***Original Research Article***

# *IN VITRO* EVALUATION AND MOLECULAR DOCKING ANALYSIS OF POTENTIAL ANTICANCER COMPOUNDS FROM *SYZYGIUM ALTERNIFOLIUM*


## Abstract

*Syzygium alternifolium* is widely distributed in tropical and subtropical regions around the world. Traditionally this plant has been used as a medicine for multiple ailments including cancer. The present study focussed on the anticancer activities of methanolic extracts of leaves of *Syzygium alternifolium* on human hepatocyte carcinoma (HepG2) cell line in comparison with standard drug doxorubicin. Doxorubicin acts in the cancer cell by intercalation into DNA and disruption of topoisomerase-II-mediated DNA repair and generation of free radicals and their damage to cellular membranes, DNA and proteins. Cytotoxicity studies have indicated that the phytoconstituents of *Syzygium alternifolium* have the ability to selectively target cancer cells ($IC_{50}$ = 185.585 µg/ml), whereas minimal or negligible cytotoxic effects were observed on normal cells. Molecular docking studies were carried out for selected toxicity filtered compounds against human Topoisomerase-2 and CDK-2 proteins (Protein Data bank-ID: 1ZXM and 1DI8) through Mcule docking software. *In silico* studies suggest seven compounds have the possibility to use as future nontoxic inhibitors. Molecular docking studies using the phytoconstituents were performed in order to gain a better understanding of the putative mechanisms of action leading to the development of improved and affordable therapies. This study paves a way to better understand the integration of molecular docking and *in vitro* studies can accelerate cancer drug discovery showing a good consistency of anticancer therapeutic drug potentials of *Syzygium alternifolium* by *in vitro* and *in silico* approaches.



**Keywords:** *Syzygium alternifolium*, Molecular docking, MTT assay, HepG2 cell lines.

## 1. Introduction

Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) are the two major forms of primary liver cancers (PLC), accounting for approximately 90% and 5% respectively [1][2]. The incidence of each is the most common widespread cancer in the world. HCC causes high annual mortality rates, particularly in Thailand, Cambodia and Laos, where viral hepatitis is endemic [3]. The induction of HCC is preceded by the occurrence of hepatocellular damage via reactive oxygen species (ROS) and the generation of chronic inflammation related to hepatocarcinogenesis [4]. Adjunctive therapies such as tumor necrosis factor and melphalan; or cisplatin, epirubicin and 5-FU; doxorubicin, interferon alpha and 5-FU have been used to overcome the HCC [5]. However, the main problem of chemotherapy to treat HCC is the cancer resistance mechanism, due to up-regulation of the multi-drug resistance protein (MDR) and a decrease of apoptotic proteins [6]. Thus, more effective chemotherapy is needed to control cancer and apoptosis induction, which is the desired effect for successful cancer treatment [7].

*Syzygium alternifolium* (Wt.) Walp. (Myrtaceae) is an endemic aromatic tree, distributed in Assam and Andhra Pradesh, states of India. Locally, it is known as mogi/movi. The plant parts were used in traditional medicine to cure various diseases viz., tender shoots and fruits for dysentery, seeds for diabetes and stem bark was used to treat gastric ulcers [8]. The anti-cancer activity of methanolic extract of *S. alternifolium* (Wt.) Walp (MESA) bark against MTT assay and *in silico* docking studies of isolated compounds from the plant have not been reported so far scientifically. Hence, the present study has been carried out to evaluate the anti-cancer activity of MESA.

## 2. Materials and Methods

### 2.1 Plant materials

*S. alternifolium* (Wt.) Walp plant bark was collected in the month of November 2014 from Sheshachalam hills, Tirupati, Andhra Pradesh, India. The plant was then taxonomically identified and authenticated by the botanist Dr. K. Madhava Chetty, Assistant Professor in S.V. University, Tirupati, India.

### 2.2 Preparation of extract

The bark was dried under shade then coarsely powdered with a mechanical grinder. The powder was passed through a sieve and stored in an airtight container for the extraction, and Extracted with methanol 70% (75-78°C) up to 72 hrs. After completion of extraction, the solvent was removed by distillation. Dark brown residue was obtained. The residue was concentrated and then stored in desiccators [9].

**2.3 Method**

The *in vitro* MTT assay was performed for the test substances on HepG2 (Human Hepatocellular Carcinoma) cell line to determine the level of cytotoxicity.

**2.4 Preparation of test compound for Cytotoxicity screening**

10 mg of test substances was weighed and dissolved in DMEM-HG medium supplemented with 2% inactivated FBS to obtain a stock solution of 10 mg/mL. Furthermore, serial two-fold dilutions were prepared from the stock solution to prepare lower concentrations for cytotoxicity testing [10].

**2.5 Cell line and culture medium**

Human Hepatocellular Carcinoma cell line cell line was procured from National Centre for Cell Sciences (NCCS), Pune. Stock cells were cultured in DMEM-HG supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/mL) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% Trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm2 culture flasks and all experiments were carried out in 96 well microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

**2.6 Determination of Cell viability by MTT Assay**

The monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using DMEM-HG containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer washed once with medium and different test concentrations were added on to the partial monolayer in the microtitre plates. The untreated cells were maintained as cell control for comparison. The plates were then incubated at 37°C for 24 h in 5% $CO_2$ atmosphere, and microscopic examination was carried out and observations were noted after 24h, the test solutions in the wells were discarded and 50 µL of MTT is added with DPBS was added to each well. The plates were gently shaken and incubated for 3 h at 37 °C in 5% $CO_2$ atmosphere. The supernatant was removed and 100

μL of Isopropanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm.

**2.7 Molecular Docking Studies**

Initially the proteins were downloaded from PDB was prepared by removing extra chains. Attributes of spheres are prepared and noted. Later molecules drawn in molecular and ligand preparation was created. Proteins are uploaded with sphere attributes and the structures were docked against 1DI8 and 1ZXM proteins. Docking indicated that some of our compounds have good binding ability with both CDK-2 and Human Topoisomerase-2 proteins. Following are the ligand interactions of compounds present in *Syzygium alternifolium* bark with 1DI8 and 1ZXM proteins [11].

**2.7.1 Protein-ligand interactions**

Docking stimulations predicts the binding orientation of drug candidates to their protein targets. Mcule was used for generating docking simulation studies.

**2.7.2 Docking simulations on** Cyclin-dependent kinase-2 (1DI8), Human topoisomerase II (1ZXM)

Cyclin-dependent kinase 2 (CDK2) drives the progression of cells into the S- and M-phases of the cell cycle. CDK2 activity is largely dispensable for normal development, but it is critically associated with tumor growth in multiple cancer types. Selective CDK2 inhibition may provide a therapeutic benefit against certain tumors, and it continues to appeal as a strategy to exploit in anticancer drug development [12]. DNA topoisomerases are nuclear enzymes that catalyze the introduction of topological changes to the DNA molecule. Replication and transcription of DNA require the unwinding of the DNA helix, which results in helical tension in the rest of the DNA molecule. Topoisomerases release this tension through the formation of transient single-stranded (i.e., type I topoisomerases) or double-stranded (i.e., type II topoisomerases) breaks in the DNA double helix, which makes topoisomerases essential for proliferating cells.

Hence, we report the molecular docking analysis of CDK 2 and TOPO II with phytocompounds.

**2.7.3 Ligand preparation**

The 2D ligands sketched in Mcule docking in the ligand imported side.

### 2.7.4 Protein preparation

The x-ray crystallised structure of Cyclin-dependent kinase-2 (PDB ID: 1DI8) and Human topoisomerase II protein (PDB ID: 1ZXM) were retrieved from RCSB protein bank. Attributes of SBD site sphere are obtained from discovery studio visualizer.

### 2.7.5 Ligand docking and scoring

Protein ligand interactions were stimulated though flexible glide-ligand docking with mCULE Docking allowed. The compounds docked displays a docking score

### 2.7.6 Visualization and analysis

The resulting docking poses were visualized through discovery studio visualizer. The ligand interactions were visualized to know the binding interactions between ligands and protein. The best docked structures were chosen using glide score function. The more negative the score the more favourable the binding. Additionally, the docked ligand poses were visualized and the different ligand receptor interactions were studied.

## 3. Results and Discussion

### 3.1 *In vitro* MTT assay

The results revealed that the cytotoxicity rate was increased when the concentrations of leaf extract increases. MTT assay measured the cell viability based on the reduction of yellow tetrazolium MTT to a purple formazan dye mitochondrial dehydrogenase enzyme. So, the amount of formazan produced reflected the number of metabolically active viable cells [13].

The CTC50 values observed for Cell line HepG2 against methanolic extract of *Syzygium alternifolium* was 297.805 µg/ml given in table 1. Test substances were assayed for *in vitro* cytotoxicity study against HepG2 cells (Human Hepatocellular carcinoma) by exposing the cells to different concentrations ranging from 8 to 1000 µg/mL. MTT assay was employed to test the cytotoxic effect of selected concentrations of the plant extract on the cell viability of HepG2 cells by measuring the metabolic activity through a colorimetric determination. Cell viability is a measure of the proportion of live, healthy cells within a population. The MTT assay is usually carried out to detect the cells with constant mitochondrial activity, thereby; an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. In the present study, the $CTC_{50}$ value of the plant extract was found to be 297.805

µg/mL. The plant extract exhibited 68.68±1.44% toxicity in HepG2 cells at the highest concentration tested (1000 µg/mL).

The plant has the enormous medicinal properties and its various parts used to cure many diseases. Quercetin exhibits direct proapoptotic effects on tumor cells and thus can inhibit the progress of numerous human cancers. The anticancer effect of quercetin has been documented in numerous *in vitro and in vivo* studies that involved several cell lines and animal models. On the other hand, the high toxic effect of quercetin against cancer cells is accompanied with little or no side effects or harm to normal cells [14]. Recently, apigenin has been widely investigated for its anti-cancer activities and low toxicity. Apigenin was reported to suppress various human cancers *in vitro* and *in vivo* by multiple biological effects, such as triggering cell apoptosis and autophagy, inducing cell cycle arrest, suppressing cell migration and invasion, and stimulating an immune response [15]. Kaempferol mechanistically can induce anticancer effects mainly through down regulation of the expressions of proteins involved in the cancer progression and formation alongside apoptosis induction, cell cycle arrest, and decreasing the expression for anti-inflammatory proteins [16]. Since the presence of these compounds in the extract might be responsible for the anticancer activity.

**Table 1: MTT Assay- Percentage cytotoxicity of MESA against Hep G2 cell lines**

| **Test substance** | **Concentration (µg/mL)** | **Percentage Cytotoxicity** | **CTC50 (µg/mL)** |
|---|---|---|---|
| Methanolic extract of *Syzygium alternifolium* | 1000 | 68.68±1.44 | 297.805 |
| | 500 | 57.54±0.44 | |
| | 250 | 48.22±0.58 | |
| | 125 | 3.45±0.18 | |
| | 62.5 | 3.27±0.32 | |
| | 31.25 | 2.72±0.07 | |
| | 15.625 | 1.81±0.79 | |
| | 7.8 | 0.70±0.57 | |

**Table 2: *In vitro* MTT assay –Percentage cytotoxicity of Doxorubicin against HepG2 cell lines**

| µM of Doxorubicin | µg of Doxorubicin | Cytotoxicity % | CTC50 (µg/mL) |
|---|---|---|---|
| 10 | 5 | 86.28 | |
| 5 | 2.5 | 84.79 | |
| 1 | 1.25 | 70.17 | |
| 0.5 | 0.625 | 57.47 | 0.485 |
| 0.25 | 0.312 | 41.63 | |
| 0.125 | 0.156 | 35.76 | |
| 0.0625 | 0.078 | 28.59 | |

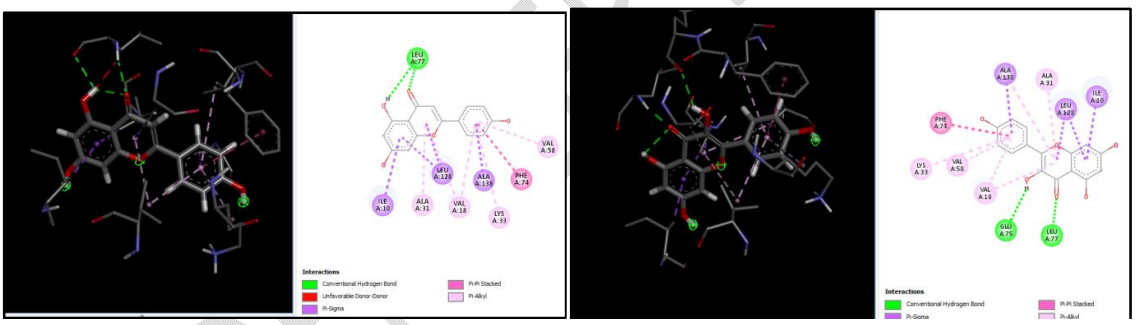
**Table 3: Molecular docking studies of isolated compounds against Topoisomerase-2 and CDK-2**

| Compounds | Cyclin Dependent Kinase II (1DI8) Docking Score | Topoisomerase II (1ZXM) Docking Score |
|---|---|---|
| 3-Hydroxy benzoic acid | -6.1 | -7.2 |
| Gentisic acid - | -5.7 | -6.5 |
| Caffeic acid | -7.1 | --6.3 |
| 2,5-monoformal-1-rhamnitol | -5.5 | -6.6 |
| 4-oxo-5-phenyl pentoic acid | -7.2 | -7.2 |
| Apigenine | -9.8 | -9.4 |
| Lutidine | -5.1 | -5.1 |
| Quercetin | -8.8 | -9.8 |
| Kaempferol | -9.4 | -9.5 |

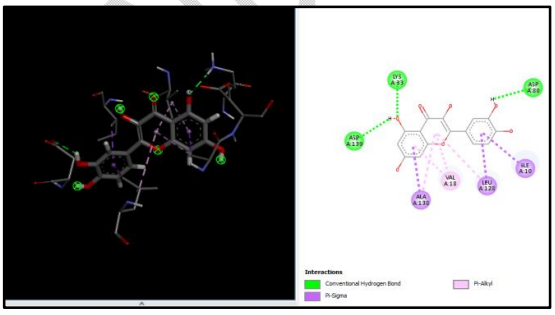
| | | |
|---|---|---|
| Acarbose | -6.2 | -7.4 |
| Squalene | -7.7 | -8.3 |
| Stigmasterol | -8.1 | -8.6 |
| Doxorubicin | | |

### 3.2 Molecular Docking Studies

Initially the proteins were downloaded from PDB was prepared by removing extra chains. Attributes of spheres are prepared and noted. Later molecules drawn in molecular and ligand preparation was created. Proteins are uploaded with sphere attributes and the structures were docked against 1DI8 and 1ZXM proteins. Docking indicated that some of our compounds have good binding ability with both Cyclin-dependent kinase-2 and Human topoisomerase II proteins represented in table 3. Following are the ligand interactions of compounds present in *Syzygium alternifolium* whole plant with 1DI8 and 1ZXM proteins represented in figure 1 and 2.



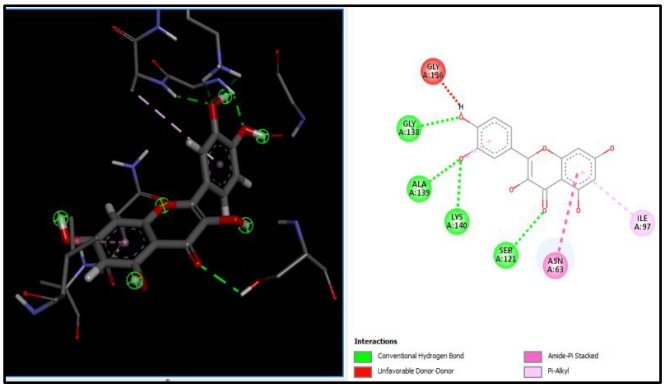
**a) Apigenine -9.8** **b)Kaempferol -9.4**



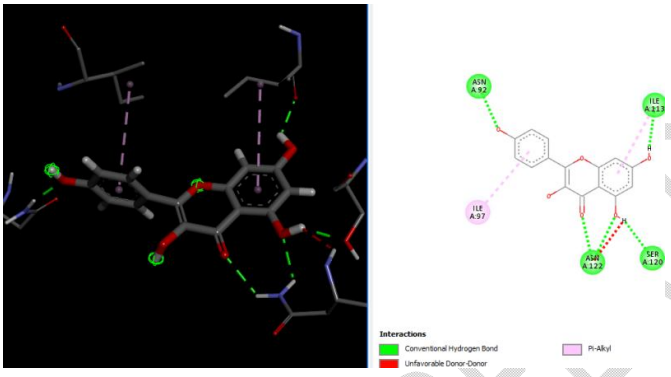
**c) Quercitin -8.8**

**Figure 1: Docking poses of phytocompounds with Cyclin-dependent kinase-2**

**Quercetin - -9.8**




**Kaempferol - -9.5**




**Apigenine - -9.4**

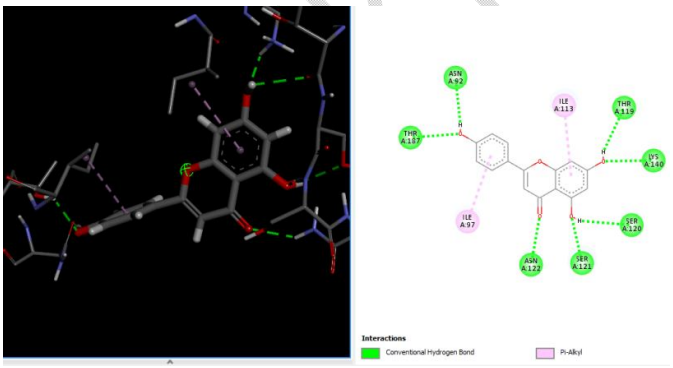



**Figure 2: Docking poses of phytocompounds with Human topoisomerase II protein**

The molecular docking in this study shows a vital role in predicting molecular interactions of phytocompounds with targeted proteins. This application is widely used in the pharmaceutical industry as a powerful tool, particularly in the analysis of structure–activity

relationship. The analysis of molecular docking outputs, such as binding affinity, are frequently applied in the determination of potential ligands. Molecular docking also has the ability to predict small molecule ligands binding toward appropriate target binding site.

Apigenine, Kaempferol and Quercitin have demonstrated remarkable binding affinity towards Cyclin-dependent kinase-2. Apigenine shown highest docking score -9.8 in comparison to other compound topwards CDK-2[17]**.** Quercetin, Kaemferol and Apigenine have demonstrated remarkable binding affinity towards TOPO-2. Quercetin shown highest docking score -9.8 in comparison to another compound towards TOPO-2. Thus, indicating that these compounds are potent inhibitor of the CDK-2 antiapoptotic family of proteins and TOPO-2 proteins [18].

The application of molecular docking studies for the compounds that are present in methanolic extract of *Syzygium alternifolium* with proteins 1DI8 and 1ZXM are considered very useful and proven anticancer activity. Our findings conclude that all phytocompounds are possibly able to act as potential inhibitors for the targeted 1DI8 and 1ZXM proteins, supported by the high binding affinities.

**4. Conclusion**

The present study scientifically established the *in vitro* MTT assay and *in silico* docking studies to predict anticancer activity. The MTT assay was performed and the results showed that the extract has anticancer activity. From docking scores, we can conclude that the isolated compounds from the extract possess anticancer activity through inhibition of CDK-2 and TOPO-2 can be further preceded to wet lab synthesis.



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