Anticancer effects of zapotin flavone in human gastric carcinoma cells are mediated via targeting m-TOR/PI3K/AKT signalling pathway

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The current study aims to investigate the anticancer effects of zapotin flavone in human gastric carcinoma cells. MTT assay was performed to determine the cytotoxicity effects of zapotin against the gastric cancer cells (SNU-1) and normal gastric cells (GES-1). SNU-1 cell morphology was analyzed through phase-contrast microscopy. Apoptosis was identified through DAPI staining assay and quantified through annexin V/propidium iodide (PI) staining. The effects on cell migration and invasion were carried out through transwell assay. Apoptosis and m-TOR/PI3K/AKT signalling pathway related proteins were analysed through western blotting. Proliferation rate of gastric cancer SNU-1 cell line declined with enhanced zapotin concentrations in comparison to normal GES-1 cells. Substantial morphological changes after zapotin exposure, including nuclear condensation and membrane rupture was observed. Further, increasing number of apoptotic cells, suppression of both cell migration and invasion was observed with increased zapotin concentrations. Finally, western blotting indicated significant blocking of m-TOR/PI3K/AKT signalling pathway. We conclude that zapotin can act as a potential drug against the gastric cancer.

Key words: gastric cancer, flavone, zapotin, caspase, apoptosis

Received: 10 December, 2021; revised: 05 March, 2022; accepted: 05 March, 2022; available on-line: 09 June, 2022

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Abbreviations: Bcl-2, B-cell lymphoma 2; DAPI, 4',6-diamidino-2-phenylindole; mTOR, mammalian target of rapamycin; NFκB, tumor necrosis factor 1; PI3K, phosphoinositide 3-kinase; PI, propidium iodide; TNF-α, tumor necrosis factor

INTRODUCTION

Flavonoids are polyphenolic compounds frequently found in a common human diet. Flavonoids are present at higher concentration in plants, including vegetables and fruits (Scalbert & Williamson, 2000). Polyphenolic phytochemicals (flavonoids) are presently of great clinical interest due to their immense pharmacological activities. Flavonoids act as antioxidants, have cardioprotective, anti-carcinogenic, and anti-inflammatory effects (Le Marchand, 2002; Tungmunnithum et al., 2018). Recent investigations of flavonoids have reported that they react with a wide range of signalling enzymes and alter their expression, like transcription factors and cellular proteins (Mansuri et al., 2014; Selvakumar et al., 2020). Apoptosis is a highly preserved process in multicellular organisms for maintaining homeostasis and orderly development of tissues. Apoptosis is a result of various extrinsic signals stimulated by Fas, radiation, TNF-α (tumor necrosis factor), and growth factor withdrawal (Elmore, 2007). Intrinsic factors that induced activation of apoptosis includes oxidative stress and p53 activation (Reed & Green, 2002, Ashkenazi & Dixit, 1998). Flavonoids have also been reported with apoptosis inducing potential against different human cancers like gastric cancer, ovarian cancer, cervical cancer, and others (Jaudan et al., 2018; Sak, 2014). Gastric cancer is a life-threatening distortion associated with the digestive system and accounts for high mortality rate across the globe. Gastric cancer incidence shows geographical variation, and in underdeveloped and developing countries higher number of gastric cancer cases have been reported (Crew & Neugut, 2006; Rawla & Barsouk, 2019). Dysregulation in the expression levels of telomerase and telomere length are associated with the progression of gastric cancer. Telomerase expression is tightly regulated in normal cells and during carcinogenesis telomerase is reactivated, which in turn maintains telomere length in rapidly proliferating cancer cells (Leao et al., 2018; Hannen & Bartsch, 2018). Gastric cancer is a lethal malignancy, and its lethality enhances due to lack of efficient treatment. Surgery is the major available strategy for the treatment of gastric cancer (Zhang et al., 2021). Chemotherapeutics commonly used against human cancers have not proved fruitful against gastric cancer (Song et al., 2017; Li et al., 2020). Thus, there is an immediate need to look for novel drugs that can prove effective in gastric cancer management. Zapotin – a flavone – is a major constituent of the tropical fruit zapote blanco (Casimiroa edulis). This fruit is consumed throughout the world, including Asia and Central America. Zapotin has been reported to activate apoptosis and differentiation potential against HL-60 leukemia cells (Maiti et al., 2007). Therefore, the current study aims to investigate the possible anticancer role of zapotin flavone in human gastric carcinoma cells.

MATERIAL AND METHODS

Evaluation of cell proliferation rate

The proliferation rate of human gastric SNU-1 cancer cells and normal gastric GES-1 cells was evaluated...
through MTT assay. Briefly, both cell lines were cultured in 96-well plates at a concentration of 6×10^4 cells/well. Cells were cultured 24 h prior to zapotin treatment at varying concentrations (0, 10, 20, 50, and 100 μM). Afterwards, both treated cells lines were incubated for 24 h at 37°C and 5% CO₂. Thereafter, media was decanted, followed by addition of 200 μl MTT stock solution (5 mg/ml) to each well. Then the cells were left untouched for about 3 h followed by dissolving of formazan crystals with DMSO. Finally, absorbance was measured at 570 nm and 650 nm using a microplate reader (Thermo Fisher Scientific Inc., Waltham, USA).

**Morphological analysis through phase contrast microscopy**

Cellular morphology was investigated via phase contrast microscopy. Gastric SNU-1 cancer cells were placed onto 6-well plates at a density of 3×10^4 cells/well. Thereafter, cancer cells were exposed to different zapotin concentrations (0, 20, 50 and 100 μM) for 24 h followed by phosphate buffered saline (PBS) washing. All the cell media was decanted, and cell morphology was observed under inverted microscope (Leica DMI 300B, Germany).

**Evaluation of apoptosis with DAPI and annexin V/PI staining**

SNU-1 cells were plated in 96-well plates for determination of apoptotic cell morphology at a density of 3×10^4 cells/well. Seeded SNU-1 cells were exposed to zapotin (0, 20, 50, and 100 μM) and incubated for 24 h. Further, DAPI solution was added to the zapotin-treated cells, followed by washing with PBS. Thereafter, DAPI-stained cells were fixed with 10% formaldehyde. Apoptosis analysis was finally carried out under fluorescence microscope. Similar procedure was followed for annexin V/PI staining assay except staining with DAPI was replaced with annexin V/PI dual staining and analysis was performed through flow cytometry.

**Observations of cell migration and cell invasion**

SNU-1 cells were treated with zapotin at varying concentrations (0, 20, 50, and 100 μM). Treated cells were transferred to upper chambers of transwell chambers containing RPMI-1640 medium at a concentration of 1×10^4 cells/well. Lower chambers were filled with DMEM medium (600 μl) containing 10% FBS (Corning Incorporated, Corning, NY, USA). Transwells were then incubated for 12 h followed by fixation with methanol at 4°C for 10 min. Removal of un-migrated cells was performed using a cotton swab, while migrated cells were stained for 5 min with crystal violet at room temperature. Finally, various sections were pictured with a light microscope under 200× magnification (TS100; Nikon Corporation, Tokyo, Japan). Invasive ability of SNU-1 cells was also observed through transwell chambers except that the transwell chambers were coated with Matrigel.

**Western blotting analysis**

SNU-1 gastric cancer cells were cultured and harvested at 70% of growth confluence. Cultured cells were subjected to zapotin exposure at changing concentrations (0, 20, 50, and 100 μM). Zapatin-treated cells were lysed with RIPA lysis buffer and protein content within each lystate was quantified by BCA assay. From each lystate about 40 μg of protein was loaded over SDS-PAGE and then electrophoretically transferred to nitrocellu-

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**Zapotin induced cytotoxicity in human gastric SNU-1 cancer cells**

Cell proliferation rate of cells (SNU-1 and GES-1) was measured by MTT viability assay. The results revealed that zapotin molecule is a potential dose-dependent proliferation inhibitor against SNU-1 cells, but not in GES-1 cells. The percent viability of SNU-1 cells at 0, 10, 20, 50, and 100 μM was nearly 90%, 80%, 60%, 25%, and 10%, respectively (Fig. 1), thus indicating dose-dependent suppression of cell viability. However, the normal cells (GES-1) were less sensitive to zapotin treatment.

The effect of proliferation suppression of zapotin was very low against normal GES-1 cells.

**Zapotin treatment modified cellular morphology of SNU-1 cells**

The morphology of SNU-1 gastric cancer cells was investigated through phase contrast microscopy after the treatment with zapotin (0, 20, 50, and 100 μM). Control cells were observed with normal morphology with regular nucleus, membranes, and organelles. Drug-exposed cells showed completely changed morphology with irregular organelles, condensed nucleus, and ruptured membranes (Fig. 2). The effects of changing morphology of zapotin increased with enhanced concentrations. These modifications also indicated that the anti-proliferative ef-
Apoptosis analysis of zapotin-treated SNU-1 cells

Apoptosis in zapotin-treated SNU-1 gastric cancer cells was analysed by DAPI staining and annexin V/PI staining. The results indicated significant dose-dependent increase in the number of apoptotic cells. DAPI staining indicated substantial morphological variations including randomly distributed chromatin along with dislocated, fragmented, condensed, and shrunken nuclei with better fluorescence (Fig. 3). In comparison to the controls no such morphological modifications were observed. Annexin V/PI staining results specified remarkable increase in apoptotic cell percentage with increased zapotin concentrations. The percentage of annexin V+/PI apoptotic cells was nearly 3%, 13%, 21%, and 25% at 0, 20, 50, and 100 μM, respectively. The annexin V+/PI+ apoptotic cell percentage was 6%, 12%, 23%, and 35% with the same drug concentrations as above (Fig. 4). Further, western blotting analysis indicated a caspase-dependent apoptosis inducing potential of zapotin. The expressions of caspase-9, caspase-8, caspase-3, and BAX proteins increased with increasing zapotin concentrations. The expression level of BCL-2 proteins reduced with enhanced zapotin concentrations (Fig. 5). Thus, these results predicted that zapotin induced caspase-dependent apoptosis in SNU-1 cells.

Zapotin reduced cell migration and invasion ability of SNU-1 cells

Transwell assay was performed to monitor cell migration ability of SNU-1 gastric cancer cells after zapotin exposure at varying concentrations (0, 20, 50, and 100 μM). The results indicated that zapotin reduced cell migration ability of SNU-1 cells dose-dependently (Fig. 6). Transwell chambers assay revealed dose-dependent reduction in the number of invasive cells (Fig. 7). Thus, both cell migration and invasion of SNU-1 cells reduced dose-dependently after zapotin exposure.

Zapotin targeted m-TOR/PI3K/AKT signalling pathway in SNU-1 cells

The effect of zapotin treatment on m-TOR/PI3K/AKT signalling pathway in SNU-1 cells was analysed through western blotting analysis. The results showed that the expressions of m-TOR/PI3K/AKT signalling pathway related proteins altered significantly with increasing zapotin concentrations. The expressions of mTOR, PI3K, and AKT remained almost unchanged in
contrast to the p-mTOR, p-PI3K, and p-AKT, which were reduced after drug exposure (Fig. 8).

DISCUSSION

Cysteine-aspartic proteases (Caspases) belong to a family of cysteine proteases and play a pivotal role in bringing about necrosis, apoptosis, and inflammation (Fink & Cookson, 2005). Either death receptor mediation (extrinsic) or mitochondrial mediation (intrinsic) activates caspases in apoptotic pathways. The extrinsic pathway gets activated through death receptors present on plasma membrane like Fas/CD95 (Fas receptor/cluster of differentiation 95) and TNFR1 (tumor necrosis factor 1). The intrinsic pathway is categorised by mitochondrial permeabilization and the release of cytochrome c into the cytoplasm. The release of cytochrome c results in the activation of caspase-9 and formation of apoposome. Thus, caspase activation plays a vital role in bringing about apoptosis in cancer cells.

Flavonoids have been shown to induce anticancer effects in a wide range of human cancers. Galangin, a flavonoid molecule, has been reported to show anticancer effects in human kidney cancer cells and these effects are mediated via mitochondrial mediated apoptosis, suppression of cell migration and invasion, and targeting PI3K/AKT/m-TOR signalling pathway (Zhu et al., 2018). Siliatin, another flavonoid, inhibits the proliferation of BGC-823 (human gastric cancer cells) mediated through induction of mitochondrial mediated apoptosis (Li et al., 2017). It has been observed that zapotin and extracts or fractions containing zapotin possess different biological activities like anticancer, antibacterial, antioxidant, antifungal, antiviral, antianxiety, antiinflammatory, and antidepressant-like effects (Mata-Greenwood et al., 2001; Cuendet et al., 2008; Toton et al., 2012; Strawa et al., 2021). In the current study the anticancer effects of zapotin flavone were investigated in human gastric carcinoma cells mediated via caspase activation, cell apoptosis, suppression of cell migration and cell invasion, and targeting mTOR/PI3K/AKT signalling pathway. MTT assay was performed to evaluate the effects of zapotin molecule on cell viability of gastric cancer SNU-1 cells and normal GES-1 cells and the results indicated that this molecule reduced the cancer cell viability (SNU-1) with a dose-dependent manner. Phase-contrast microscopy was carried out to analyse morphological changes induced by zapotin in SNU-1 cells, which showed significant variations in the morphology indicating that the anti-proliferative effects of zapotin might be due to its apoptosis-inducing potential. This was not the case in GES-1 normal cells. Therefore, DAPI and annexin V/PI staining assays were performed to identify and quantify apoptosis. The results indicated remarkable morphological alterations representing apoptosis and it was found that the number of apoptotic cells was enhanced with increasing drug concentration. Further, western blotting analysis indicated that zapotin induced caspase-dependent apoptosis in SNU-1 cells. Next, cell migration and invasion ability of gastric cancer SNU-1 cells was investigated by transwell assay showing zapotin dose-dependent suppression of both migration and invasion. Finally, western blotting assay was carried out to determine the effects of zapotin on mTOR/PI3K/AKT signalling pathway. The results depicted no change in mTOR, PI3K, and AKT activity but

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**Figure 7.** Pictures of transwell chambers invasion assay presenting the inhibitory effects of zapotin on cell invasion. Results indicated a decrease in cell invasion with increasing dose of zapotin.

**Figure 6.** Pictures of transwell chambers migration assay presenting the inhibitory effects of zapotin on cell migration. Results indicated a decrease in cell migration with increasing dose of zapotin.

**Figure 8.** Western blotting assay results presenting the activity of the m-TOR/PI3K/AKT signalling pathway associated proteins. The expressions of mTOR, PI3K and AKT remained almost unchanged after drug exposure in contrast to the level of p-mTOR, p-PI3K and p-AKT, which were reduced after drug exposure.
the activity of phosphorylated mTOR, PI3K, and AKT was observed to be declining with enhanced zapotin concentrations.

CONCLUSION

All the above results indicate that zapotin flavone induces anticancer effects in human gastric carcinoma cells. The anticancer effects were associated with caspase activation, cell apoptosis, suppression of cell migration and invasion, and targeting m-TOR/PI3K/AKT signalling pathway.

Consent for publication

All the authors agree for publication.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

YC and JH; designed and performed the experiments; GW and BQ analyzed and interpreted the results of the experiments; and HZ wrote and revised the manuscript.

Acknowledgements

Not applicable

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