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# Hesperetin and Naringenin sensitize HER2 positive cancer cells to death by serving as HER2 Tyrosine Kinase inhibitors

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ABSTRACT

*Aim:* Aberrant human epidermal growth factor receptor-2 (HER2) expression and constitutive mutant activation of its tyrosine kinase domain account for tumor aggression and therapy resistance in many types of cancers with major share in breast cancer cases. HER2 specific treatment modalities still face challenges owing to the side effects and acquired resistance of available therapeutics. Recently, the anti-proliferative and pro-apoptotic potential of phytochemicals, especially of flavonoids have become increasingly appreciated as powerful chemo preventive agents. Consequently, the major goal of our study is to identify flavonoids capable of inhibiting HER2 Tyrosine Kinase (HER2-TK) activity and validate their anti-tumor activity against HER2 positive tumors.
*Main methods:* Molecular docking studies for identifying flavonoids binding at HER2 kinase domain, ADP-Glo™ Kinase Assay for determining kinase activity, MTT assay to measure growth inhibition, various apoptotic assays and cell cycle analysis by FACS were performed.
*Key findings:* Among the flavonoids screened, Naringenin (NG) and Hesperetin (HP) possessed high glide scores from molecular docking studies of enzyme-inhibitor mode. The interaction analysis revealed their ability to establish stable and strong interaction at the ATP binding site of HER2-TK. These compounds also inhibited *in vitro* HER2-TK activity suggesting their role as HER2 inhibitors. The study also unraveled the anti-proliferative, pro-apoptotic and anti-cancerous activity of these flavonoids against HER2 positive breast cancer cell line.
*Significance:* The **s**tudy identified two citrus fruit flavonoids, NG and HP as HER2-TK inhibitors and this is the first report on their potential to target preferentially and sensitize HER2 positive cancer cells to cell death.


## 1. Introduction

HER2 is an integral member of epidermal growth factor receptor family (HER/EGFR/ERBB) and plays pivotal function in cell survival, proliferation and resistance to apoptosis [7,9]. HER2 receptor protein possesses a Tyrosine Kinase domain (TK). HER2 gets activated by phosphorylation upon the dimer formation with the other members of the EGFR [9,11]. Atypical expression of HER2 protein or the deregulated tyrosine kinase activation promotes carcinogenic events including initiation, progression and aggression of tumors, and are termed as HER2 positive cancers [5]. HER2 positive cancer accounts for one third of all breast cancer incidences and is the second most aggressive breast cancer subtypes with poor prognosis and survival outcome [13]. Therefore, functional inactivation of HER2 Tyrosine Kinase (HER2-TK) or the receptor protein expression as such is recognized as promising therapeutic strategy [24,31,32,36,38]. Tumors with HER2 phenotype are often resistant to conventional chemotherapy and thus very specific therapeutic formulations from monoclonal antibody approach (Trastuzumab) or small molecule kinase inhibitors (Lapatinib) are only found effective [14,15,22,23]. Advent of potential therapeutic agents to tackle HER2 positive cancers found promising initially. However, it suffers from the disadvantages of being toxic and getting insensitive over time in certain cases [21,26,47]. This scenario thus demands the development of better HER2-TK inhibitors with minimal toxicity issues to be introduced as targeted anti-cancer therapeutics.

Taking into account of consumption of fruits and vegetables, natural products have a huge influence on human health. Almost 80% of drugs up to now are either derived or modified from plant products. Of these, flavonoids as a class of compounds are well acclaimed for their anti-cancerous, anti-proliferative, pro-apoptotic activities with minimal toxicity issues. Flavonoids are one of the common components in the human diet and generally found as O-glycosides in fruits, vegetables and seeds with sugars bound at C3 position. Flavonoids are widely present in the Rutaceae family of plants such as citruses. Flavonoids exhibit anti-inflammatory, antioxidant, anti-mutagenic and enzyme modulating activities [35,52]. Several flavonoids like resveratrol, targeretin and

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quercetin possess chemopreventive and anti-cancerous properties against a variety of cancer cell lines and mouse models [16].

The flavonoids present in the common fruits and vegetables were considered for *in silico* docking studies to screen the flavonoids capable of establishing interaction with HER2-TK at the ATP binding site. Molecular docking studies and the subsequent *in vitro* kinase assay identified NG and HP as structural analogues of HER2-TK inhibitors. Cytotoxic and apoptotic vigor of these compounds in HER2 positive breast cancer cell line were then assessed in comparison with HER2 negative cell line. The results clearly suggested the potential of the above flavonoids to differentially target and inhibit proliferation of HER2 positive cancer cells.

## 2. Materials and methods

### *2.1. Computational modeling for HER2-TK inhibitors*

#### *2.1.1. Ligand preparation*

The two-dimensional structure of phytoflavonoids were selected from pubchem library and prepared low energy, 3D structures with precise chiralities of various ionization states, tautomers, stereochemistries and ring conformations of all the flavonoids at Optimized Potential for Liquid Simulation version 2005 (OPLS-2005) force field using ligprep module. These structures were used as initial materials during computational docking procedure to study interactions with the binding site of the HER2-TK domain.

#### *2.1.2. Protein preparation*

The atomic coordinates of HER2-TK are obtained from the crystallographic structure, accession no 3PP0 of Protein Data Bank (PDB). This structure contains asymmetric dimer of HER2-TK complex with selective inhibitor of HER2-TK, pyrrolo[3,2-*d*]pyrimidine-based compound, SYR 127963. In order to perform the docking calculations, only chain A was selected and the co-crystallized ligand (s) and crystal water molecules were removed. Hydrogen atoms were incorporated geometrically with a pH of 7.0 and parameterized with OPLS-2005 force field. [33]. The crystal structure was processed using the Protein Preparation Wizard in Maestro 9.1 [34]. Then the structure was energy minimized with an RMSD cutoff of 0.3 Å by the impref module in the Schrödinger package. The receptor grid was created with the centroid of the crystal ligand (SYR) as the center. The structures of relevant flavonoids were obtained from pubchem for *in silico* docking study with the kinase domain of HER2.

#### *2.1.3. Molecular docking*

Molecular docking is performed between the optimized ligands with ATP binding site of Tyrosine Kinase domain of HER2 in extra precision mode (XP mode) of maestro 9.1 module of Schrodinger. Glide scores suggested the possibility of the interaction between ligand to protein at the site of protein of interest. Based on glide score, the flavonoids were ranked and selected for further studies.

#### *2.1.4. HER-2 kinase assay*

ADP-Glo™ Kinase Assay kit (Promega, CAT # V9381) and HER2 kinase enzyme system (Promega, CAT # V3891) were used for the assay and performed the experiment as per manufacturer's protocol. However, the concentration of ATP used in the experiment was marginally decreased to 10 μM by critical standardizations to estimate the lowest HER2 kinase inhibitory concentration of HP and NG. The ADP-Glo™ Kinase Assay is a luminescent ADP detection assay that provides a universal, homogeneous, high-throughput assay method to measure kinase activity by quantifying the amount of ADP produced during a kinase reaction. In short, the kinase reaction was allowed to take place over a period of 30 min incubation time. In this reaction ATP was used up and ADP generated proportionally and the rest of the unconsumed ATP was to be depleted. After 30 min of incubation, an equal volume of ADP-Glo™ reagent was added to terminate the kinase reaction and deplete the remaining ATP. Then, the kinase detection reagent was added to convert ADP to ATP simultaneously and allowed the newly synthesized ATP to be measured using a luciferase/luciferin reaction. The light generated was measured using a luminometer. Luminescence was correlated to ADP concentrations from an ATP-to-ADP conversion curve. The result is shown as graph in percentage of kinase activity/inhibition in comparison with positive and negative control.

#### *2.1.5. Cell lines and reagents*

SKBR3 and MDA-MB-231 cell lines were purchased from NCCS, Pune, India. SKBR3 cell line was maintained in McCoy's 5 A medium (Gibco, Cat# 16,600–082) supplemented with 10% heat inactivated fetal bovine serum (FBS), and MDA-MB-231 cell line was grown in 10% heat inactivated FBS containing DMEM (Cat# 11,960,044). 100 U/mL Penicillin and 100 μg/mL streptomycine (Invitrogen) were added to cell culture medium to prevent contamination and both the cells were grown at 37 °C in humidified incubator constantly maintained at 5% $CO_2$. Trypsinization and sub-culturing of the cell lines were done as per the standard procedures.

Hesperetin(Cat # H4125) and Naringenin (Cat # W530098) and Etoposide (Cat#E1383) were purchased from sigma. Lapatinib (Cat #sc-202205B) was procured from Santa Cruz.

#### *2.1.6. MTT cell viability assay*

Cell viability was assessed by intensity based measurement of blue formazan metabolized from colorless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by the action of mitochondrial dehydrogenase enzyme, active only in live cells. Both SKBR3 and MDA-MB-231 cell lines were seeded at a density of $5 \times 10^3$ cells per well in 96 well plate for 24 h and then treated with various concentrations of HP and NG for concentration dependent as well as time dependent MTT assay. At the end of the treatments, 20 μL, 0.5 mg/mL MTT reagent diluted in 1× PBS was added at 37 °C for 4 h. To solubilize the formazan crystals, 200 μL of DMSO (dimethyl sulfoxide), was transferred to each well after aspirating MTT containing medium from the wells. Absorption values were read at 540 nm on microtiter plate reader (spectra MAX 340, Molecular Devices, Sunnyvale, *CA*, USA). Data were expressed as the mean percent of viable cells *vs.* control.

#### *2.1.7. TMRM staining for mitochondrial membrane potential loss determination*

TMRM (tetramethylrhodamine) is a cell permeable, positively-charged, red-orange dye that readily accumulates in active mitochondria owing to their relative negative charge. Depolarized or damaged mitochondria have decreased membrane potential and fail to sequester TMRM. Mitochondrial membrane potential loss is an early marker of apoptosis and can be determined using TMRM staining. Cells at seeding density of $5 \times 10^3$ cells per well were cultured in 96 well plate and initiated drug treatment at 70% cell confluency. 50 nM concentration of TMRM was preloaded prior to the treatment for 30 min. The images were captured with excitation wavelength 514 nm and emission at 570 nm using fluorescent microscope equipped with CCD camera.

#### *2.1.8. Chromatin condensation by Hoechst staining and imaging*

Cells were harvested and seeded in 96 wells plate. At about 80% confluency, treatment is ensued for different time intervals as specified in the result part. Stained with Hoechst at the concentration (5 μg/mL) prior to the drug treatment and the images were captured under fluorescent microscope equipped with CCD camera. The excitation wavelength of 350 nm and the emission of 461 nm filters were used for imaging.

#### *2.1.9. Cell cycle analysis*

Cell cycle analysis was performed by propidium iodide staining of harvested cells after treatments with HP and NG. This analysis was carried out by flow cytometry. Briefly, the cells were seeded in 6 well plate

**Table 1**
XP glide score of top 10 phytochemicals.

| Sl No | Compounds | Glide score (kcal $mol^{-1}$) |
|---|---|---|
| 1 | Taxifolin | −9.62 |
| 2 | Naringenin | −9.02 |
| 3 | Hesperetin | −8.91 |
| 4 | Licoleafol | −7.72 |
| 5 | Eriodictyol | −7.43 |
| 6 | Sakuranectin | −7.32 |
| 7 | Tangeretin | −7.31 |

and treated with 500 μM concentration of HP and 250 μM concentration of NG for 24 h. The treated cells were harvested, washed with PBS and fixed with 70% ice cold ethanol. Then cells were washed with cold PBS and suspended in RNase solution incubated at 37 °C for 30 min and then stained with Propidium Iodide (50 μg/mL) for cell cycle analysis. DNA content of the cells was measured by BD flow cytometer Arya at 650 nm with suitable band pass filter.

*2.1.10. Western blotting*

Whole cell extract for western blotting was prepared in phospholysis buffer containing protease inhibitor cocktail. Protein estimation was performed by Bradford assay and 60 μg of protein samples were resolved on 10 or 12% SDS-polyacrylamide gels. Wet transfer for 2 h at 100 V to a PVDF membrane was carried out each time, and the membranes were blocked with 3% blocking buffer for an hour. Blots were probed with specific primary antibodies and incubated overnight in cold. Caspase 3 (Cat #9662), caspase 8 (Cat #9746), and HER2 (Cat # 2165) were purchased from cell signaling technology and Beta Actin (Cat #A 5316) from Sigma followed by specific secondary antibodies conjugated with HRP and developed with the ECL reagent (Thermo fisher).

*2.1.11. Statistics*

Results are represented as mean ± SEM. Comparisons between the experimental values were done with one-way analysis of variance (ANOVA) to comprehend the significance by GraphPad Prism, version 5.04. $P$ value $<0.05$ is treated as statistically significant.

## 3. Results

### 3.1. Molecular docking

The crystal structure of the kinase domain of HER2 in complex with HER 2 inhibitor SYR (pdb id 3PP0) was selected for the study [2]. The crystal data indicated that SYR binds to HER2-TK in ATP competitive manner and established interactions through 8 hydrogen bonds (Met801, Gly865, Glu778, Asp863, Gly865, Phe864, Asn774 and Lys753). A total of 150 flavonoids were selected for the study based on reports of their anti-cancer activity and their abundance in the common fruits and vegetables. From this, the first 50 flavonoids with higher glide scores were selected for extra precision (XP) docking analysis. The top 7 flavonoids based on glide scores are provided in Table 1. Top three flavonoids from XP docking were considered for induced fit docking and binding free energy assessment by MMGBSA method. As the HER2 inhibitory potential of quercetin derivatives were published, we omitted it from the present study, and hence considered HP and NG as the candidates for further study based on *in silico* analysis. Both these compounds exhibited binding energy comparable with the binding energy of Lapatinib (Lap), a known potent inhibitor of HER2-TK as per *in silico* analysis of the interaction between HER2-TK. The flavonoids showed hydrogen bonding and van der Waals interactions with HER2-TK. Hydrogen bonds were visualized by pymol. HER2-TK established five hydrogen bonds with HP and four hydrogen bonds with NG (Table 2, Fig. 1A and B respectively). The details of interacting amino acids and the hydrogen bond lengths are shown in Table 3. Among the eight hydrogen bond forming residues of HER2-TK with SYR, Met801 and Lys753 were found forming hydrogen bonds with HP where as NG had formed only one hydrogen bond with Met801. It was also found that both HP and NG interacted within the same region where SYR occupied in the crystals structure of HER2-TK following similar hydrogen bonding scheme. The complexes of HP and NG with the HER2-TK domain are shown in Fig. 1A and B respectively. HER2 kinase activity is associated with the kinase domain where the ATP molecules bind to transduce phosphorylation. XP docking with Lap, a potent HER2-TK inhibitor and approved as chemotherapeutic drug has also been carried out. The results indicated that, it formed four hydrogen bonds through amino acid residues Met801, Asp808, Leu726 and Lys724 with the HER2-TK, suggesting that the region spanning between residues 700 to 900 are crucial for the kinase activity of HER2 (data not shown). According to the present study, NG and HP were found to foster interactions with the residues of the region spanning 700 to 900, which as per the crystal structure comprised of the part of HER2-TK activation loop. The binding of ligands at this site may prevent ATP binding and thereby reduce the kinase activity of the protein and phosphorylation.

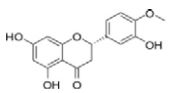
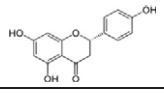
### 3.2. NG and HP inhibited HER2-TK activity

In order to validate *in vitro* HER2 kinase inhibitory potential, luminescence based HER2 kinase activity measurement was done by ADP-Glo™ Kinase assay kit using specific HER2 protein fraction comprising the active tyrosine kinase domain. The maximum HER2-TK activity was assessed without any inhibitor/ligand and Etoposide was kept as negative control. The *in vitro* kinase assay presented a clear evidence for the potential of the compounds to inhibit HER2 kinase activity whereas the Etoposide at various concentrations ranging from 10 μM to 40 μM did not alter HER2 kinase activity. Equimolar concentration of HP and NG with that of substrate (ATP at 10 μM concentration) induced 15% kinase inhibition. However, the higher concentrations of HP and NG triggered inhibition of kinase enzyme directly proportional to their concentration. When the concentration of inhibitors doubled, 50% HER2-TK inhibition was observed. According to the experiment, $IC_{50}$s of HP and NG were observed to be around 20 μM. The results of kinase assay are represented graphically as percentage inhibition of kinase activity in comparison with the maximum kinase activity (Fig. 2).

### 3.3. NG and HP triggered growth inhibition in HER2 positive cancer cells

Inhibitory potentials of NG and HP towards HER2 kinase activity were confirmed from kinase assay experiment, and subsequently the

**Table 2**
Glide score and hydrogen bonds from induced fit docking.

| Sl no. | Compounds | Structure | No. of hydrogen bonds | G score (Kcal $mol^{-1}$) |
|---|---|---|---|---|
| 1 | Hesperetin |  | 5 | −10.4 |
| 2 | Naringenin |  | 4 | −9.6 |

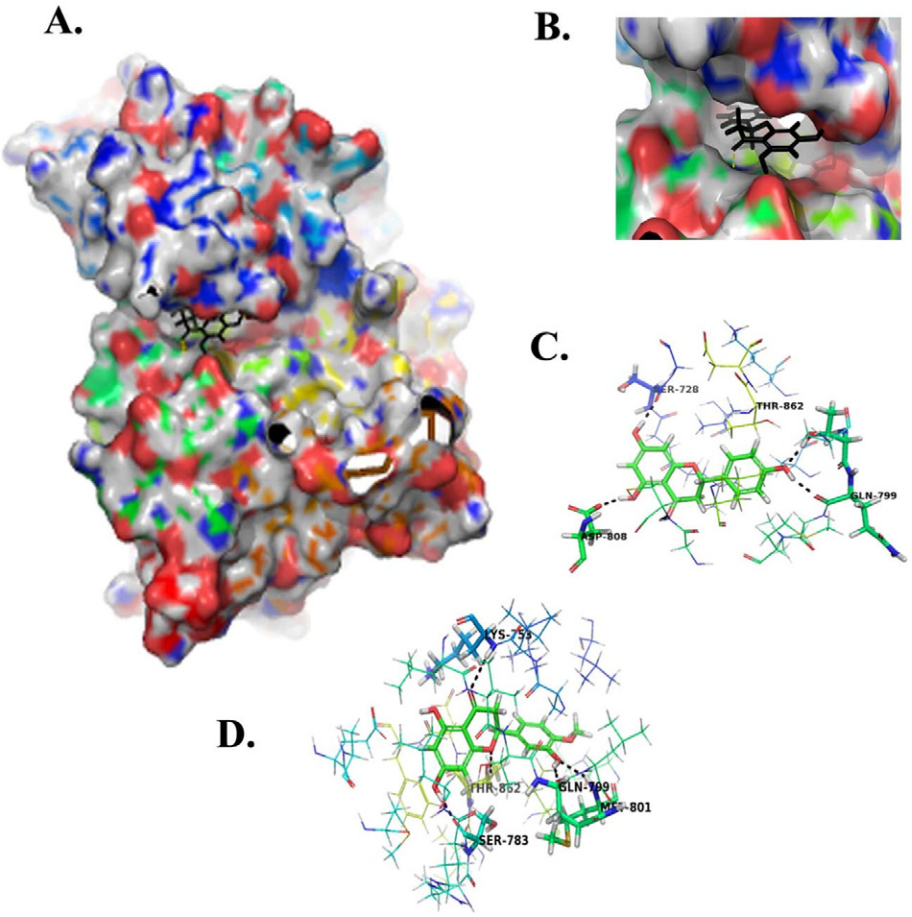



**Fig. 1.** Molecular docking. A). Surface diagram of HER2-TK and NG complex from induced fit docking. B). Close up view of the ATP binding pocket with NG. C). Hydrogen bonds formed between NG and the residues at the HER2-TK binding pocket. D). Hydrogen bonds formed between HP and the residues at the HER2-TK binding pocket.

growth inhibitory potentials of these compounds in HER2 positive breast cancer cell line SKBR3 were determined. Cell viability/growth inhibition measurement by MTT assay using various concentrations of HP and NG was initially carried out to determine $IC_{50}$ of these agents in HER2 positive cancer cell line SKBR3. Concentration dependent MTT assay with varied concentration of HP and NG was initially carried out. SKBR3 cells were treated with 150 μM, 250 μM and 500 μM of HP and NG for 24 h and MTT assay was performed. It has been found that 150 μM concentrations of both HP and NG were too far below the required concentration to trigger significant growth inhibition or death. However, 250 μM of NG triggered 50% growth inhibition/cell death while HP at 500 μM concentration only brought comparable growth inhibition. Fig. 3A represents the concentration dependent MTT assay result, revealing $IC_{50}$s of the flavonoids as 500 μM and 250 μM for HP and NG respectively. Ability of these agents to inhibit growth in HER2 positive cancer cell SKBR3 and HER2 negative cell line MDA-MB-231 was analyzed in a time dependent MTT assay for 12 h, 24 h, 36 h and 48 h time points. Both HP and NG induced more anti-proliferative activity towards SKBR3 at their $IC_{50}$ concentrations in comparison with MDA-MB-231. Fig. 3B shows the time dependent MTT assay results of HP and Fig. 3C represents the results of NG treatment for the set time points. Growth inhibition was negligible by NG and HP in both HER2 positive and negative cell lines at 12 h treatment. And upon 24 h onwards, the clear distinction of growth inhibitory pattern became evident, with

**Table 3**
Details of hydrogen bonds and bond length.

| Sl no. | Compound | Hydrogen bonds | Interacting atom from the protein | Interacting atom from the flavonoid | Bond length (Å) |
|---|---|---|---|---|---|
| 1 | Hesperetin | Met801 | H | O4 | 2.3 |
| | | Gln799 | O | H10 | 1.9 |
| | | Lys753 | H | O6 | 2.6 |
| | | Thr862 | HG1 | O1 | 2.4 |
| | | Ser783 | O | H11 | 1.5 |
| 2 | Naringenin | Gln799 | O | H12 | 1.5 |
| | | Thr798 | HG1 | O4 | 2 |
| | | Asp808 | OD2 | H10 | 1.9 |
| | | Ser728 | O | H11 | 1.9 |

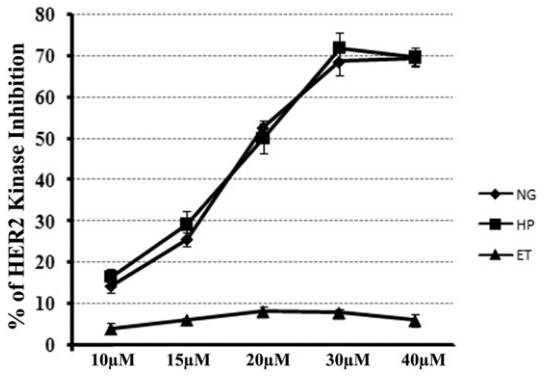



**Fig. 2.** Kinase assay. Kinase assay was done with purified active fragment of tyrosine kinase domain of HER2 by ADP-Glo™ Kinase Assay kit. The percentage inhibition of the activity of HP and NG were determined in comparison with untreated, and represented as graph. *$p \leq 0.05$ is compared with control (without any inhibitor) or inhibitors.

50% growth inhibition at 24 h in SKBR3 cell line with NG. However, the same treatment induced only 10% growth inhibition in MDA-MB-231 cell line. The same trend has been observed with higher time points of 36 h and 48 h. At 500 μM concentration HP induced 50% growth inhibition at 24 h in SKBR3 cell line and the similar treatment gave only 10% growth inhibition in MD-AMB-231. The above discussed results suggest the potential of these flavonoids to target and inhibit HER2-TK activation to render growth inhibition.

### 3.4. NG and HP exhilarated apoptotic cell death

The apoptotic potential of these flavonoids in SKBR3 cancer cell lines was determined on the basis of mitochondrial membrane potential (MMP) loss and chromatin condensation. Loss of MMP is an early apoptotic event and condensation of chromatin is regarded as the later event in apoptosis. Therefore, TMRM together with Hoechst staining would provide index of apoptosis similar to Annexin-PI staining experiment for scoring apoptosis. Both MD-AMB-231 and SKBR3 cells were treated with HP at 500 μM concentration or NG at 250 μM or Lap at 10 μM for 24 h for this experiment. MMP loss together with chromatin condensation by Hoechst staining in both MD-AMB-231 and SKBR3 cells were compared to analyze the HER2 dependency of HP and NG to trigger apoptosis. The result indicated that both these agents triggered significant MMP loss and chromatin condensation in HER2 positive SKBR3 cells than HER2 negative MD-AMB-231 cells. MMP loss together with chromatin condensation was performed in SKBR3 cells alone to further compare the apoptotic potential of HP and NG. The results indicated that NG is more potent than HP to trigger cell death as the loss of MMP started at 12 h with NG, while this was began to appear only at 24 h onwards with HP. This data has been shown in the supplementary section (Supplementary Fig. 1) where TMRM staining images for MMP loss and chromatin condensation with the corresponding DIC images of SKBR3 cells treated with HP and NG for 12 h, 24 h and 48 h are provided.

Chromatin condensation at higher time points 24 and 36 h was carried out in SKBR3 in comparison with two HER2 negative cell lines, MD-AMB-231 and MCF-7 to confirm HER2 dependent apoptotic induction. The condensation of the chromatin was analyzed from microscopic images and quantified the condensed nuclei *versus* normal nuclei to determine the extent of cell death. The result was expressed as percentage of condensed or fragmented nuclei against total number of nuclei as in graph (Fig. 4B) and the representative images are furnished as supplementary data (Supplementary Fig. 1). HP treatment at 500 μM induced around 11% chromatin condensation at 24 h and around 21% at 48 h in MDA-MB-231 cell line while the same concentration of HP induced 35% and 50% chromatin condensation at 24 h and 36 h respectively in SKBR3 cell line. On the other hand, NG at 250 μM for 24 h and 36 h induced 22% and 33% chromatin condensation in HER2 negative MDA-MB-231 cell line which were far below the percentage apoptosis by similar treatment in SKBR3. MCF7 cells which is negative for HER2 also demonstrated the apoptotic tendency similar to MD-AMB cells towards HP and NG treatment. Statistical analysis implied significant induction of apoptosis of HER2 positive cell line by these flavonoids with respect to HER2 negative cell line. Altogether, the results clearly indicated that these

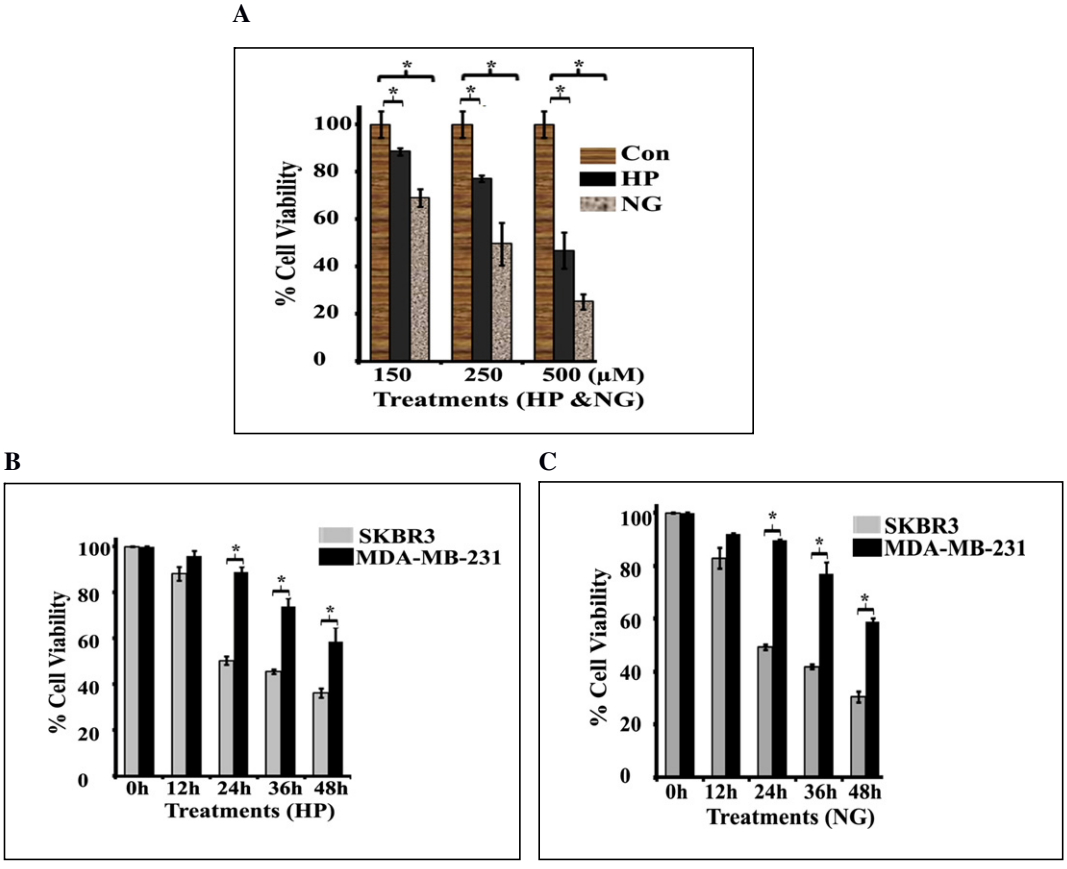



**Fig. 3.** MTT assay. A) SKBR3 cells were treated with different concentrations of HP and NG (150 μM, 250 μM and 500 μM) for 24 h. Cell proliferation was measured by MTT assay, and the concentration of each compound that induced 50% growth inhibition ($IC_{50}$ of HP and NG) was determined. Result is expressed as graph on the basis of percentage of growth inhibition from three independent experiment values. $^{*}p \leq 0.01$ compared with HP or NG treatment on SKBR3 **B**) SKBR3 and MDA-MB-231 cells were treated 500 μM HP for different time points 12 h, 24 h, 36 h and 48 h. Growth inhibition at each time point is measured as percentage inhibition and represented graphically from three independent experiments. $^{*}p \leq 0.001$ compared with HP treatment in SKBR3 and MDA-MB-231 cells C) SKBR3 and MDA-MB-231 cells were treated 250 μM NG for different time points 12 h, 24 h, 36 h and 48 h. Percentage growth inhibition at each time point is measured and represented graphically from the average values from three independent experiments. $^{*}p \leq 0.001$ compared with NG treatment in SKBR3 and MDA-MB-231 cells.

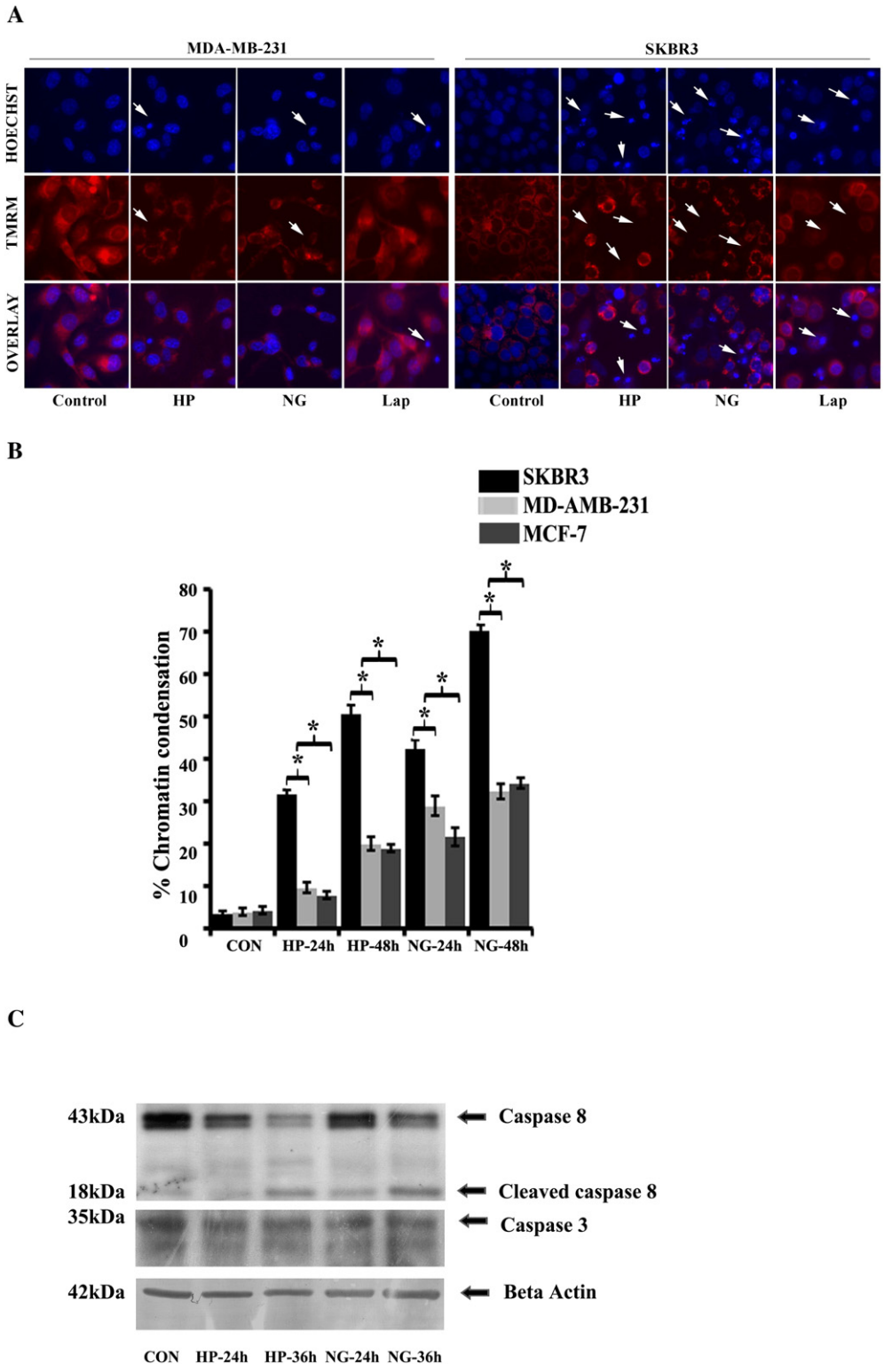



**Fig. 4.** TMRM and Hoechst staining. A) SKBR3 and MD-AMB-231 cells were treated with 500 μM HP or 250 μM NG or 10 μM LAP for 24 h. TMRM and Hoechst staining images with overlay are provided. B) SKBR3, MDA-MB-231 and MCF-7 cells were treated with 500 μM HP and 250 μM NG for 24 and 36 h. The number of fragmented or condensed nuclei was counted manually and determined the percentage of chromatin condensation in comparison with untreated and represented as graph. The results represent the means of three independent experiments. $^{*}p \leq 0.05$ compared with HP or NG treatment in SKBR3 and MDA-MB-231 cells. C) SKBR3 cells were treated with HP and NG for 24 and 48 h and western blot analysis of caspase 8, caspase 3 and Beta Actin are shown.

compounds triggered more apoptosis in HER2 positive SKBR3 cells than HER2 negative MD-AMB-231 and MCF-7 cancer cell lines.

Apoptosis induction of HP and NG in HER2 positive cancer cell line SKBR3 was confirmed by western blotting for caspase 3 and caspase 8 activation, which act as important mediators of apoptosis as executioner and initiator caspases respectively. Caspase8 activation was very prominent in both HP and NG treatment. HP triggered significant caspase 8 activation at 36 h whereas NG induced caspase 8 activation at 24 h itself, suggesting NG is stronger apoptosis inducer than HP against HER2 positive cells (Fig. 4B). Activation of caspase 8 indicates

that cell death occurs through the involvement of death receptor pathway of apoptosis as well. Caspase 3 is an important executioner caspase which can be triggered by both mitochondrial and death receptor pathway of apoptosis. Both HP and NG induced caspase 3 activation as evident from the reduction in mother band intensity of caspase 3 in the treated lanes (Fig. 4C).

### 3.5. HP and NG interrupted cell cycle progression in SKBR3

Finally, cell cycle analysis using propidium iodide (PI) staining by flow cytometry was carried out to determine the effect of these flavonoids on cell cycle. HP and NG were treated in MD-AMB-231 and SKBR3 for 24 h. The effect of these agents in cell cycle was very evident and consistent with an n of 3 experiments with >10% of difference in S and G2 phases in HER2 positive cell SKBR3. HP treatment brought about evident G2 arrest in SKBR3 and NG induced S phase arrest. However, these agents does not bring out any significant changes in G1, S and G2 cell cycle phases in MD-AMB-231. This suggests the existence of HER2 dependent action of these agents on regulating cell cycle progression at S and G2 phases. Percentage of cells in each phase was estimated and plotted as graph in Fig. 5A and B. Quantification of apoptotic cells upon treatments based on Sub G0 population was not precise as a major portion of apoptotic cells were eliminated as debris during the sample preparation.

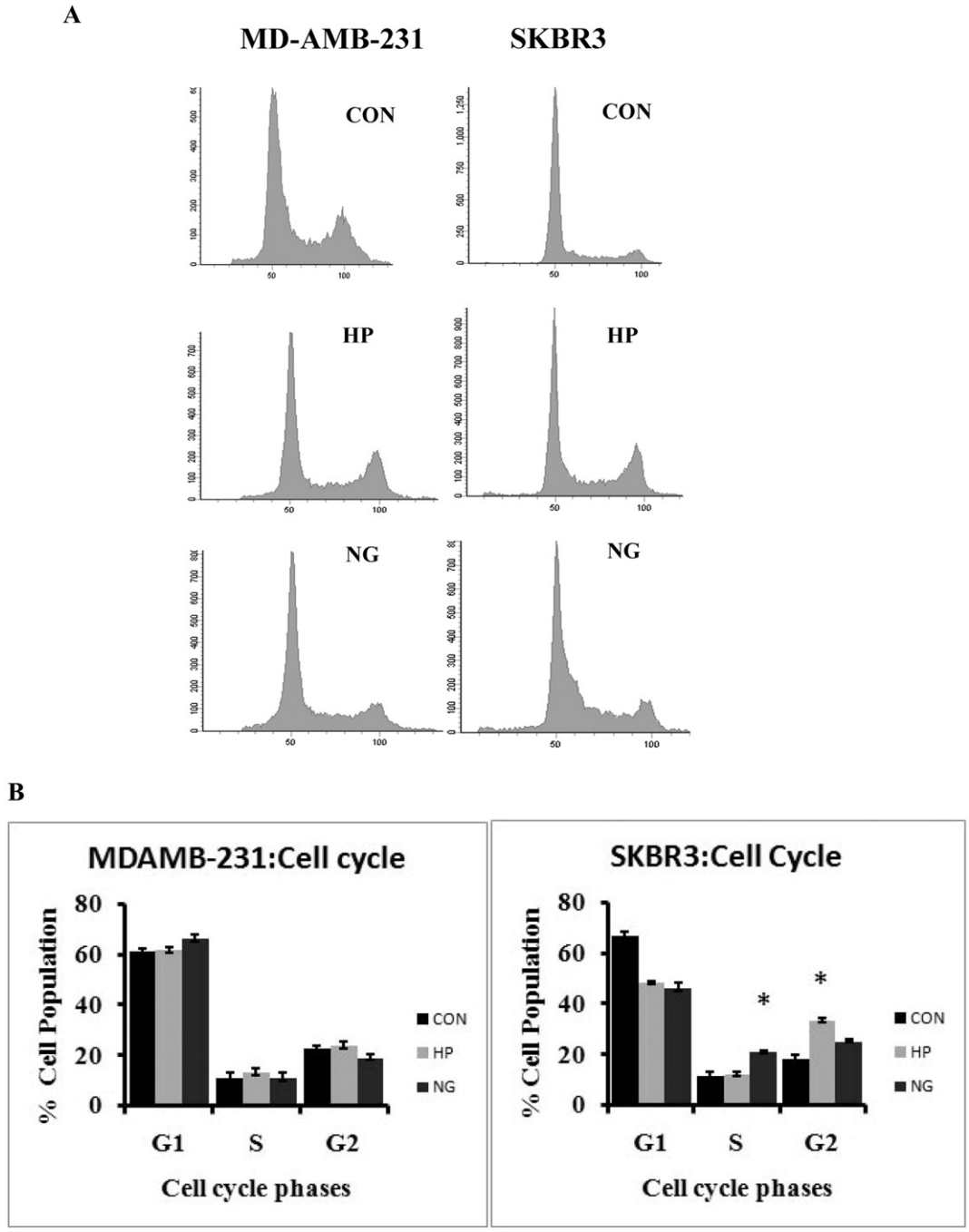



**Fig. 5.** Cell cycle analysis by FACS. A) SKBR3 and MDA-MB-231 cells were treated with 500 μM HP and 250 μM NG for 24 h and cell cycle analysis was carried out. Histogram representation of the cell cycle is provided. Cell count on Y axis and Intensity of PI on X axis. B) The percentage of cell population in G0/G1, S and G2 phases was determined and represented graphically for MD-AMB-231 and SKBR3. The graph is plotted from the values of three independent experiments. *$p \leq 0.05$ compared with cell death induced in MDA-MB-231 and SKBR3 cells.

## 4. Discussion

HER2 receptor protein possesses a tyrosine kinase domain. Homo or hetero dimerization with the members of EGFR family leads to kinase activation and phosphorylation resulting in the induction of downstream signaling pathways such as PI3K-Akt, MAPKs regulating cell survival, proliferation, migration, differentiation and in carcinogenesis [37]. Elevated HER2 expression is a major hallmark of 30% of breast cancer incidences worldwide contributing to cell death resistant and aggressive cancer phenotype. The tumors with HER2 phenotype in general are insensitive to conventional treatment modalities and hence targeted chemotherapy and immunotherapy are highly recommended to downregulate HER2 expression or inhibition of its kinase activity [46]. These therapeutic approaches were found promising initially but, later might become insensitive owing to the development of acquired resistance mechanisms to overcome HER2 inhibition by the cancer cells. Moreover, the available therapeutics against HER2 positive tumors suffer from toxicity issues ranging from potential skin, abdominal and cardiac side effects [4,27,30,45]. Clinical experience has been shown that only a small percentage of patients respond to targeted therapies. Primary resistance to targeted therapy is often due to the constitutive activation of downstream signal transducers and mutations at the gatekeeper residues of the drug targets [17,20,25]. Thus very specific targeted treatment regimens are often found failing in this context. Considering the issue of acquired resistance to TK inhibitors and interplay of immune system against immunotherapy, the effective treatment of HER2 positive tumors demands the establishment of better therapeutic alternatives. Thus, we hypothesize that flavonoids in general, which are well acclaimed for anti-proliferative and pro-apoptotic activities could be evaluated for anti-HER2 action to design safe, efficient and cost effective therapeutic agents against HER2 positive tumors.

Flavonoids are phenolic phytochemicals, having long been appraised for their health benefits in resisting many chronic diseases and so are extensively employed in folk medicines [49]. They also possess anti-oxidant, pro-apoptotic and anti-cancerous properties. Anti-tumor activity of sophoranone, EGCG, curcumin, genistein, quercetin, apigenin, kaempferol, chrysin, luteolin, biochanin, daidzein, myrecetin, catechin, and silymarin are well demonstrated in cancer cell line and mouse models [35,49,51,54,58,59,61]. Chemo-preventive potential of flavonoids has long been exploited non-specifically in traditional medicines in Asian countries. Diet with soy flavones containing food has been found to reduce the number of breast cancer and prostate cancer incidences in Asian countries in comparison with western countries in meta-analysis based studies [29]. Many flavonoids such as catechin, nobiletin, quercetin and apigenin have been reported to prevent tumor invasion and metastasis as well [8,12,40].

*In silico* results of the present study demonstrated that both NG and HP could establish stable interaction at the ATP binding site of the tyrosine kinase domain of HER2 (Fig. 1A and B) to where SYR, a potential inhibitor of HER2-TK, was found to interact in the co-crystallized complex [2]. The ATP binding pocket of HER2-TK adopted a semi closed tunnel shape. The flavonoid molecules, HP and NG found to get inside to act as HER2-TK substrate structural analogues at this pocket. The binding free energy calculation by MMGBSA, suggested the potential of these flavonoids to form a thermodynamically stable complex with the protein. Previously, curcuminoid analogues were found to serve as HER2-TK inhibitors (Yim im et al., 2014). $IC_{50}$s of two of the flavonoids, HP and NG were found to be around 20 μM according to the *in vitro* HER2 kinase assay suggested their prospective efficacy as promising HER2-TK inhibitors (Fig. 2). Several flavonoids are reported for enzyme modulatory activities as well [53]. The potential of these flavonoids, HP and NG to inhibit other kinases like CDK, MMP2 and Akt important in cancer signaling has been previously established [19,42,43,48]. These reports suggest the ability of the flavonoids, HP and NG to inhibit a range of kinases important in carcinogenesis. However, their activity as HER2-TK inhibitors has not been reported so far. Therefore we claim this observation as the first report on the ability of HP and NG to distinctly sensitize HER2 positive cancer *via* serving as HER2-TK inhibitors. According to the recent studies, the acquired resistance of HER2 positive tumors is the major drawback of current treatment regimens. Therapy with highly selective HER2 targeted drugs may render complete HER2 inhibition, compelling the cancer cells to initiate alternative signaling cascades to bypass this inhibition [18]. As the flavonoids, HP and NG are not very specific in their action may leave a residual HER2 kinase activity, thereby it can restrict the induction of alternative signaling events that render cancer cell survival.

Rutaceae family is rich in flavonoids and hence the citrus fruit extract itself have been reported for their anti-cancerous activity towards a variety of cancers. Thus it can be stated that the presence of NG and HP in abundance in citrus fruit peel and pulp might account partly for anti-proliferative or pro-apoptotic activities of citrus fruit extract. Many reports expressed the pro-apoptotic potential of NG and HP in many cancers including breast cancer [1,3,6,10,28,42,43,50,56,57,60]. Cytotoxicity of these agents in HER2 positive cell lines were assessed based on MTT assay and the results revealed that both HP and NG significantly reduced cell viability in HER2 positive breast cancer cell line SKBR3 than HER2 negative breast cancer cell line MDA-MB-231 (Fig. 3). Here, the study highlighted the ability of HP and NG to inhibit HER2-TK and thereby to limit proliferation and sensitize cells to death. Quercetin and its derivatives have already been established as HER2-TK inhibitors and omitted from this study [41].

Mitochondrial membrane potential loss is an early event in the apoptotic cascade. Initially the pro-apoptotic tendency of the agents to trigger cell death in breast cancer cells SKBR3 and MDA-MB-231 were determined by measuring MMP loss. The results revealed increased tendency of these compounds to trigger MMP loss in SKBR3 than MDA-MB-231 cancer cell lines (Fig. 4A). As per the TMRM data, NG induced more MMP loss starting at 12 h of treatment onwards, whereas, HP triggered MMP loss only at 24 h in HER2 positive cancer cell line. Though both compounds induced comparable growth inhibition, MMP loss was more when SKBR3 cells were treated with NG than HP at early time points. This could be because; NG might be better in triggering apoptosis while HP might be inducing anti-proliferative activity only at early hours of treatment in SKBR3 cell lines. Chromatin condensation or fragmentation is regarded as a late and prerequisite hallmark of apoptosis. Therefore, apoptotic potential of NG and HP was scored based on chromatin condensation by Hoechst staining followed by imaging and manually counting the condensed nuclei *versus* normal. Caspase 8 and caspase 3 activation upon HP and NG treatment in SKBR3 cell evidently support the ability of HP and NG to trigger apoptosis based cell death mechanism. This data also clearly indicates the preferential capability of these compounds to trigger more apoptotic cell death in HER2 positive SKBR3 cells in comparison with HER2 negative MDA-MB-231 cell line (Fig. 4B). All these experimental evidences validated the *in silico* and *in vitro* experiment results of the flavonoids, HP and NG as HER2 Tyrosine Kinase inhibitors. This strongly suggests the biased activity of these agents in HER2 positive cancer cell line in comparison with HER2 negative cells and would only be possible if these agents potentially inhibit HER2-TK. Interestingly, the expressed cell cycle analysis presented a HER2 dependent influence of these agents on S and G2 cell cycle phases. NG induced S phase arrest where as HP induced G2 arrest of HER2 positive cells, while any such tendency was absolutely absent in MD-AMB-231 cell line. It is interesting to note that how these structurally similar compounds with same target affinity could differentially influence cell cycle phases in HER2 dependent manner (Fig. 5).

Identification of these flavonoids, HP and NG as HER2-TK inhibitors, facilitates the scope for the development of better inhibitors by either through their derivatives or by chemical modifications *via* considering the structural advantages of HER2-TK or biotransformation. Recently, much advancement has been made in the development of flavonoid based anti-cancer drugs. Many flavonoids including flavopiridol, silybin and their derivatives or their analogues are in cards to be tested for their

efficacy and potency as anti-cancer agents in clinical trials [39,44,55]. Altogether, these advances pin point the potential of flavonoids, HP and NG as potent anti-cancer agents against HER2 positive tumors and their capability to serve as a frame for the development of novel and effective chemotherapeutic agents in future.

## 5. Conclusions

Two citrus fruit flavonoids, HP and NG have been demonstrated to inhibiting tyrosine kinase activation of HER2 receptor protein on the basis of both *in silico* and *in vitro* studies. Although the anti-proliferative or pro-apoptotic potential of these compounds against a variety of cancers have previously been demonstrated, none of the studies suggested their function as HER2-TK inhibitors or their preferential action against HER2 positive breast cancer cell line. Thus we claim this as the first report on their distinguished capability to target and inhibit tyrosine kinase activity of HER2 to facilitate targeted therapy. Thus, we hope that flavonoids of our study may serve as a framework for the development of more efficient and safe therapeutic combinations for HER2 over-expressing cancers.

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