the relative modal stiffness.

Variance

The variance associated to each normal mode is inversely related to the eigenvalue. Coloured bars show the individual (red) and cumulative (green) variances.

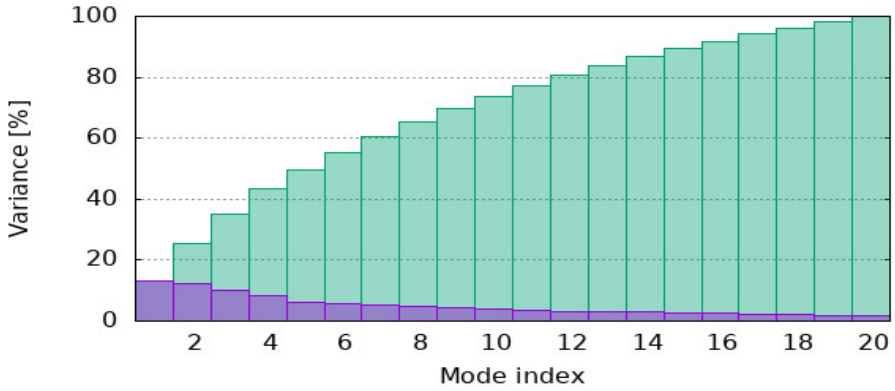


Fig 14: Variance of 6O0K

Above figure represents the reverse behaviour than Eigen values. The variance associated to the modes indicates their relative contribution to the equilibrium motions.

Covariance map

Covariance matrix indicates coupling between pairs of residues, i.e., whether they experience correlated (red), uncorrelated (white) or anti-correlated (blue) motions.

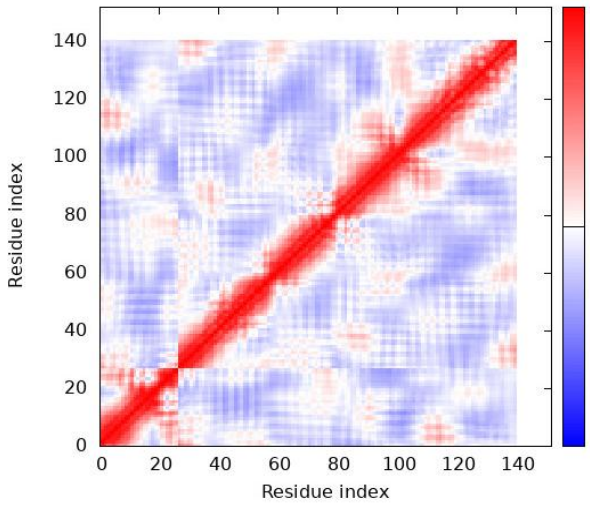


Fig 15: Covariance Map

Covariance matrix indicates which parts of the macromolecule move in a correlated, uncorrelated or anti-correlated fashion. Red colour shows the correlation between residue pairs; white shows no correlation and blue colour shows autocorrelations.

Elastic network

The elastic network model defines which pairs of atoms are connected by springs. Each dot in the graph represents one spring between the corresponding pairs of atoms. Dots are coloured according to their stiffness; the darker grey indicates stiffer spring and vice versa.

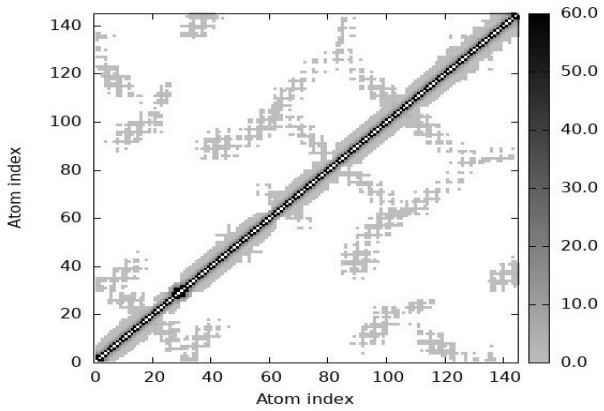


Fig 16: Elastic Network

The elastic network model used to compute the normal modes can be illustrated as a linking matrix.

SUMMARY:

Number of Molecules.....1

Number of Chain..... 1

Number of Segments..... 2

Number of Groups..... 141

Number of Atoms 1172

Number of Hetero Mol. 0

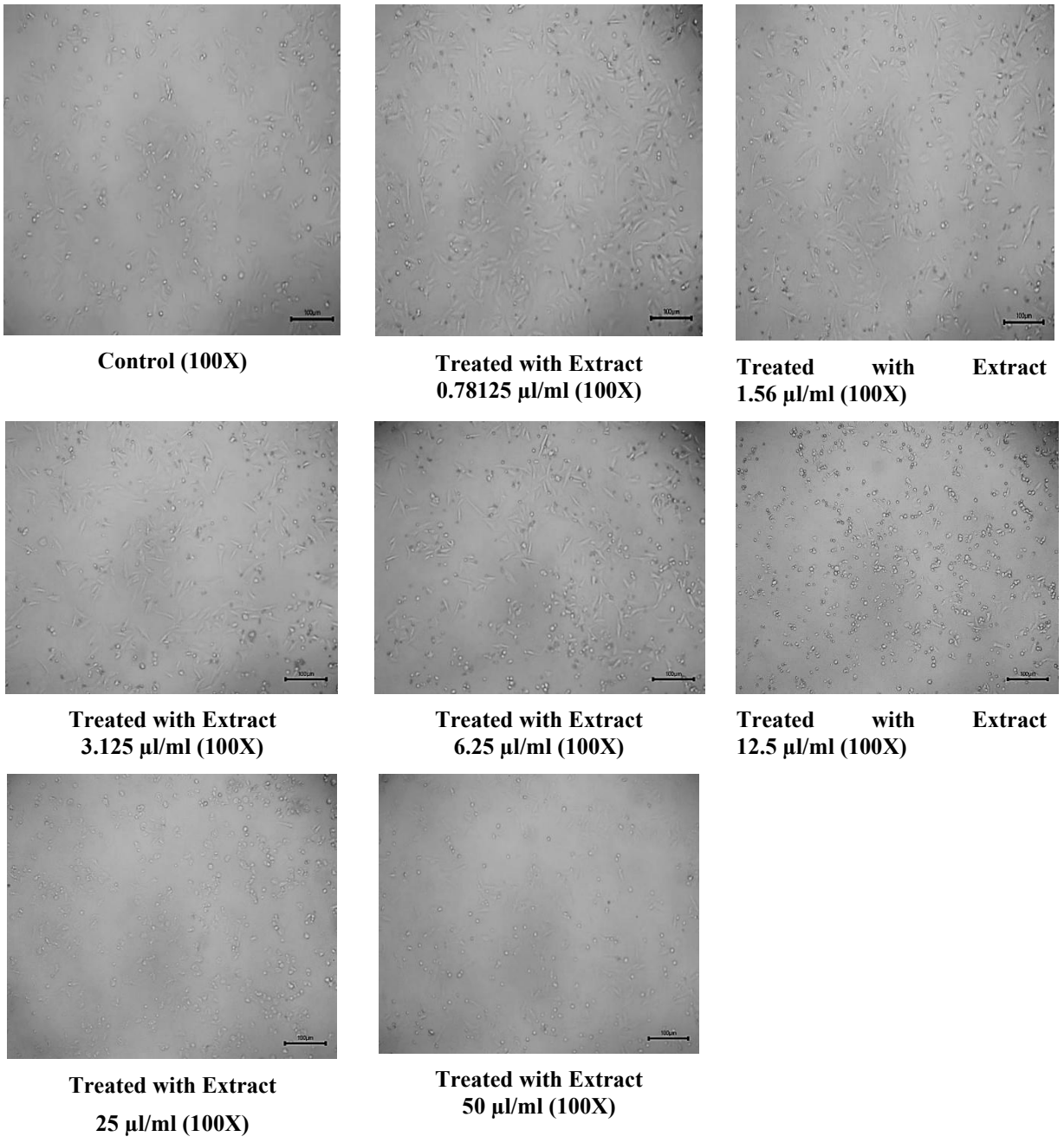
Number of Hetero Atoms….....0

Cytotoxicity study: MTT assay

Cell line: MCF-7

Extract: *Annona squamosa* L leaf extract

Conc. (µl/ml) against Paclitaxel as a standard drug



Control (100X)

Treated with Extract 0.78125 µl/ml (100X)

Treated with Extract 1.56 µl/ml (100X)

Treated with Extract 3.125 µl/ml (100X)

Treated with Extract 6.25 µl/ml (100X)

Treated with Extract 12.5 µl/ml (100X)

Treated with Extract 25 µl/ml (100X)

Treated with Extract 50 µl/ml (100X)

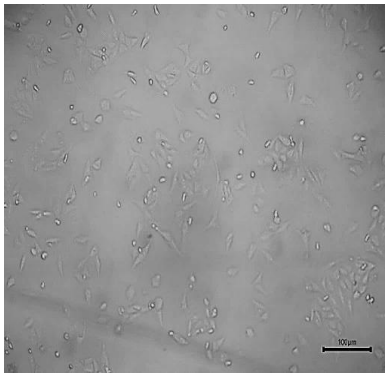
Fig 17: Microscopic image of control & treated MCF-7 cell line With *Annona squamosa* L leaf extract Conc. (µl/ml)

Cytotoxicity study: MTT assay

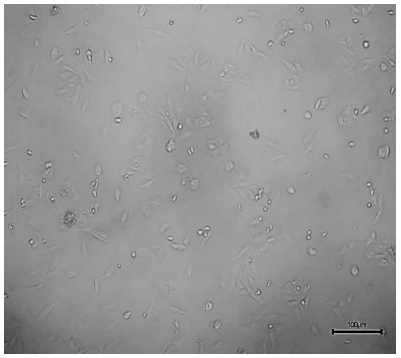
Cell line: MCF-7

Standard Drug: Paclitaxel

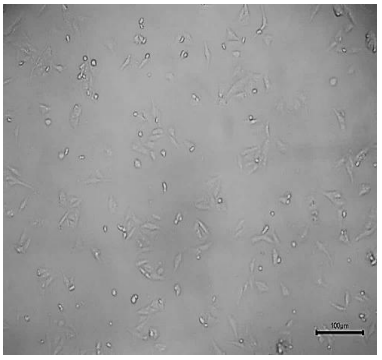
Conc. Paclitaxel Conc. (nM)



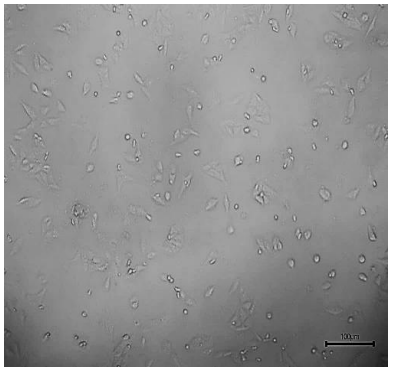
Control (100X)



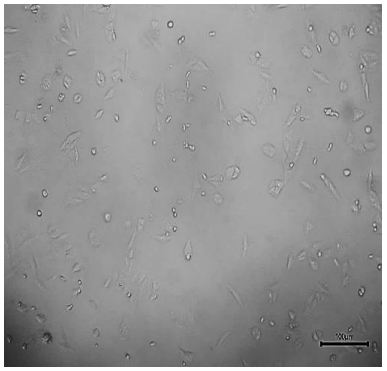
Treated with paclitaxel 0.1 nM (100X)



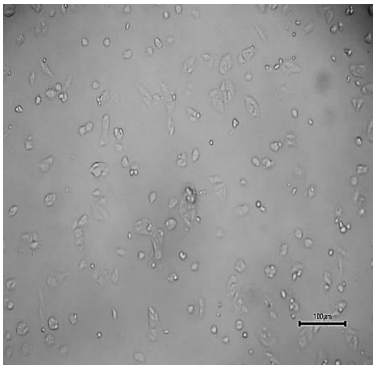
Treated with paclitaxel 1 nM (100X)



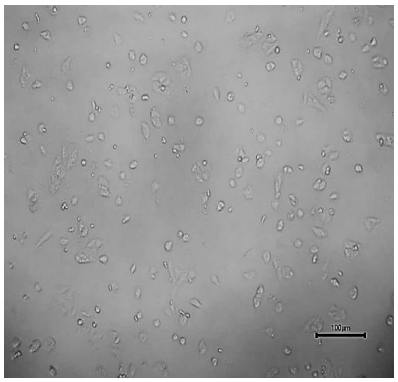
Treated with paclitaxel 10 nM (100X)



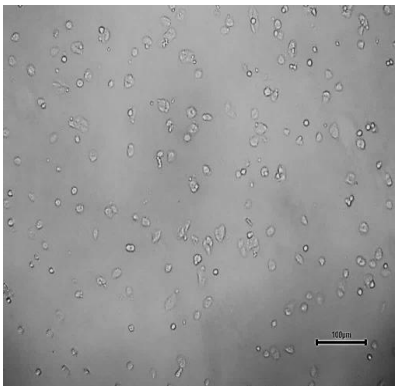
Treated with paclitaxel 50 nM (100X)



Treated with paclitaxel 100 nM (100X)



Treated with paclitaxel 500 nM (100X)



Treated with paclitaxel 1000 nM (100X)

Fig 18: Microscopic image of control & treated MCF-7 cell line With Paclitaxel Conc. (nM)

% Viable cells = (A_{test} / $A_{Control}$) *100

(A_{test} = Absorbance of test sample)

($A_{Control}$ = Absorbance of Control)

Table 9: Comparatives results (IC50 value) Treated MCF-7 Cell line

1. **Treated with**	2. ***IC*$_{50}$ value** 3. Mean ± SEM
4. Paclitaxel	5. 75.2 ± 0.09 nM
6. Annona squamosa leaf extract	7. 3.872 ± 0.08 µl/ml

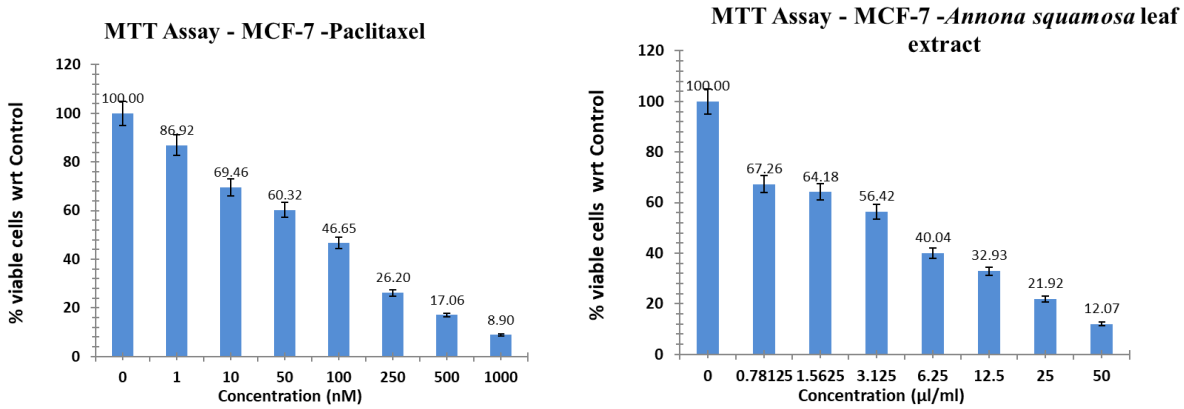


Fig 19: Comparative Histogram of Paclitaxel & Annona squamosa Leaf Extract

Treated MCF - 7 Cell Line

Control (Untreated cells) – Cells look healthy, round/spread out, high density.

Treated with 0.78125 µl/ml – Fewer cells visible, some are rounded/shrunken, indicating early cytotoxic effects.

Treated with 1.56 µl/ml – Even fewer cells, many appear damaged or dead

Treated with 3.125 µl/ml- More than 56% of MCF -7 cells appear damaged or dead

So, it can be concluded that

The extract reduces cancer cell viability.

The effect is dose-dependent – higher concentration causes more cell death.

To visually compare the effect of the treatment: One image shows healthy control cells. Two others show cells after treatment with increasing concentrations. This helps demonstrate how the extract progressively kills or damages the cancer cells

In the current study the anticancer activity and cytotoxic activities of A. Squamosa leaves extracts were performed against MCF 7 breast cancer cell line [23, 26]. The outcome of the results revealed that the cytotoxicity of MCF 7 cell lines occurs in a does dependent manner The MTT assay also revealed the extract's cytotoxicity against MCF-7 breast cancer cells at concentrations from 0.78125 to 50 µl/ml. microscopic images showed a dose-dependent reduction in cell viability, with morphological changes (e.g., cell shrinkage, rounding) indicative of apoptosis at higher concentrations. However, specific absorbance or IC50 values were not provided, limiting quantitative conclusions. The visual evidence suggests significant anti-cancer potential, particularly at higher doses. The cytotoxicity against control showed that the A Squamosa leave extract have lower activity against non-tumour cell. This remarkable anti-cancer activity as compared to the standard drug Paclitaxel may due to the high cons. Of flavonoid presence. **[24,25]**

4. CONCLUSION

In this study we have attempted to elucidate ethnobotanical activities of *A. Squamosa* L leaves extracts and find out the major bioactive compounds which is responsible for its antioxidant, cytotoxic, scavenging and anticancer activity. The above study may contribute to the development of new remedies in associated with common chemotherapeutic drugs for the treatment of breast cancer. In silico study with the breast cancer cell receptor will assist to determine the molecular mechanism for MCF-7 breast cancer cell apoptosis

5. ACKNOWLEDGMENT

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Aakaar Biotechnologies Pvt. Ltd. (Kanpur)

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