Study to Elucidate the Inhibitory Potential of Selected Flavonoids against Jab1 in Cervical Cancer

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Abstract: Jab1 has gained tremendous attention as a potent target in cancer therapeutics. Jab1 has been associated with the progression of numerous carcinomas by exhibiting several negative roles, such as degradation of tumor suppressor genes. We have mainly focused our research on elucidating inhibitory phytocompound (flavonoid) that could inhibit the Jab1 expression in cervical cancer, one of the most globally recognized gynecologic malignancies. Moreover, this study would provide a better targeted therapeutic approach for the management of cervical cancer. Although numerous flavonoids have presented potent cytotoxic and antitumor efficacy against HeLa cells, the inhibitory potential of some flavonoids against Jab1 has not been explored yet. Therefore, the current research aims to explore the inhibitory potential of flavonoids against Jab1 in cervical cancer. A total of eleven potent phytocompounds have been selected for screening potent phytocompound that could inhibit HeLa cancer cell growth via Jab1 downregulation. In silico findings demonstrated baicalein as the best Jab1 inhibitory phytocompound. Additionally, MTT assay and RT-PCR analysis have also strongly validated its inhibitory potential against Jab1 in cervical cancer. However, the further detailed analysis needed to be done to elucidate a novel mechanism that could help identify a novel and potent therapeutic phytocompound for drug development against cervical cancer.

Keywords: Jab1; Flavonoids; molecular docking; MTT; RT PCR; cervical cancer.

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1. Introduction

Jab1 has emerged as a potential oncetarget in the management of several chemotherapeutic approaches [1]. Aberrant increase in cervical cancer incidences has demanded extreme attention globally [2]. Considering the numerous side effects of conventional therapies, the current research is focused on identifying novel phytocompounds targeting these crucial oncetargets involved in cancer progression [3-4]. Interestingly, Jab1 (CSN5) has been known to play a significant role in cell cycle regulation, DNA damage repair, cellular differentiation, and apoptosis [5]. Indeed, our previously published study has also demonstrated its significant involvement in gall bladder cancer progression [6]. This multifunctional oncoprotein has played a crucial role in the progression of several carcinomas, including cervical cancer, making it imperative to screen phytocompounds that can downregulate its expression in cervical cancer [7,8].

Natural compounds have displayed significant potential in apoptotic induction in cancerous cells by altering various crucial components of signaling pathways and emerged as
potent chemopreventive agents [9,10]. Phytocompounds exhibit several benefits, including limited toxicity, easy availability, and minimal cost, which supported their potential in managing numerous carcinomas [11]. Flavonoids have gained wider attention by displaying significant apoptosis-inducing efficacy in several cancer cell lines such as LNCaP (human prostate cancer cell), MDA-MB-231 (human breast cancer cell), HT-29 (human colon cancer cell) [12,13]. Although flavonoids have been well known for their anticancerous potential in HeLa cells, the inhibitory potential of Flavonoids against Jab1 in HeLa cancer cells remains unelucidated [14,15]. Thus, our study is mainly targeted to elucidate the inhibitory efficacy of selected Flavonoids against Jab1 in HeLa cancer cells.

The development of novel anticancerous drugs is a very time-consuming and costlier task; therefore, a systematic and planned approach is required for rational drug discovery to conquer the limitations associated with chemotherapeutic approaches. *In silico* approaches, employing medicinal benefits of phytocompounds has been a fundamental element of drug designing and development in this era of personalized medicine, cost, and effective health outcomes [16-19]. To date, very few studies have reported the inhibitory potential of these selected Flavonoids against Jab1 in cervical cancer. In this research report, eleven potent Flavonoids were selected for finding their inhibitory potential against Jab1 in cervical cancer using several *in silico* and *in vitro* approaches. However, more *in vitro* experimentation needed to be performed to gain detailed insight into the mechanism involved.

2. Materials and Methods

2.1. Tools required for *in silico* study.

Windows, MGL tools, Autodock 4.2, Discovery Studio Visualizer, PatchDock online server, Cygwin and Binary files

2.2. Ligand (Flavonoids) preparation.

Eleven potent flavonoids have been selected through literature sources (Table 1), and their 3D structures were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) database. Energy minimization of selected phytoligands was performed before docking [20].

2.3. Preparation of target protein (macromolecule).

The 3D crystal structure of Jab1 (PDB ID: 4F7O) was obtained from PDB (Protein Data Bank) (http://www.rcsb.org/pdb). Energy minimization and refinement of the 3D structure of Jab1 were done before performing docking. The refinement process was performed by adding Kollman charges and polar hydrogen atoms and removing crystallographic water molecules from the protein.

2.4. Molecular docking analysis using AutoDock 4.2 and PatchDock software.

Molecular docking of selected eleven phytocompounds and two standard drugs against Jab1 (target proteins) was done using AutoDock 4.2 [21]. Autogrid was utilized to identify the position of ligand on the binding site of Jab1 protein with grid spacing 0.375 Å and grid coordinates (X, Y, and Z) axes. After analyzing the binding of eleven phytocompounds with Jab1 using AutoDock 4.2 and PatchDock software, baicalein has displayed maximum binding affinity against Jab1.
Table 1. List of ten screened phytocompounds (Flavonoids).

| S. No | Name of compounds | Pubchem ID | 3d structure |
|-------|-------------------|------------|--------------|
| 1.    | Hesperidin        | 10621      | ![3d structure](image1) |
| 2.    | Curcumin          | 969516     | ![3d structure](image2) |
| 3.    | Apigenin          | 5280443    | ![3d structure](image3) |
| 4.    | Naringin          | 442428     | ![3d structure](image4) |
| 5.    | Quercetin         | 5280343    | ![3d structure](image5) |
| 6.    | Luteolin          | 5280445    | ![3d structure](image6) |
2.5. **PASS (prediction of activity spectra for substances) analysis.**

PASS analysis was done to predict the biological activity spectrum of phytocompound based on its SAR (structure-activity relationship) with a known chemical entity [22]. Several online and offline tools were utilized to perform PASS analysis, as mentioned below.

2.6.1. **Lipinski’s rule of five.**

Drug likeness of selected eleven phytocompounds and two standard conventional drugs were elucidated using Lipinski’s rule of five [23]. Parameters considered for analyzing drug likeness were MW, number of H (hydrogen) bond donors, logP, H (hydrogen) bond acceptor sites, number of rotatable bonds, and TPSA (topological polar surface area). Molinspiration
2.7. Bioactivity score (BAS) prediction.

BAS values predict the overall ability of phytocompound to be a potent lead (drug) candidate. Molinspiration chemoinformatics (an online tool) was utilized to assess the drug score of prospective phytocompound with respect to various human receptors, including ion channels, GPCRs, enzymes, kinases, proteases, and nuclear receptors. In general, a higher bioactivity score exhibits a greater probability of the active compound being active [24].

2.8. Pharmacokinetic (PK) parameters analysis.

ADMET properties of all the components, including and standard drugs and selected phytocompounds were predicted using SwissADME software (http://www.swissadme.ch/). This online software analyses several key pharmacokinetic properties of a compound such as BBB (blood-brain barrier), LogKp (skin permeability), and its metabolism in terms of it being a Cytochrome P450 (CYP2C19, CYP1A2, CYP2D6, CYP2C9, CYP3A4) inhibitor, P-gp (P-glycoprotein substrate) [25].

2.9. Cell line and cell culture.

HeLa cancer cells were procured from NCCS (National Centre for Cell Science, Pune), India. HeLa cells were maintained in RPMI-1640 medium (supplemented with 100 U/mL of penicillin, 10% Fetal Bovine Serum (FBS), and 100 μg/mL of streptomycin) and were grown in an incubator (at 5% CO₂ and 37 °C temperature).

2.10. MTT analysis.

The effect of best-screened flavonoid amongst selected flavonoids on HeLa cell viability was investigated by MTT assay as described earlier [26]. HeLa cells were stored for 24 h in an incubator and after 24 h of overnight incubation, cells were treated with baicalein and thereafter with MTT dye. Lastly, the treated cells were left incubated for 4 h, and absorbance was recorded at 540 nm (using a microplate reader, BioRad, USA).

2.11. RT-PCR analysis.

To further validate whether screened compounds via in silico analysis possess significant inhibitory potential against Jab1 mRNA expression, RT-PCR was performed on baicalein-treated HeLa cells [27]. HeLa cancer cells (1 x 10⁶) were grown in 75 cm² flasks and left for adherence for 24 h. Afterward, the cells were then treated with selective doses of a screened compound or DMSO control for 24 h. Treated cells were then washed with 5 µL PBS and trypsinized. The cancer cell suspension was then centrifuged (at 350 x g for 3 minutes), and the obtained cell pellet was resuspended in PBS (ice-cold) buffer. The cell suspension was further centrifuged (at 17 000 x g for 3 min at 4 °C). Obtained pellet was stored at -80 °C for future analysis. Total RNA was extracted after 24 h of baicalein treatment using TRIzol Reagent (Invitrogen) as per the manufacturer's protocol. RT-PCR was used using one-step RT-PCR (SuperScript III) with Platinum Taq DNA polymerase kit (12574-018; Invitrogen).
Relative expression was calculated by the $2(\Delta\Delta Ct)$ method. Primers used in this study are (Table 2):

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| Jab1 | 5'-GGCGCCTTAGGACATACC-3' | 5'-CATGAAACTCCCTCGTCCC-3' |
| β-actin | 5'-GTCTGTGATGCCCTTAGATG-3' | 5'-AGCTTATGACCCGCACCTAC-3' |

3. Results and Discussion

3.1. Docking analysis of selected Flavonoids against Jab1.

AutoDock 4.2 and PatchDock were utilized for the docking study of selected phytocompounds against Jab1 (Table 1 and Table 2). As is evident from Table 3, all selected eleven phytocompounds exhibited significant binding affinity to Jab1 based on the dissociation constant (Kd) and best binding energy of ligand-protein interactions. However, baicalein displayed the highest binding energy against Jab1 in comparison to standard drugs. Obtained docking results were visualized in the Discovery Studio Visualizer.

3.2. PASS analysis of selected phytocompounds (Flavonoids) using Lipinski’s rule of five.

Lipinski’s rule of five explains the molecular properties of a compound that are crucial for lead optimization and selectivity in clinical applications. Table 5 displays PASS analysis of selected phytocompounds versus standard drugs in terms of their physicochemical properties.

Table 2. Primer used for RT-PCR analysis.

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| Jab1 | 5'-GGCGCCTTAGGACATACC-3' | 5'-CATGAAACTCCCTCGTCCC-3' |
| β-actin | 5'-GTCTGTGATGCCCTTAGATG-3' | 5'-AGCTTATGACCCGCACCTAC-3' |

Table 3. Docking analysis of selected eleven phytocompounds with Jab1 using AutoDock and PatchDock software.

| S.NO | Ligand | Binding energy kcal/mol | Total internal energy | Inhibitor constant | Score | ACE |
|------|--------|-------------------------|-----------------------|--------------------|--------|-----|
| 1. | Hesperidin | -5.75 | 1.45 | 61.35 | 3768 | -84.32 |
| 2. | Curcumin | -5.59 | 1.12 | 79.36 | 4150 | -80.01 |
| 3. | Apigenin | -6.29 | 0.7 | 24.37 | 6444 | -147.12 |
| 4. | Naringin | -5.72 | 1.69 | 64.54 | 6690 | -66.87 |
| 5. | Quercetin | -6.47 | 0.99 | 18.04 | 5800 | -100.49 |
| 6. | Luteolin | -6.48 | 0.43 | 17.68 | 4338 | -43.60 |
| 7. | Myricetin | -6.45 | 1.25 | 18.72 | 4466 | -63.28 |
| 8. | Cyanidin | -6.95 | 0.54 | 7.99 | 4368 | -46.39 |
| 9. | EGCG | -6.21 | 1.02 | 28.21 | 5584 | -91.66 |
| 10. | Baicalein | -7.2 | 1.26 | 531 | 4406 | -80.93 |
| 11. | Genistein | -6.59 | 0.74 | 14.65 | 4292 | -47.51 |
| 12. | Doxorubicin | -7.19 | 1.9 | 3.21 | 4406 | -80.93 |
| 13. | 5 fluorouracil | -3.46 | 0.0 | 2.9 | 2548 | 38.81 |

Table 5 displays PASS analysis of selected phytocompounds versus standard drugs in terms of their physicochemical properties.
Usually, any orally active lead candidate should not have more than 1 Lipinski's violation, or else its bioavailability would be compromised. Interestingly, baicalein exhibited no Lipinski's violation in comparison to all other screened phyto compound and standard drugs, thereby strongly representing it as a strong drug candidate for the management of cervical cancer.

**Table 4.** AutoDock and PatchDock analysis.

| S.No. | Compound | AutoDock | PatchDock |
|-------|----------|----------|-----------|
| 1.    | Hesperidin | ![AutoDock](image1) | ![PatchDock](image2) |
| 2.    | Curcumin | ![AutoDock](image3) | ![PatchDock](image4) |
| 3.    | Apigenin | ![AutoDock](image5) | ![PatchDock](image6) |
| 4.    | Naringin | ![AutoDock](image7) | ![PatchDock](image8) |
| S.No. | Compound | AutoDock | PatchDock |
|-------|----------|----------|-----------|
| 5.    | Quercetin| ![AutoDock](image1.png) | ![PatchDock](image2.png) |
| 6.    | Luteolin | ![AutoDock](image3.png) | ![PatchDock](image4.png) |
| 7.    | Myricetin| ![AutoDock](image5.png) | ![PatchDock](image6.png) |
| 8.    | Cyanidin | ![AutoDock](image7.png) | ![PatchDock](image8.png) |
| 9.    | EGCG    | ![AutoDock](image9.png) | ![PatchDock](image10.png) |
| S.No. | Compound      | AutoDock       | PatchDock       |
|-------|---------------|----------------|-----------------|
| 10.   | Baicalein     | ![AutoDock Image](image1) | ![PatchDock Image](image2) |
| 11.   | Genistein     | ![AutoDock Image](image3) | ![PatchDock Image](image4) |
| 12.   | Doxorubicin   | ![AutoDock Image](image5) | ![PatchDock Image](image6) |
| 13.   | 5 fluorouracil| ![AutoDock Image](image7) | ![PatchDock Image](image8) |

**Table 5.** PASS analysis of selected phytocmpounds (Flavonoids).

| S.No | Ligand   | No of heavy atoms | Molecular weight g/mol | TPSA | Log p-value | H-bond donor | H-bond acceptor | Number of rotational bonds | Lipinski's violation |
|------|----------|-------------------|------------------------|------|-------------|--------------|------------------|----------------------------|---------------------|
| 1.   | Hesperidin| 43                | 610.56                 | 234.25 | -1.1        | 8             | 15               | 7                          | 3                   |
| 2.   | Curcumin  | 27                | 368.38                 | 93.06  | 3.2         | 2             | 6                | 8                          | 0                   |
| 3.   | Apigenin  | 20                | 270.24                 | 90.90  | 1.7         | 3             | 5                | 1                          | 0                   |
| 4.   | Naringin  | 41                | 580.53                 | 225.06 | -0.5        | 8             | 14               | 6                          | 3                   |
| 5.   | Quercetin | 22                | 302.24                 | 131.36 | 1.5         | 5             | 7                | 1                          | 0                   |
| 6.   | Luteolin  | 21                | 286.24                 | 111.13 | 1.4         | 4             | 6                | 1                          | 0                   |
| 7.   | Myricetin | 23                | 318.24                 | 151.59 | 1.2         | 6             | 8                | 1                          | 1                   |
| 8.   | Cyanidin  | 21                | 287.24                 | 114.29 | ------      | 5             | 6                | 1                          | 0                   |
Kp (blood-skin) 

Skin that... 3.4. ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of phytocomponents.

The pharmacokinetics feasibility of all eleven selected phytocompounds (as a prospective lead candidate) was calculated using online SwissADME software (Table 7). LogP value indicated the lipophilic (lipid-soluble) and good absorption nature of phytocompound across the skin. Interestingly, none of the phytocompounds displayed BBB (blood-brain barrier) permeability as well as behaved as P-gp (permeability-glycoprotein) substrates except three phytocompound and standard drug. P-gp (an ATP-dependent bioavailability protein pump) removes drugs from biological systems. Normal excretion of potential drugs back into the lumen (gut) by P-gp reduces the pharmacokinetics efficacy of pharmaceutical drugs (that are known to be P-gp substrates). CYPs (Cytochromes P450) are a superfamily of metabolic enzymes associated with xenobiotics biotransformation. Compounds that inhibited five classes of CYPs (CYP3A4, CYP1A2, CYP2C9, CYP2C19, and CYP2D6) would cause enhanced plasma concentrations, thereby contributing to improved bioavailability. Kp (Skin permeability) is mainly used to describe quantitative chemical permeation through the epidermis or outermost layer of skin.
| S. No | Ligand       | Lipophilicity | BBB Permeant | p-gp Substrate | Cyp1A2 Inhibitor | Cyp2C19 Inhibitor | Cyp2C9 Inhibitor | Cyp2D6 Inhibitor | Cyp3A4 Inhibitor | Log kp (cm/s) |
|-------|--------------|---------------|--------------|----------------|------------------|------------------|------------------|------------------|------------------|---------------|
| 9     | EGCG         | 1.01          | No           | No             | No               | No               | No               | No               | No               | -8.27         |
| 10    | Baicalein    | 2.24          | No           | No             | Yes              | No               | No               | Yes              | Yes              | -5.70         |
| 11    | Genistein    | 2.04          | Yes          | No             | Yes              | No               | Yes              | Yes              | Yes              | -6.05         |
| 12    | Doxorubicin  | 0.44          | No           | Yes            | No               | No               | No               | No               | No               | -8.71         |
| 13    | 5 fluorouracil | 0.13      | No           | No             | No               | No               | No               | No               | No               | -7.73         |

### 3.5. MTT analysis to study growth arrest in baicalein-treated HeLa cells.

Cytotoxic or growth arrest potential of baicalein in HeLa cancer cells was determined by treating cancer cells with selective baicalein doses (0, 25, 50, 75, 100, and 200 µM) for 24 and 48 h. Evaluated data displayed significant growth arrest in baicalein-treated HeLa cancer cells compared to control (Figure 1) according to earlier research reports [28].

![Figure 1](https://biointerfaceresearch.com/)

**Figure 1.** Dose and time-dependent efficacy of Baicalein on HeLa cancer cells. Percent (%) cell viability of baicalein treated HeLa cells (0–200 µM) for 24 and 48 h assessed by MTT assay. Obtained results are visualized as mean ± SEM of experiments performed independently in triplicate (*P < 0.01, **P < 0.001 denoted considerable difference in comparison to control).

### 3.6. RT-PCR analysis to study gene expression

Because baicalein exhibited significant Jab1 inhibitory potential in silico analysis, therefore, we further performed RT-PCR analysis to validate the inhibitory potential of baicalein against Jab1 mRNA expression in HeLa cancer cells. Baicalein treatment resulted in significantly downregulated Jab1 mRNA expression in HeLa cells (Figure 2) in comparison to untreated (control) cells.

![Figure 2](https://biointerfaceresearch.com/)

**Figure 2.** Differential expression analysis of JAB1 mRNA in baicalein treated cells by real-time PCR in HeLa cancer cells values are presented as mean ± SEM of three independent experiments (*P<0.05, ** P<0.01 compared to negative control).
Altogether, both in silico and in vitro findings have strongly revealed that baicalein could be a potential Jab1 inhibitor. Since Jab1 has been positively associated with cervical cancer cell growth [29], hence our experimental findings provided a potent phytocompound as a potent lead candidate that can not only display growth arrest in HeLa cancer cells but also suppress the overexpression of Jab1 oncogene that has been crucially involved in the progression of cervical cancer. However, more in vitro experiments are needed to explain the mechanism associated with the inhibitory potential of Baicalein against Jab1 in cervical cancer.

4. Conclusions

Our experimental findings endeavored to elucidate the inhibitory potential of phytocompounds (Flavonoids) against Jab1 oncogene that has been crucially associated with several cancer progressions. We have selected eleven Flavonoids for our investigation, and interestingly, both in silico and in vitro findings strongly validated the Jab1 inhibitory potential of baicalein amongst all other selected phytocompound, thereby revealing it as a strong lead candidate for the management of cervical cancer disease.

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Conflicts of Interest

The authors declare no conflict of interest.

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