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Evaluation of In silico and In vitro pharmacological activities of 1,3,8-trihydroxyanthraquinone

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Abstract

A trihydroxy anthraquinone component from *Cassia fistula* fruit pulp belonging to Legumenosae family was isolated from the toluene extract of fruit pulp by the process of hot extraction and characterized as 1,3,8–trihydroxyanthraquinone (**AQ**) using various spectroscopic techniques like I.R, U.V, NMR,and Mass. It is the first report of the presence of 1,3,8 – trihydroxyanthraquinone in *Cassia Fistula fruit pulp*. In vitro antioxidant activity of the compound was studied by superoxide and hydroxide radical scavenging assay. This phytoligand was also subjected to in silico molecular docking studies with CK2, P53 and ERK protein using iGEMDOCK software to investigate their cytotoxic activity. The in vitro cytotoxic activity of this compound was then studied to substantiate the data obtained from the docking studies in HT-29, DLA and A375 cell lines. Its Chorio Allantoic Membrane (CAM) assay was also performed in chick embryo to find the antiangeogenic activity.

Key words: *Cassia fistula*, Fruit pulp, 1,3,8–trihydroxyanthraquinone, iGEMDOCK, antioxidant, HT–29, DLA, A375, antiangeogenic, CAM.

1. Introduction

Cassia fistula belongs to the Legumenosae (1) family and is found mostly in southern parts of India. It is being widely used for preparing traditional medicines in India and highly reputed for its strong laxative and purgative properties. In Ayurvedic medicine, it is used against various disorders such as hematemesis, pruritus, leucoderma and diabetes. The antipyretic and analgesic effect of *Cassia fistula* has been reported with its antifungal, antibacterial and anti-inflammatory activities (2). The fruit pulp of this tree was investigated and a trihydroxy component containing anthraquinone moiety was isolated. The literature review reveals the presence of phytochemical constituents in pods which are derivatives of hydroxyanthraquinone and its glycosides (3, 4). As there exist only few reports on the chemical examination of pods, it was selected as the subject of the present investigation. In this communication, we report the isolation and characterization of 1,3,8–trihydroxyanthraquinone (Figure 1) in *Cassia fistula* fruit pulp. Its various pharmacological activities like antioxidant, cytotoxicity and antiangiogenic assay are also a part of the investigation.

Antioxidants are vital substances that possess the ability to protect the body from damages caused by free radical-induced oxidative stress. The results from various experiments and a survey of literature revealed a relationship between antioxidant and antitumor promoting activities in the cases of anthraquinones (5). Nordamnacanthal being a strong antitumor promoter was found to be strong antioxidant too. In the literature it was found that two hydroxyl groups arranged at either the *meta*- or *ortho*-positions are required for an anthraquinone to inhibit lipid peroxidation (6). However, anthraquinone derivatives that have no hydroxyl groups as well as two hydroxyl groups affixed to different rings did not inhibit lipid peroxidation (7, 8). The isolated compound in our investigation contains three hydroxyl groups, two of them being attached to a single ring. So it was subjected to antioxidant activity by superoxide and hydroxide scavenging activity. Its analogue, 1,4,8–trihydroxyanthraquinone obtained from soil sample is reported to have antitumor and antibiotic activity (9). Column chromatography of the toluene extract of *Cassia fistula* fruit pulp using silica gel gave compound **AQ**. Presence of the same compound in *Senna reticulata* has been reported earlier (10).

Before performing direct in vitro and in vivo studies a green chemistry approach is to do computational analysis to get early information on pharmacokinetic properties like absorption, distribution, metabolism, excreation and toxicity which can be obtained from various bioinformatic tools like MedChem designer, ADMET SAR etc. In this present study we have used a computational tool MedChem Designer to gather information related to these pharmacokinetic properties. After doing the in silico analysis for the bioviability, the compound was subjected to in silico docking studies using iGEMDOCK software. This is performed to find the binding affinity/activity of the compound with various targets like CK2, P53 and ERK which are proteins over expressed in cell lines HT-29, DLA and A375 in our investigation. All the proteins are selected based on literature survey (11, 12, 13)

Colon cancer, also known as colorectal cancer, ranks second in the cancer death causes in both men and women according to United State Centre for Disease Control and Prevention (CDC). There is an urgent need for new therapeutics of natural origin possessing less toxicity. The utilization of conventional drugs like doxorubicin is usually limited due to systemic toxicity and lack of specificity.

Melanoma is the most dangerous and deadly type of skin cancer. It develops in melanolytes, the pigment cells present in the skin. It can be more serious than the other forms of skin cancer because it may spread to other parts of the body (metastasize) causing serious illness and death. A significant number ranging to about 50,000 new cases of melanoma are diagnosed in the United States every year. Lymphoma is a type of blood cancer that occurs when lymphocytes, white blood cells that help protect the body from infection and disease began to show abnormal behavior. Division of abnormal lymphocytes happen faster than normal cells or in other words, they may survive longer than they are supposed to. Lymphoma may develop in various parts of the body, including the lymph nodes, spleen, bone marrow, blood or other organs.

In our present study we focus our attention to find the cytotoxicity of isolated AQ in colon (HT-29), Melanoma (A375) and in Dalton's Lymphoma Acites (DLA). In vitro analysis will give ample proof to verify and substantiate the in silico studies. The in vitro studies were conducted on the above mentioned cell lines by means of MTT assay and Tripan blue method.

CAM (Chorio Allantoic Membrane) assay (14) is widely utilized as an *in vivo* system to study anti-angiogenesis. In our present work the isolated hydroxyanthraquinone (AQ) was subjected to CAM assay in chick embryo to examine the angeostatic activity.

2. Materials and Method

2.1. Extraction and isolation

The mature pods of *Cassia fistula* were collected from College of Engineering, Thiruvananthapuram Campus, Kerala, India during the month of January. It was then authenticated by the Department of Botany, Government College for Women, Thiruvananthapuram, Kerala, India. The pulp alone was used for the extraction process. A 25% solution of methanol in water was added to 500 g of fruit pulp, acidified with dil. HCl and warmed slightly. This was followed by the addition of toluene and the extraction was done as per literature reports (9). About 100 mg of a yellowish brown crude mass was obtained and was subjected to silica gel column chromatography. Isocratic elution was carried out with ethylacetate as solvent to obtain 30 mg of yellowish orange amorphous solid, whose purity was checked by Thin Layer Chromatography (TLC). It was then recrystallized from methanol, characterized by various spectroscopic techniques and was identified as AQ. To the best of our knowledge, this is the first report of the said compound in *Cassia fistula* fruit pulp. The compound AQ also gave a pink colour with Borntragers reagent (15) confirming the presence of an anthraquinone moiety in it.

2.2. Characterisation

Melting point determinations of the isolated compound were done using a capillary melting point apparatus. A Shimadzu IR Prestiege–2I FT IR spectrometer using KBr (neat) was used to obtain the IR spectral data. ^{1}H and ^{13}C NMR spectra were recorded on a 500 MHz Bruker advanced DPX spectrometer with dimethyl sulphoxide d_6 as solvent and TMS as the internal standard. The absorption spectra of the isolated compounds were measured in acetone solvent on a UV–vis–NIR spectrophotometer (Shimadzu UV–3600). The mass spectra were recorded on a Shimadzu QP–2010+ mass spectrometer (GCMS). TLC analyses were performed on pre-coated aluminium plates of silica gel 60 F 254 Plates (0.25mm. Merck) and the developed TLC plates were visualized under short and long wavelength UV lamps. Column chromatography was performed using silica gel G (mesh 100-200) and silica gel G (Merck) respectively.

2.3. In silico analysis

Various medium and high throughput ADMET (16) screenings are now in practice. A flexible scoring system that encompasses many components of risk like absorption, distribution, metabolism, excretion and toxicity can be predicted. The ADMET test was done using the software MedChem designer.

In this study we have employed a graphical–automatic drug discovery system called iGEMDOCK (17) for integrating docking, screening, post-analysis and visualization. iGEMDOCK software is one of the robust and well developed tool for predicting the favourable poses of the molecule. The poses generated are directly visualized by a molecular visualization tool and can be analyzed by a post analysis tool.

The docking/ screening were performed by preparing two files, the protein structure file and the ligand file. The protein CK2, Bcl2 and ERK were selected from protein data bank (PDB) having ID 1M2R, 3ZM4 and 2XOW and the docking was performed using AQ, as ligand. The measured free energy values were then compared with that of the standard drug, Cyclophosphamide and Doxorubicin.

2.4. Antioxidant Studies

The antioxidant activities of hydroxyquinone were done by means of superoxide and hydroxide radical scavenging assay with ascorbic acid as standard.

2.4.1. Hydroxyl radical scavenging assay

DNA damage induced by free radicals has been related to ageing and various diseases, which includes cancer and chronic inflammations. The hydroxyl free radical is by far the most potent and therefore the most dangerous oxygen metabolite. Elimination of such radicals is one of the major aims of antioxidant administration. This assay is based on the quantification of the degradation product of 2–deoxy ribose by condensation with TBA. The studies were performed as per literature (18) in varying concentrations both for the standard ascorbic acid (AA) and the sample. The absorbance was measured at 532 nm against an appropriate blank solution. The percentage inhibition was calculated as

$$\% \text{ inhibition of } [OH^{-\cdot}] = \frac{[\text{Absorbance of control} - \text{Absorbance of test}]}{\text{Absorbance of control}} \times 100$$

2.4.2. Superoxide radical scavenging assay

Superoxide radical is known to have harmful effects to cellular components as it is a precursor of the more reactive oxygen species, which contribute to various diseases and tissue damage. These radicals may also play an important role during the peroxidation of unsaturated fatty acids and other potential susceptible substances (19). Super oxides are biologically important as it can form singlet oxygen and hydroxyl radical. Over production of super oxide anion radical contribute to redox imbalance. Super oxide assay was done as reported elsewhere in literature (20). Absorbance of the sample and standard was

measured at 560 nm in UV visible spectrometer after incubating for 30 minutes. The percentage of inhibition was calculated as

$$\% \text{ inhibition of } [O_2^{-}\cdot] = \frac{[\text{Absorbance of control} - \text{Absorbance of test}]}{\text{Absorbance of control}} \times 100$$

2.5. In vitro cytotoxicity study

The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with Phosphate Buffered Saline (PBS). Cell viability was determined by Trypanblue exclusion method (21). Viable cell suspension (1×10^6 cells in 0.1 ml) was added to tubes containing various concentrations of AQ and the volume was made up to 1ml using PBS. The control tube contained only cell suspension. These assay mixture were then incubated for three hours at 37 °C. Further cell suspension was mixed with 0.1ml of 1% trypanblue and kept for two to three minutes. Sample solution and standard drug in micro molar concentrations were added to the grown cells at 10 µg, 20 µg, 50 µg 100 µg and 200 µg from a stock of 10 mg/ml and incubated for 24 hours. The dead cells alone take up the blue colour of trypanblue, sparing the live cells. The percentage of cell death was calculated as per literature.

A375 and HT-29 Cells were seeded to each well of a 96 well microtitre plate (5000 cells/well). The plates were kept incubated overnight at 37 °C in a humidified 5% CO_2 incubator. Outer most wells of the 96 well microtitre plates were covered with PBS buffer in order to prevent the evaporation of the sample. 100 µL of 1,3,8-trihydroxy -9,10-Anthraquinone in Dulbeccos modified Eagle's medium (DMEM) at a different concentration was added to corresponding wells in triplicates. The negative control wells were loaded with 100µL of 10% DMEM. In the positive control well, 100 µL of Doxorubicin drug at various concentration were added to corresponding wells in triplicates. The plates were kept for 48 hours of incubation period. Media was discarded and the wells were washed with 100 µL PBS buffer. Hundred micro litre of the working MTT dye in DMEM was added. It was then incubated for 2 hours. Hundred micro litre of MTT lyses buffer was added and incubated for 4 hours. The absorbance was measured at 570 nm and the proliferation rate (PR) was calculated using the formula.

$$\% \text{ cytotoxicity} = \frac{\text{Number of dead cells}}{\text{Total number of cells}} \times 100$$

2.6. CAM assay

Fertilized chick embryos were brought from poultry farm and incubated in a humidified incubator at 37 °C. After four days fertilization, a small hole was made on the egg shell and 100µg of compound was concentrated in a gelatinous sponge and inserted inside the CAM. The hole was properly sealed and kept in the incubator. The eggs were taken on the 12^{th} day and the CAM was opened and photographed.

3. Results and Discussion

3.1. Spectral analysis

M.P. 280–283 °C. The molecular weight determination from mass spectrum established its molecular formula as $C_{14}H_8O_5$. $[M]^+$ at m/z (rel.int.%) 256 and other fragments (22): 228 [2.5, M–C=O]$^+$; 239[2.5, M–OH]$^+$ & 207 [3.5, M–3OH]$^+$. Absorption bands at 1627 cm^{-1} and 1693 cm^{-1} in the IR spectrum of the compound revealed the presence of hydrogen bonded and free carbonyl groups respectively (23) The IR absorption band obtained at 1608 cm^{-1} was assigned for the trihydroxy system (24) The absorptions for free hydroxyl group and for the hydrogen bonded hydroxyl group (25) were observed at 3313 cm^{-1} at 3072 cm^{-1} respectively. The ^{1}H NMR spectrum gave peaks for aromatic protons at δ8.13 [s, H–2], δ7.76 [s, H–4], δ7.73 [d, H–5, J=7.4 Hz], δ7.85 [t, H–6, J=7.7 Hz], δ7.74 [d, H–7, J=8.25 Hz]. The peaks at δ values 11.88, 11.97 & 12.05 for C–1, C–8, and C–3 respectively were assigned for the phenolic protons. ^{13}C NMR: δ137, δ124.1, δ124.6, δ119 & δ118 correspond to the five aromatic carbon atoms C–2, C–4, C–7, C–5 and C–6 respectively. The peaks at δ137.9 and δ137.5 correspond to [C–8'&C–1'] and [C–4' & C–5'] which are assigned to two types of quarternary carbon atoms present in the quinone moiety. The two carbonyl carbon atoms at C–9 and C–10 show absorptions at δ181 and δ191 respectively. The peaks observed at δ161.03, δ165, δ161.4 are assigned to the hydroxy substituted carbon atoms C–1, C–3 & C–8 respectively. The twelve different peaks observed in the ^{13}C NMR spectra of the compound correspond to twelve different carbon environments. ^{13}C DEPT90° was taken to get further confirmation of the C–H environments. The five peaks observed at δ137, δ124.1, δ119, δ118 & δ124.6, established the presence of five C–H environments and the presence of three substituents in the anthraquinone ring. Additional evidence for the regio chemistry of the compound was drawn from proton connectivity established by the 2D-COSY ^{1}H NMR. The ring proton at δ7.74 [H–7] is connected to the proton at δ7.85 [H–6]. Similarly the ring proton at δ7.85 [H–6] is connected to the proton at δ7.73 [H–5] and δ7.74 [H–7]. The protons at δ8.13 [H–2] and δ7.76 [H–4] have no correlation with other protons and are found to be isolated, thus establishing the proposed structure. The UV–vis maxima in acetone were obtained at 374 nm and 417 nm (quininoid group) (26) corresponding to n – π*& π – π* transition. A shift in absorption to 487 nm in the presence of alkali suggested the presence of 1, 8 – dioxygenated (24) system.

Based on the above observations, the compound was identified as 1,3,8–trihydroxyanthraquinone. This compound is being reported for the first time in the species *Cassia fistula* fruit pulp. The presence of this compound in another species, *Senna reticulate* has been reported earlier. The characterization of this compound is in conformity with the above reported structure (10).

3.2 Antioxidant activity

The ability of the isolated compound to scavenge radicals was assessed through hydroxide radical scavenging assay and superoxide radical scavenging assay. Ascorbic acid was used as the standard in both cases. The IC_{50} values were also measured. All the measurements were done in triplicate and the results are given as mean ± SD.

The results of hydroxide radical scavenging activity are represented a**s** Figure 2. It is clear from the figure that at higher concentrations, the compound AQ has almost the same antioxidant property as that of the standard under test. The IC_{50} value of AQ was found to be 248.28±1.44 µg /ml.

Figure 3 depicts the superoxide radical scavenging activity of the the compound and compared with ascorbic acid standard. The IC_{50} value of the compound AQ was 1117.05± 28.23 µg/ml . The study reveals that the the super oxide free radical abstraction activity for the compound under investigation was inferior when compared to the hydroxide scavenging activity.

3.3. In silico pharmacological studies

3.3.1.ADMET

The properties that make a drug different from chemicals are its molecular mass, partition coefficient, number of hydrogen bond donors and number of hydrogen bond acceptors. Partition coefficient is a function of lipophilicity measured in terms of partition between octanol/water system and it is expressed in three different approaches S + log P, S + log D and M log P. The other pharmacokinetic properties are the number of hydrogen bond donors and hydrogen bond acceptors. All these properties are generalised in the form of Lipinski's rule of five. For a compound to quailify as a drug, it should have a comparatively low molecular weight and should be relatively non-polar. Again, it should have a partition between an aqueous and the lipid phase. MedChem designer is an advanced molecular design software application that combines an intuitive sketch interface with fast and accurate ADMET property that can predict whether the drug obeys the rule. The results of the various pharmacokinetic properties along with their limiting values are given in Table 1. It is evident from the table that, the isolated compounds obeyed the Lipinski's rule of five and have good pharmacokinetic parameters. The drug likeness of the sample in this investigation showed values less than 5. Thus it pose to be promising candidate for invitro analysis.

3.3.2. Docking study

Molecular docking study was performed using the iGEMDOCK software against the target P53 protein (PDB ID: 2XOW), CK2 protein (PDB ID:1M2R) and ERK protein (PDB ID:3ZM4) and the free energy values were obtained (Table 2). Free energy is the sum total of energy due to hydrogen bonding, van der Waals (VWD) and electrostatc interactions. The higher negative value of free energy indicates a better activity of the drug. The free energy value of the ligand is then compared with the standard drug cyclophosphamide

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(CP) and Doxorubicin using same target.It was observed that the activity of AQ was much higher than that of the standard cyclophosphamide and comparable to that of the standard Doxorubicin. The docked poses are given in the Figures 4-6.

3.4. In Vitro Cytotoxicity

The compound AQ, was studied for its short term in vitro cytotoxicity using Dalton's Lymphoma Ascites cell lines (DLA) by taking cyclophosphamide (CP) as the standard after incubating for a period of 24 hrs by tripan blue assay as shown in Figure 7. Similarly the cytotoxicity of the drug in HT-29 and A375 cell line was done by MTT assay which was compared with the standard Doxorubicin and are given in the Figures 8 and 9. It can be seen that the percentage of cell death in the case of AQ was higher than that of the standard at all concentrations in DLA cell lines and the IC_{50} value was found to be 109 µg /ml. IC50 value of AQ in HT-29 and A375 were 96 µg/ ml and 108 µg/ ml respectively. The results reveal that the compound under investigation is found to be more active than the standard drug, cyclophosphamide in DLA. In the case of HT-29 and A375 it can be understood that the AQ exhibits comparable activity as that of the standard at higher concentrations.

3.5 In vivo Antiangeogenic Assay

CAM assay is an in vivo method by which the antimetastatic effect of the drug is studied on the chick allantoic membrane of a four day old embryo. CAM assay using 100µg concentration of AQ revealed that there was a significant reduction in the formation of new blood vessels. Figures 10 and 11 show the assay with the sample and the control.

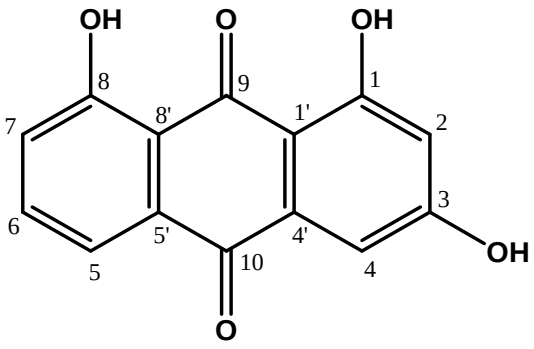

Figure 1. 1, 3, 8–trihydroxyanthracene–9, 10–dione

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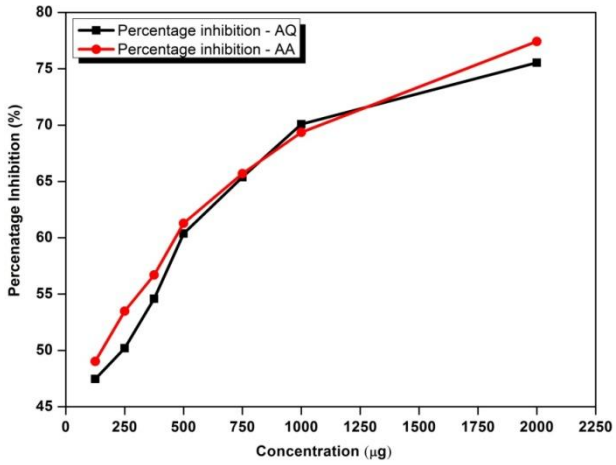

Figure 2. Hydroxide radical scavenging assay

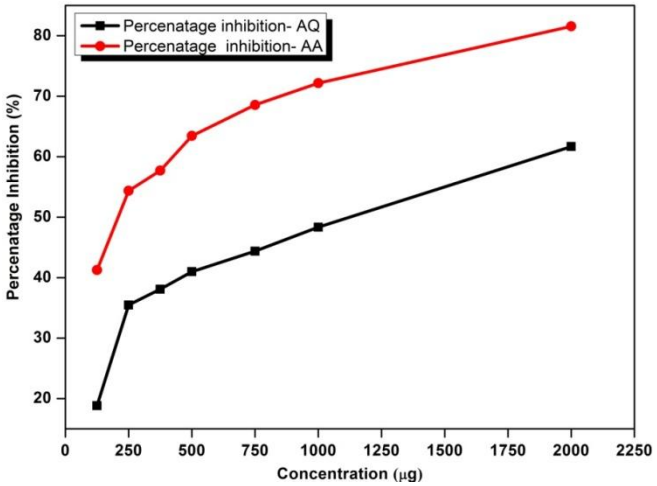

Figure 3. Superoxide radical scavenging assay

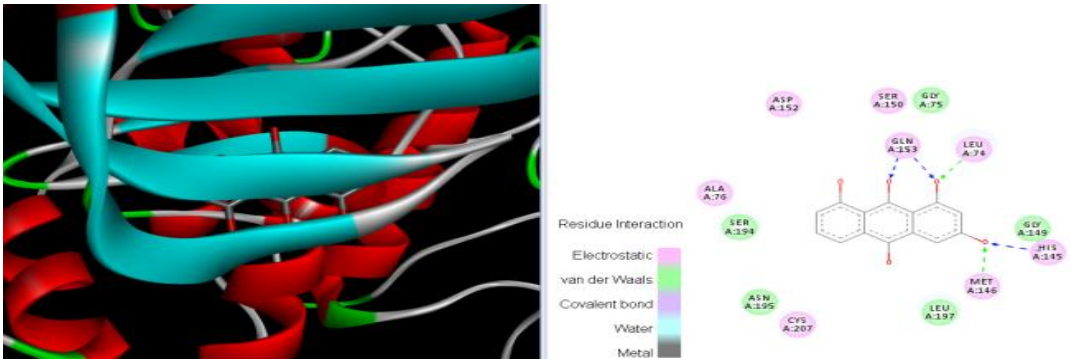

Figure 4. Docking of AQ with ERK protein (PDB ID: 3ZM4) over expressed in Melanoma

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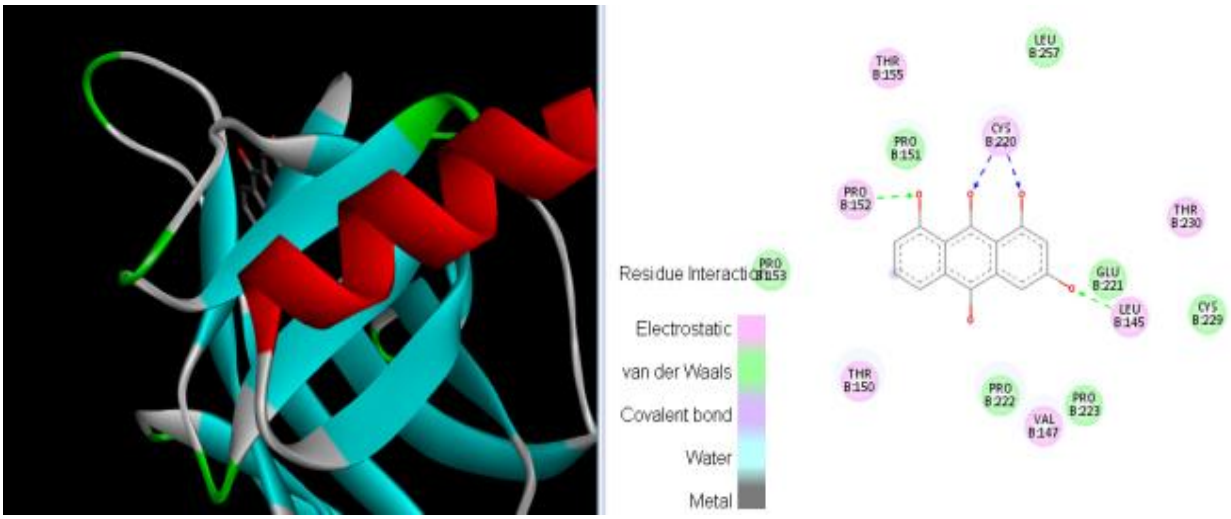

Figure 5 Docking with P53 protein (PDB ID: XOW) over expressed in DLA

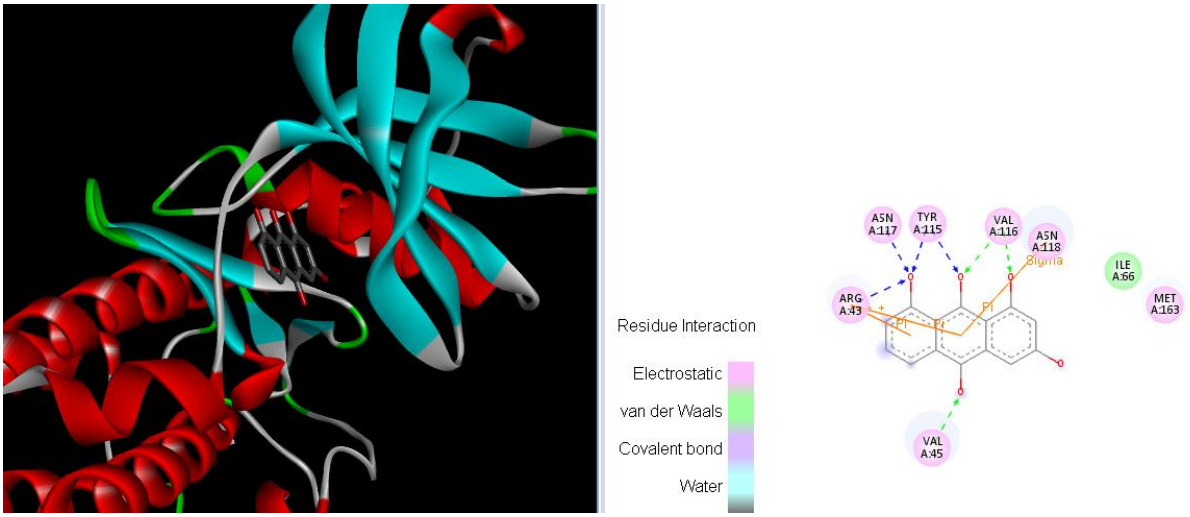

Figure 6. Docking with CK2 protein (PDB ID:1M2R) over expressed in HT-29

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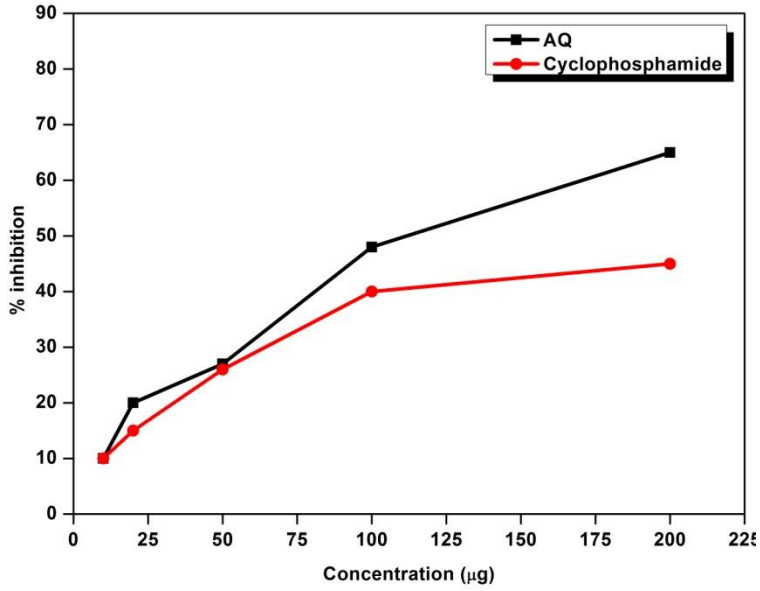

Figure 7. In vitro cytotoxicity in DLA cell lines

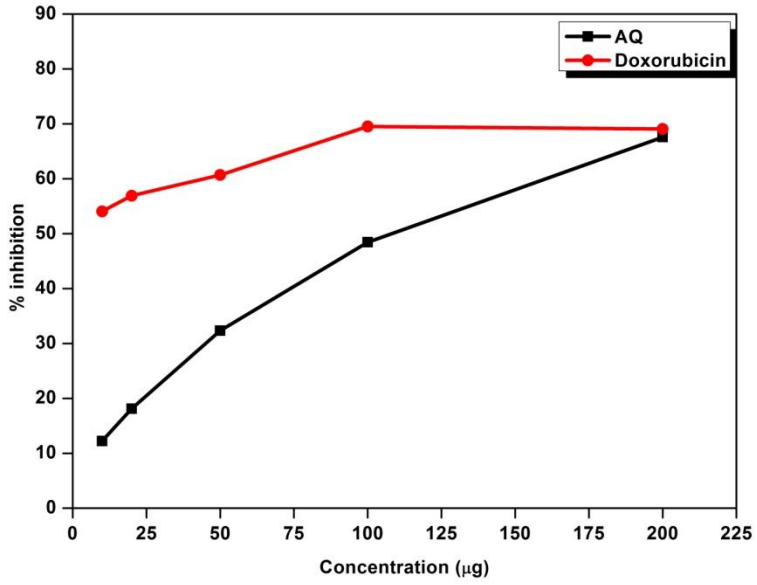

Figure 8. In vitro cytotoxicity in A375 cell lines

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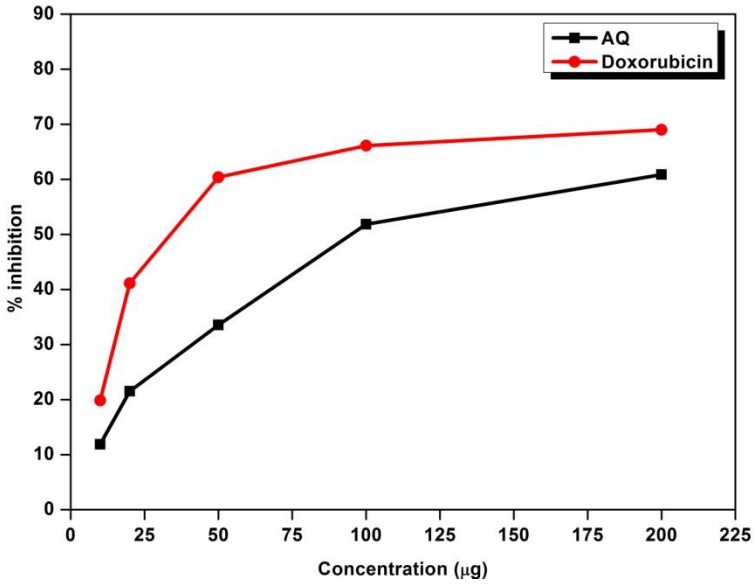

Figure 9. In vitro cytotoxicity in HT-29 cell lines

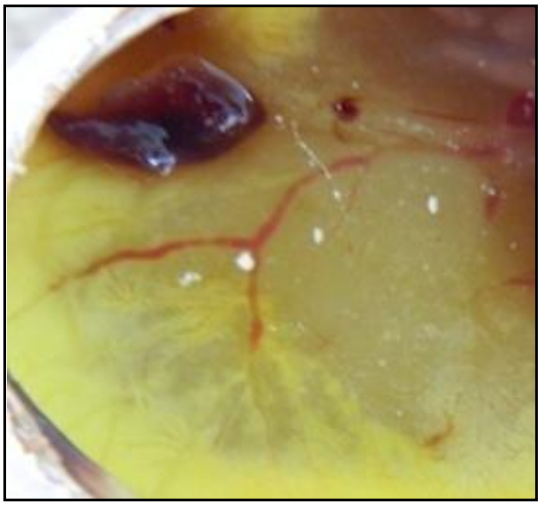

Figure 10 CAM assay of 1,3,8-anthraquinone

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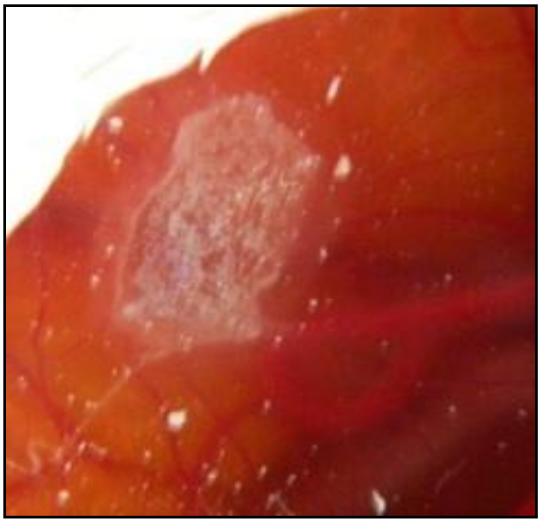

Figure 11 Negative Control (PBS) 1 Ml

Table.1 ADMET values of AQ

Pharmacokinetic properties	Values	Limitting value
S + log P	2.663	5
S + log D	1.789	< 4.1
M logP	1.116	< 4.15
Molecular weight	256.216	< 500
No. of hydrogen bond Donors	3	< 5
No. of hydrogen bond Acceptors	5	< 10
Rule of 5	0.000	0.000

Table 2 Docking results

Ligand Interaction	Target	Total energy	VWD	Hydrogen bond	Electrostatic
1,3,8-AQ	2XOW(P53)	–100.63	–86.3	–14.32	0
Cyclophosphamide	2XOW(P53)	–75.71	–69.87	–5.84	0
1,3,8-AQ	3ZM4(ERK)	–101.63	–74.2	–27.2	0
Doxorubicin(Std)	3ZM4(ERK)	–149.09	–119.59	–29.5	0
1,3,8-AQ	1M2R(CK2)	–86.94	–66.84	–20.1	0
Doxorubicin(Std)	1M2R(CK2)	–135.33	–118.56	–16.76	0

Conclusions

1,3,8–trihydroxyanthraquinone was isolated from *Cassia fistula* fruit pulp and characterized by various physicochemical techniques. This is the first report revealing the presence of 1,3,8 – trihydroxyanthraquinone in *Cassia fistula fruit pulp*. The in vitro antioxidant activity of the compounds was investigated by superoxide and hydroxide radical scavenging assay. The phytoligand was also subjected to in silico molecular docking studies to investigate its cytotoxic activity. The in vitro cytotoxic activity of these compounds were also performed to substantiate the data obtained from the docking studies in various cell lines. The antiangeogenic activity of the compound was also a part of the investigation. The CAM assay performed with chick embryo also exhibited pronounced activity as evident by the reduction in new blood vessel formation at its IC_{50} value. The results confirms that 1,3,8–trihydroxyanthraquinone which is a naturally occurring hydroxyquinone can be a potential candidate as a lead molecule for drug discovery if subjected to further studies.

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