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# Anti-Proliferative Effect of Curcumin on Eukaryotic Cells and Their Plausible Modes of Action

**Priya Josson Akkara[1*], Joel P. Joseph[2], Nandini Damodaran[2], Supriya Prakash[2], Amarnath[2], Rashmi Kv[2], Jagadeesh Kumar[2]**

**[1]Department of Life Sciences, KristuJayanti College (Autonomous), Bengaluru-560077**
**[2]Department of Biotechnology, Sir M. Visvesvaraya Institute of Technology, Bengaluru-562157**

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**Corresponding Author Email:** priyajosson@kristujayanti.com

## Abstract

*Curcuma longa* (commonly known as turmeric) is a rhizomatous herb of the Zingiberaceae family native to South India and Indonesia. Traditional knowledge about turmeric throws light on its wound healing properties. In the recent years, there has been an outburst of interest in the use of traditionally known herbs like turmeric as sources of alternative medicine in the treatment of several maladies like cancer. In the present study, an attempt was made to investigate the effect of Curcuminas an anti-proliferative agent on eukaryotic cells using BHK-21 cells and onion root tips. Various dilutions of Curcumin were found to exert anti-proliferative effect on BHK-21 cells proportional to the concentration of Curcumin, with which the cells were treated.After treating BHK-21 with Curcumin, MTT assay was done to check its viability post treatment.

The mitotic activity of the Curcumin treated onion root tip model was studied. Mitotic anomalies were observed at various steps of cell division after treatment with Curcumin. *In silico* assessment of the mechanism of action of Curcumin by docking studies revealed that Curcumin bound to three cell cycle enzymes (Cyclin Dependent Kinase 2, Aurora Kinase, and Haspin Kinase) and possibly brought about the anti-proliferative effect by the inhibition of these cell cycle enzymes.

**Keywords**
Curcumin, anti-proliferative, cell cycle

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## INTRODUCTION

*Curcuma longa*, commonly known as turmeric, is an herbaceous perennial plant of the Zingiberaceae family [1], whose native is southwest India, and requires 20 - 30°C (68 and 86°F) temperature and a considerable amount of annual rainfall to thrive [2].

Curcumin is a major constituent in turmeric which not only imparts it the yellow color, but also is responsible for its many medicinal properties. The percentage of Curcumin in turmeric depends on the geographical area of cultivation and is mostly about 3% in general. Chemically named as ditenilymethane, Curcumin comprises of Curcumin I (94%), Curcumin II (6%), Curcumin III (0.3%). It was first isolated in 1815, following which Roughly and Whiting deciphered its chemical structure in 1973. It has the molecular formula $C_{21}H_{20}O_6$.

Curcumin has been shown to exhibit a wide range of pharmacological activities including anti-inflammatory activity, anti-oxidant activity, anti-atherosclerotic activity, anti-microbial activity, and wound healing effects [ 3,4]. *In vitro* and *in vivo* studies have unraveled these vital properties of Curcumin.. These properties have been conferred to it due to the complex molecular structure it possesses and the abililty that it has to interact with numerous signaling molecules. Cancer is a malignant growth or tumour resulting from an uncontrolled division of cells. As a matter of fact, the term is a collective one representing over 100 different types of diseases that fall under this broad spectrum of diseases. According to WHO, the risk factors for cancer include: tobacco use, alcohol use, overweight and obesity, dietary factors, insufficient fruit and vegetable intake, physical inactivity, infections from *H. pylori*, Hepatitis C virus (HCV), Hepatitis B virus (HBV), and some types of Human papilloma virus (HPV), environmental and risks including ionizing and non-ionizing radiation. The cancer-causing agents, carcinogens, can exist in the food, water, air, chemicals and sunlight. Carcinogens interact with the inherited and acquired constitutions of a person and cause cancer. These agents include chemical carcinogens, ionizing radiations, viral and bacterial infections, hormonal changes, genetic factors and immune dysfunction [5].

While certain risks cannot be eliminated, a change in lifestyle would help prevent the occurrence of cancer. Individuals restricting their exposure to tobacco products, sunlight and pollution can decrease their risk of developing cancer. Besides, chemoprevention of cancer is also possible by the use of certain chemotherapeutic agents. WHO has defined traditional medicine as therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine [2].Curcumin was found to be a potential anticancer agent due to its antiproliferative and cytotoxic effect in tumor cells[6,7].Recently curcumin has been found to possess anti-cancer activities via its effect on a variety of biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis and metastasis [8]Anti-precancerous outcome of curcumin was found to be mediated through anti-oxidant as well as pro-oxidant pathways [9].Clinical trials with 1125-2500 mg per day of curcumin in humans has confirmed safety of it [10].The aim of this this study is to investigate the anti-proliferative effect of curcumin on eukaryotic cells and analyses its possible modes of action.

## MATERIALS AND METHODS:

**Cell culture:** BHK-21 cells were cultured in GMEM medium supplemented with 10% FBS and 1X Anti-Anti (Sigma, USA). The cells were cultured in a humidified incubator at 37$^o$C and 5% $CO_2$ [11,12] The medium was changed every alternate day and the cells were trypsinized when they reached 80% confluence.

**Serum Optimization studies:** BHK-21 cells were seeded at an initial density of 0.5*10$^5$cells/ml. Three aliquots of the cell suspension were prepared with 5%, 7%, and 10% FBS respectively. In a 24-well plate, 1 ml of the 5% aliquot was added to each of the first two columns in the 24-well plate, 1 ml of the 7% aliquot was added to the next two columns in the 24-well plate, and 1 ml of the 10% aliquot was added to the last two columns in the 24-well plate. The 24-well plate was incubated in a humidified incubator at 37$^o$C and 5% $CO_2$.At every 24-hour intervals, cells were harvested consecutively, and the cell viability was determined [13].

**Growth curve analysis:** BHK-21 cells were seeded at an initial seeding density of 1*10$^5$cells/ml. In a 6-well plate, 2 ml of the cell suspensions of initial seeding density of 1*10$^5$cells/ml was added into each plate. The cells were incubated in a humidified incubator at 37$^o$C and 5% $CO_2$. Cells were harvested from two wells consecutively at every 24-hour time interval and the cell count was determined.

**MTT Assay:** BHK-21 cells were seeded at an initial seeding density of 0.5*10$^5$cells in a 24 well plate. When the cells were about 60% confluent, we treated them with various concentrations of Curcumin (10µM,

20µM, 30µM, 40µM, 50µM, 75µM, 100µM, 150µM, 200µM, 300µM) diluted in DMSO. The cells were incubated in a humidified incubator at 37°C and 5% $CO_2$. Post 24 hours of treatment, 100 µL of MTT working solution was added to each well and the plates were incubated for 2h in a humidified incubator at 37-degree C and 5% $CO_2$. The formazan crystals were then dissolved in a detergent containing TritonX-100, isopropanol and HCl. The absorbance at 570 nm was noted and the graph of Absorbance vs. cell concentration was plotted [14].

**Analysis of mitotic anomalies in onion root tips:** Fresh uniformly sized onions, procured from the market, were washed thoroughly and the loose outer scales of bulbs were removed with the help of sharp and pointed forceps to expose the root to media. A series of bulbs were then placed on beakers containing different concentrations of Curcumin - 10 µM, 30 µM, 50 µM, 75 µM, 150 µM at 25 °C for about 72 hours. One of the beakers was filled with water and kept as control. Post treatment, the bulbs were washed thoroughly under running tap water and the root tips from each bulb were plucked and fixed using Glacial acetic acid: Ethanol in 1:3 ratio for 24 hours to arrest the stages of cell division. For chromosomal analysis, the root tips were transferred to a watch glass with 0.1N HCl. This was heated intermittently for 2 minutes to soften the tips. The root tip was cut and placed on a glass slide in a drop of distilled water and a drop of aceto-orcein stain was added. The slide was then covered with a cover slip and the cells were observed under the microscope for chromosomal aberrations.

**Molecular Docking studies**: Protein-Ligand docking was performed for Curcumin with three important cell cycle enzymes – CDK2, Aurora Kinase, and Haspin Kinase using FlexX v2.1.3. The molecular structure of Curcumin was retrieved from Zinc database and the structure of the enzymes were retrieved from Protein Data Bank (PDB) and the ligand binding sites were chosen based on the data on the ligand interactions of the protein in PDB. The interactions were visualized in Discovery studio 3.5 and the results were captured and recorded.

## RESULTS AND DISCUSSION

**Serum Optimization studies:** The growth of BHK-21 was tested at three different serum concentrations (5%, 7%, and 10%). The optimum serum concentration essential for the optimum growth of BHK-21 cells was determined from the results obtained as described in **Table 1** and **Figure 1**. The optimum serum concentration was found to be 10%.

| Day | Time elapsed (hours) | Viable Cell Count (*$10^5$ cells/ml) | | |
|---|---|---|---|---|
| | | 5% FBS | 7% FBS | 10% FBS |
| 0 | 0 | 0.5 | 0.5 | 0.5 |
| 1 | 24 | 0.15 | 0.2 | 0.3 |
| 2 | 48 | 0.45 | 0.6 | 0.95 |
| 3 | 72 | 2.8 | 2.95 | 2.6 |
| 4 | 96 | 5 | 5.7 | 7.15 |
| 5 | 120 | 18.9 | 19.8 | 22.5 |
| 6 | 144 | 18.5 | 19.3 | 22.3 |

**Table 1: Viable cell count of BHK-21 cells at different FBS concentrations for different incubation times**

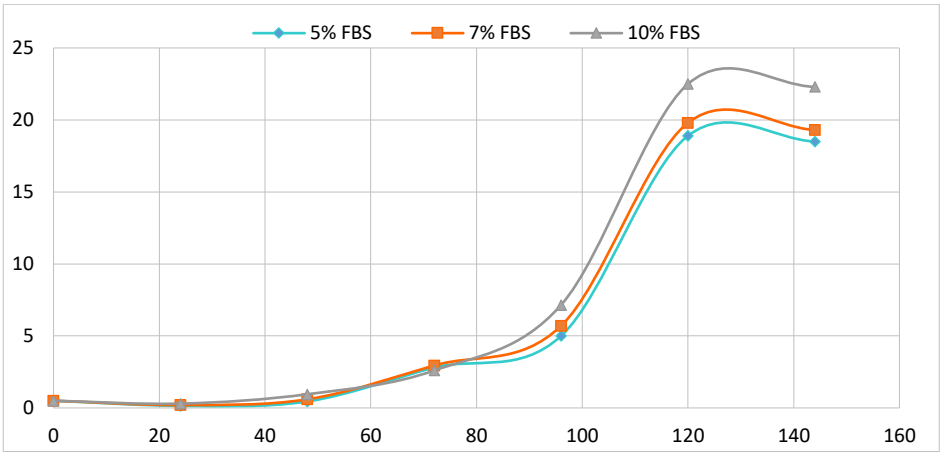



**Figure 1: Viable cell count of BHK-21 cells at different FBS concentrations for different incubation times: hrs elapsed on x-axis and (*$10^5$ cells/ml) on y-axis**

| Day | Time elapsed (in hours) | Viable Cell Count (*$10^5$ cells/ml) |
|---|---|---|
| 0 | 0 | 1.0 |
| 1 | 24 | 0.35 |
| 2 | 48 | 1.65 |
| 3 | 72 | 7.75 |
| 4 | 96 | 22.8 |
| 5 | 120 | 22.5 |
| 6 | 168 | 6.5 |

**Table 2: Table containing the data from standard growth curve studies recording the cell concentration at specific time intervals**

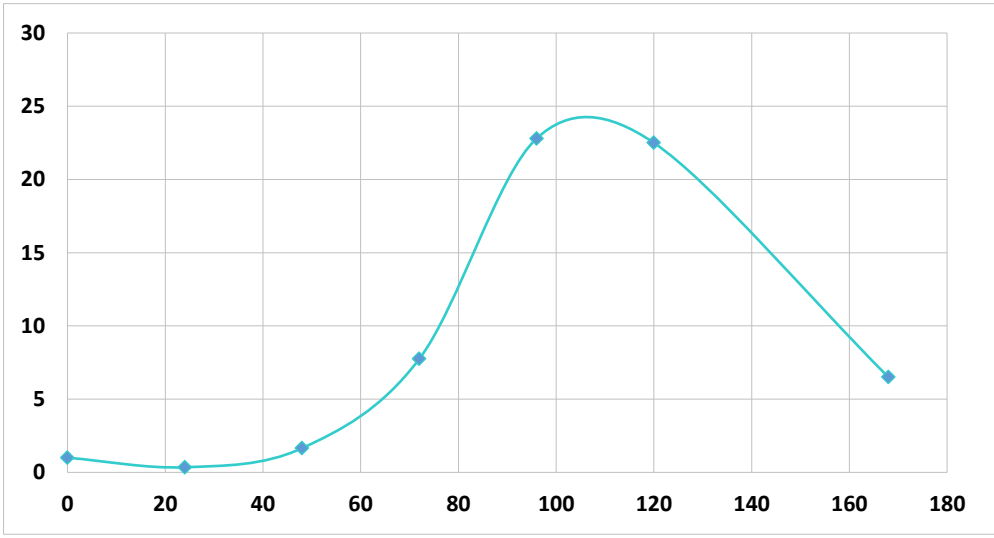



**Fig. 2: Standard growth curve of BHK-21 cells cultured in GMEM+10 % FBS: hrs elapsed on x-axis and (*$10^5$ cells/ml) on y-axis**

**Growth curve analysis:** BHK-21 cells cultured in the optimized serum concentration were observed for their growth characteristic and a graph of cell concentration vs. time was plotted as shown in **Table 2** and Figure **2**.

**MTT Assay:** BHK 21 cells were treated with various concentrations of Curcumin (10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 75 µM, 100 µM, 150 µM, 200 µM, 300 µM) and the assay was performed as described in the methods. Absorbance at 570 nm was tabulated and a graph of absorbance versus cell concentration was plotted as shown in **Table 3** and **Figure 3**. The viability of cells were inversely proportional to the concentration of curcumin and was nonviable beyond 50 µM emphasizing the antiproliferative effect of curcumin which was directly proportional to its concentration.

| Sl. No. | Curcumin concentration (µM) | Absorbance at 570 nm |
|---|---|---|
| 1 | 10 | 0.323 |
| 2 | 20 | 0.051 |
| 3 | 30 | 0.018 |
| 4 | 40 | 0.013 |
| 5 | 50 | 0.010 |
| 6 | Control | 2.63 |

**Table 3: The samples for MTT assay (BHK 21 cells treated with various concentrations of Curcumin)**

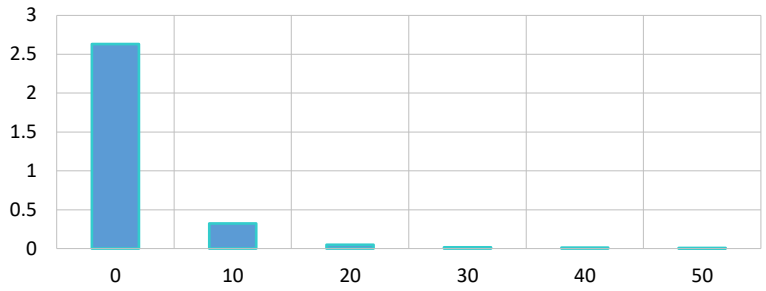



**Figure 3: A standard graph of Absorbance vs. Curcumin Concentration in cells: Curcumin (µM) on x-axis and absorbance on y-axis**

**Mitotic anomalies in onion root tips:** The slides prepared from the root tips of Onions treated with various concentrations of Curcumin and also with water were studied and the observations were noted. Mitotic anomalies were observed in the Curcumin treated samples, especially in the metaphase and anaphase stages of cell division, wherein there were abnormalities observed in the alignment of the metaphase plate and there were formations of anaphase bridges and fragments of chromosomes observed in the anaphase stage. At higher concentrations of Curcumin (75 µM and 150 µM), chromosomes were found to be dense and scattered and the particular divisional stage that the cell was in, wasn't distinctly clear. **Figure 4** shows the mitotic anomalies that are found on Curcumin treatment.

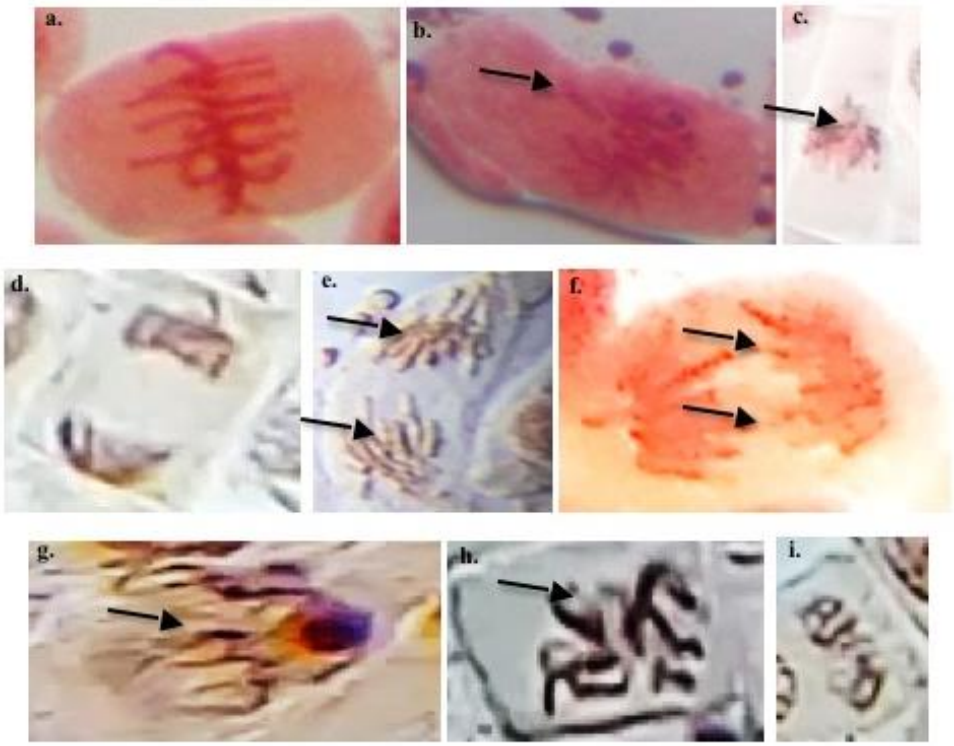



**Figure 4. Mitotic anomalies in curcumin treated onion rot tips**. **a.** A normal metaphase stage observed when the onion root tips were grown in control.**b.** A metaphase abnormality (lack of metaphase plate alignment) observed when the onion root tips were grown in Curcumin concentration of 10 µM.**c.**A metaphase abnormality (lack of metaphase plate alignment) observed when the onion root tips were grown in Curcumin concentration of 50 µM. **d.**A normal anaphase stage observed when the onion root tips were grown in control. **e.**An anaphase abnormality (anaphase bridge formation and fragments) observed when the onion root tips were grown in Curcumin concentration of 10 µM.**f.**An anaphase abnormality (anaphase bridge formation) observed when the onion root tips were grown in Curcumin concentration of 50 µM.**g.**A cell division abnormality (fragmented chromosomes) observed when the onion root tips were grown in Curcumin concentration of 75 µM. **h.** A cell division abnormality (scattered chromosomes) observed when the onion root tips were grown in Curcumin concentration of 150 µM. **i.** A cell division abnormality (chromosomes scattered from metaphase plate) observed when the onion root tips were grown in Curcumin concentration of 150 µM.

**Docking Curcumin with critical cell cycle enzymes:** Curcumin was docked with cell cycle enzymes Cyclin Dependent Kinase 2 (CDK2), Aurora Kinase and Haspin Kinase at the respective binding sites and three best poses were analyzed. Curcumin interacted with CDK2 with best dock scores of -27.283, -24.9436 and -24.3309 at the ATP binding site. There were several bonding interactions that were found between the atoms of Curcumin and the amino acids of CDK 2 and the hydrogen bonding distances between them ranged between 2.5 $A^o$ to 3 $A^o$ on an average. This implies that Curcumin may be hampering the action of the enzyme by blocking ATP binding to the enzyme for its catalytic action, thus exerting anti-proliferative effect. **Figure 5** shows the best dock pose.

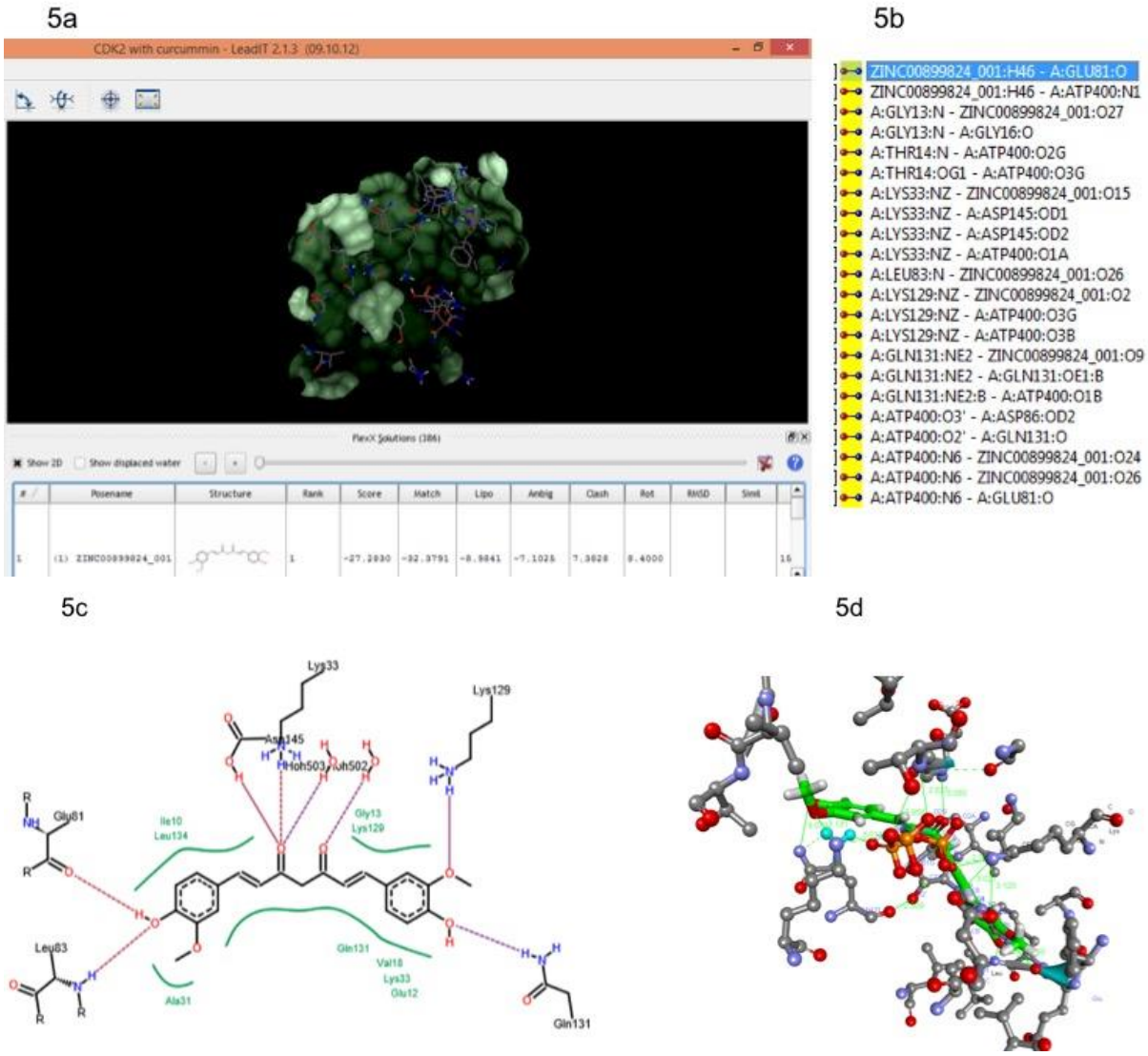



**Figure 5: Best docking pose of CDK2-Curcumin docking**. **a.** Screenshot of the dock pose and score **b.** A list of interactions of CDK 2 with Curcumin **c.** Amino acid interactions between CDK2 and Curcumin **d.** Amino acid interactions between Curcumin and CDK 2 and the hydrogen bond distances of interactions

Curcumin interacted with Aurora Kinase with best dock scores of -16.7136 and -16.4344 at its adenosine binding site. There were several bonding interactions that were found between the atoms of Curcumin and the amino acids of Aurora Kinase and the hydrogen bonding distances between them ranged between 2.5 $A^o$ to 3 $A^o$ on an average. This implies that Curcumin may be hampering the action of the enzyme by blocking Adenosine binding to the enzyme for its catalytic action, thus exerting anti-proliferative effect. **Figure 6** shows the best dock pose.

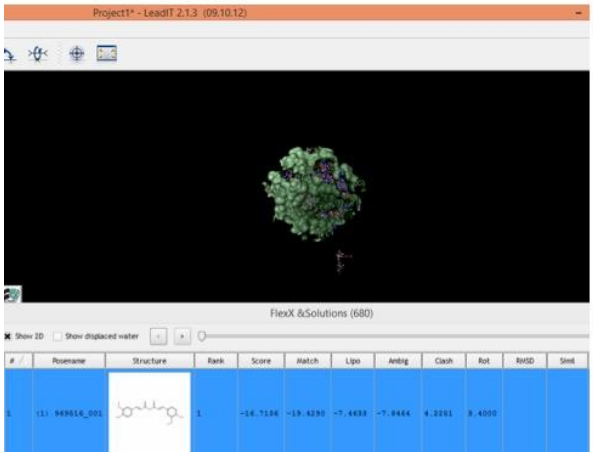
From the observations made through docking studies of Curcumin with Aurora Kinase, we could infer that the Curcumin could be possibly binding to Aurora Kinase thereby hindering the Microtubule formation or stabilization at the spindle pole during chromosome segregation. Therefore, Curcumin could be exerting its anti-proliferative effect on eukaryotic cells by restricting the microtubule formation or stabilization at the spindle pole during cell division. This could have been the possible reason for the observation of mitotic anomalies in the Anaphase of cell division. Although Aurora Kinase has lesser binding affinity to Curcumin when compared to the binding affinity of CDK 2, the dock score indicates that it still has very high binding affinity.

Curcumin interacted with Haspin Kinase with best dock scores of -11.6397 and -10.7947 at its binding site. There were several bonding interactions that were found between the atoms of Curcumin and the amino acids of Haspin Kinase and the hydrogen bonding distances between them ranged between 2.5 $A^o$ to 3 $A^o$ on an average.

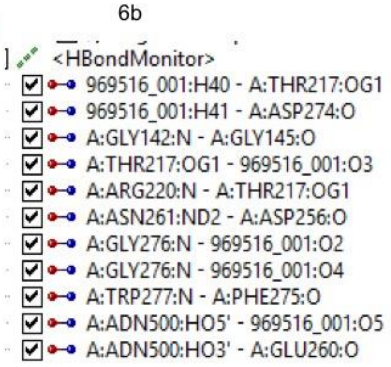
**Figure 7** shows the best dock pose.

Thus, from the observations made through docking studies of Curcumin with Haspin Kinase, we could infer that the Curcumin could be possibly binding to Haspin Kinase thereby hindering the phosphorylation histone H3 at 'Ser-3' (H3T3ph) during mitosis. The implication of this activity is that the positioning and activation of Aurora Kinase B and other components of the chromosomal passenger complex (CPC) at centromeres is affected. This has an effect on chromatid cohesion, metaphase alignment and normal progression through the cell cycle. This could be the reason why there was an abnormality observed in the metaphase plate alignment in the experiment on onion root tips. Although Haspin Kinase has lesser binding affinity to Curcumin when compared to the binding affinities of CDK 2 and Aurora Kinase enzymes, the negative dock score indicates that it still has very high binding affinity. In brief, Curcumin was found to have good binding affinities to CDK2, Aurora Kinase, and Haspin Kinase as the docking scores were negative. This indicates that Curcumin possibly binds to one or many of these enzymes and inhibits them, thereby stopping the cell division and exerting an anti-proliferative effect.

6a




6b

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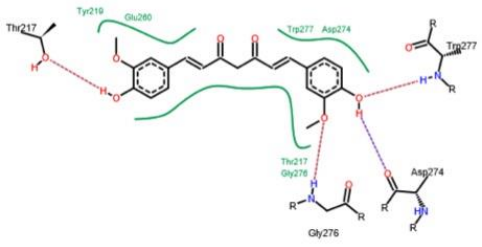


6d

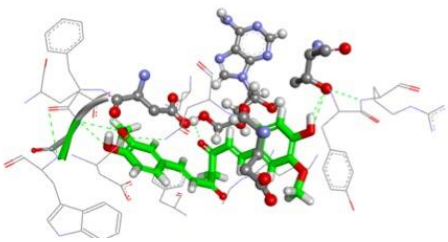

**Figure 6: Best docking pose of Aurora Kinase-Curcumin docking**. **a.** Screenshot of the dock pose and score **b.** A list of interactions of Aurora kinase with Curcumin **c.** Amino acid interactions between Aurora kinase and Curcumin **d.** Amino acid interactions between Curcumin and Aurora kinase and the hydrogen bond distances of interactions

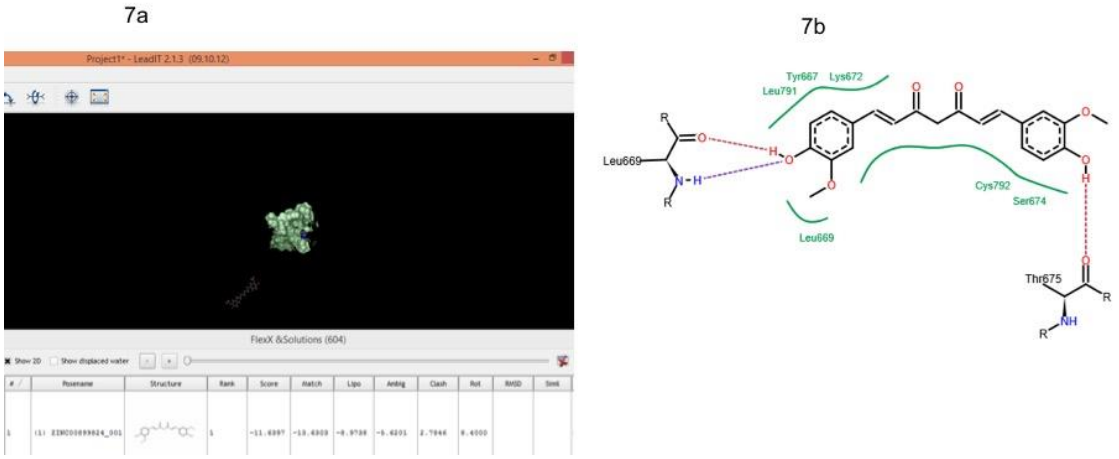


**Figure 7: Best docking pose of Haspin kinase-Curcumin docking**. **a.** Screenshot of the dock pose and score **b.** Amino acid interactions between Haspin kinase and Curcumin

## CONCLUSION

From the experiments conducted on BHK-21 cell line, it was evident that the cells were sensitive to the cytotoxic activity of Curcumin. On using Curcumin to grow the onion roots, certain mitotic anomalies were observed in the onion root tips showing that Curcumin hampered the cell division, thereby leading the cell towards death rather than division.

*In silico* assessment of the binding properties of Curcumin to three cell cycle enzymes (CDK 2, Aurora Kinase, and Haspin Kinase) by docking showed that Curcumin binds to all of these enzymes and inhibits their action thereby affecting the various divisional stages in mitosis. Curcumin was found to have good binding affinities to CDK 2, Aurora Kinase, and Haspin Kinase as the docking scores were negative. This indicates that Curcumin possibly binds to one or many of these enzymes and inhibits them, thereby stopping the cell division and exerting an anti-proliferative effect.

Therefore, from the above-mentioned observations and results one could conclude that Curcumin exerts and anti-proliferative effect on eukaryotic cells. This anti-proliferative effect is brought about possibly by the inhibition of CDK 2, Aurora Kinase, and Hasspin Kinase thereby interrupting the metaphase and the anaphase stages of the cell cycle by affecting the metaphase plate alignment and hindering spindle formation in anaphase, and also by affecting the passage of the cells through various phases of cell cycle (transition from G1 phase to S phase). Because Curcumin exerts an anti-proliferative effect on cells, it could be viewed as an effective alternative medicine for chemoprevention of cancer.

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