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PII: S0944-7113(18)30507-5
DOI: https://doi.org/10.1016/j.phymed.2018.09.224
Reference: PHYMED 52689

To appear in: *Phytomedicine*

Received date: 4 May 2018
Revised date: 7 August 2018
Accepted date: 25 September 2018

Please cite this article as: Nishtha S Srivastava , Rai Ajit K Srivastava , Curcumin and Quercetin Synergistically Inhibit Cancer Cell Proliferation in Multiple Cancer Cells and Modulate Wnt/β-catenin Signaling and Apoptotic Pathways in A375 Cells, *Phytomedicine* (2018), doi: https://doi.org/10.1016/j.phymed.2018.09.224

Curcumin and Quercetin Synergistically Inhibit Cancer Cell Proliferation in Multiple Cancer Cells and Modulate Wnt/β-catenin Signaling and Apoptotic Pathways in A375 Cells

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[1]Part of the studies reported here was presented at the INTEL Science Fair and orally presented by N Srivastava at Penn State University as part of a competition for PA Junior Academy of Sciences, and at University of Pennsylvania Nano Day Competition.

Abstract

Background: Traditional therapy using natural products, especially flavonoids and alkaloids have been in practice for a long time. Among flavonoids, curcumin, quercetin, berberine, and epigallocatechin have been studied in greater detail in terms of their anticancer and anti-inflammatory activities. Although many studies focused on the PI3K, MAP kinase and NF-κB pathways, a thorough investigation of modulation of players in the apoptotic and Wnt/β-catenin signaling pathway by curcumin and quercetin has not been done. Also, only few studies have been carried out on curcumin and quercetin co-treatment studies.

Hypothesis/Purpose: We hypothesized that the combination of natural products will have synergistic effects and the antiproliferative effect will be attenuated via apoptotic as well as Wnt/β-catenin signaling pathways.

Study Design and Methods: To test our hypothesis, we compared potency of natural anticancer agents in four cancer cell lines, A549, HCT116, MCF7, and A375 by MTT and colony proliferation assays and investigated mechanism of anticancer activities by analyzing players in apoptotic and Wnt/β-catenin signaling pathways in A375 cells treated with test agents individually or in combination.

Results: Epicatechins, up to 100 μM concentration, did not inhibit cancer cell proliferation, while curcumin inhibited proliferation in A549 and HCT116 cancer cell lines with an IC_{50} of 3 to 8.5 μM. Quercetin showed stronger inhibition of cell proliferation than berberine. Combination study with two most potent agents, curcumin and quercetin, in 4 cancer cell lines, suggested synergistic effect on cell proliferation with several fold decreases in IC_{50}. Further investigation of the mechanism of action of curcumin and quercetin in melanoma cells, A375, suggested that inhibition of cell proliferation occurred through down-regulation of Wnt/β-catenin signaling pathway proteins, DVL2, β-catenin, cyclin D1, Cox2, and Axin2. In addition, both curcumin and quercetin induced apoptosis by down-regulating BCL2 and inducing caspase 3/7 through PARP cleavage.

Conclusion: These results demonstrate that curcumin and quercetin inhibit cancer cell proliferation synergistically and Wnt/β-catenin signaling and apoptotic pathways are partly responsible for antiproliferative activities.

Key words:

Curcumin, Quercetin, Synergistic, Wnt signaling, Apoptosis, Caspase

Abbreviations:

NASH, non-alcoholic steatohepatitis; PI3K/Akt, phosphatidylinositol 3-kinase/protein kinase B; NF-κB, nuclear factor- κB; mTOR, mammalian target of rapamycin; IKK : ROS, reactive oxygen radicals; TNF-α,tumor necrosis factor;IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; JAK-STAT, Janus kinase/signal transducers and activators of transcription; ERK, extracellular signal-regulated kinase; DVL2, dishevelled 2; PARP, poly(ADP-ribose) polymerase; COX2, cyclooxygenase 2; DKK1, Dickkopf-related protein 1; GSK-3β, glycogen synthase kinase- 3β; APC, adenomatous polyposis coli; CK1,casein kinase 1; GPC3,heparan sulfate proteoglycans.

Introduction

In the past, a significant number of anticancer drugs approved for cancer therapy were derived from natural products (Newman *et al.*, 2003). The reason for natural products gaining interest as anticancer agents appears to be their low tissue toxicity. Among these traditional natural products, curcumin, quercetin, epicatechins, and berberine are shown to have multiple beneficial properties, including anti-inflammatory, antioxidant, anti-hyperlipidemic, anti-atherosclerotic, and anticancer activities (Fukuda *et al.*, 1999; Harris *et al.*, 2016; Maithilikarpagaselvi *et al.*, 2016; Abdulkhaleq *et al.*, 2017). One of these traditional medicines curcumin is a major active component of the spice turmeric (Curcuma longa Linn, (Zingiberaceae). Both in vitro and in vivo studies have shown that curcumin exhibits potent antioxidant, anti-inflammatory and anticancer activities (Bengmark, 2006). The anti-inflammatory activity of curcumin has shown protection of non-alcoholic steatohepatitis (NASH) (Shapiro & Bruck, 2005; Jang *et al.*, 2008; Tang *et al.*, 2009), which, if not treated, progresses to hepatocellular carcinoma (Asgharpour *et al.*, 2016). Besides anti-inflammatory activities (Kurup *et al.*, 2007; Varalakshmi *et al.*, 2008), curcumin also exhibit antitumor and apoptotic activities in a wide variety of human cancer cell lines (Huang *et al.*, 2008). Several clinical trials have been performed with curcumin for treatment of pancreatic cancer (Swamy *et al.*, 2008), multiple myeloma (Milacic *et al.*, 2008; Jiao *et al.*, 2009), Alzheimer's (Wang *et al.*, 2005), and colorectal cancer (Half & Arber, 2009). In terms of mechanism of action, curcumin is suggested to play its anticancer role partly via suppressing phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signal transduction pathway in several tumor models (Wang *et al.*, 2011; Qiao *et al.*, 2013; Jiang *et al.*, 2014). Curcumin significantly inhibited NF-κB and attenuated the effect of irradiation-induced prosurvival signaling through the PI3K/Akt/ mammalian target of rapamycin (mTOR) and nuclear factor- κB (NF-κB) pathways (Rafiee *et al.*, 2010). Curcumin was shown to inhibit NF-κB activation and NF-κB-regulated gene expression through inhibition of IKK and Akt activation (Aggarwal *et al.*, 2006).

Quercetin is a dietary polyphenol with particular therapeutic interest because of its potential to prevent and treat cancer. Quercetin derivatives accounting for 60% of the total daily intake of flavonoids are commonly found in many vegetables and fruits, such as red onions, apples, parsley, olive oil, cocoa, citrus fruits, tea, and red wine (Harwood *et al.*, 2007; Spagnuolo *et al.*, 2012). More than 4,000 varieties of flavonoids are known (Boly *et al.*, 2011) with seven

main categories based upon variations in their heterocyclic C-ring (Aherne & O'Brien, 1999; Boly *et al.*, 2011). Biological and pharmacological studies suggest that quercetin exhibit antioxidant, anti-inflammatory, antiproliferative, and anticancer properties (Rice-Evans *et al.*, 1996; Wang *et al.*, 2006; Nam *et al.*, 2016). Beneficial effects of quercetin on normal tissues have been attributed to several mechanisms, especially antioxidant effects through quenching reactive oxygen radicals (ROS) that play important roles in pathophysiological and degenerative processes (Ahmad *et al.*, 2017). Quercetin has shown anticancer effects against colon cancer, hepatoma, and melanoma cells (Ranelletti *et al.*, 2000; Tanigawa *et al.*, 2008). Human studies confirmed very low toxicity of quercetin (Gugler *et al.*, 1975; Harwood *et al.*, 2007; Egert *et al.*, 2008; Moon *et al.*, 2008). Quercetin inhibits the release of tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and other inflammatory mediators from monocytes (Andersson *et al.*, 2000; Rendon-Mitchell *et al.*, 2003) via mitogen-activated protein kinase (MAPK) signaling pathway (Degryse *et al.*, 2001). Other studies suggest that quercetin inhibits the Janus kinase/signal transducers and activators of transcription (JAK-STAT) signaling pathway in various inflammatory disorders (Muthian & Bright, 2004; Liao & Lin, 2014). Inhibition of curcumin-mediated cancer cell proliferation is suggested to occur through phosphatidylinositol 3-kinase (PI3K)-Akt/PKB (protein kinase B) pathway (Gulati *et al.*, 2006). Both Raf as well as MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) are also shown to be involved in quercetin-mediated inhibition of cancer cell proliferation (Gulati *et al.*, 2006; Lee *et al.*, 2008). Quercetin is suggested to act by lowering the reactive oxygen species (ROS) inside the cell (Russo *et al.*, 1999) leading to the induction of apoptosis (Russo *et al.*, 2007).

The accurate mechanism through which quercetin exerts its effect in controlling the signal transduction pathway is still not fully understood. While few studies on the mechanism of action of quercetin have been carried out, the specific targets of quercetin are not well characterized. In terms of molecular mechanisms of curcumin, published reports suggest that curcumin-induced apoptosis in cancer cells are varied and depend on cell type and concentration used in the experiments (Duvoix *et al.*, 2005; Karunagaran *et al.*, 2005). Among mechanisms suggested for curcumin-induced inhibition of cancer cell proliferation include induction of apoptotic signal (Karunagaran *et al.*, 2005; Tuorkey, 2014), suppression of antiapoptotic proteins (Reuter *et al.*, 2008), modulation of micro RNAs (Zhang *et al.*, 2010a) and protein kinases (Saini *et al.*, 2011), inhibition of NF-κB (Aggarwal *et al.*, 2006), and epigenetic regulation (Zhang *et*

al., 2010b). Some studies also suggested curcumin's anticancer activity through the players in the Wnt/β-catenin pathways (Choi *et al.*, 2010; Teiten *et al.*, 2011; Sundram *et al.*, 2012). Despite two clinical studies with combination of curcumin and quercetin (Cruz-Correa *et al.*, 2006; Zhang *et al.*, 2015a), the synergistic effects have not been reported and the mechanism of combination efficacy has not been explored. The present study was undertaken to investigate the mechanism of anticancer effect of curcumin and quercetin alone or in combination. Our results show that both co-treatment with curcumin and quercetin in four cell lines show synergism in inhibiting cancer cell proliferation partly via modulation of Wnt/β-catenin signaling and apoptotic pathways.

Materials and methods

Cell Culture

Three human CRC cell lines, A549 (lung adenocarcinoma cells), HCT116 (human colon cancer cells), MCF7 (human breast cancer cells), and A375 (human melanoma cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in IMDM medium (Invitrogen, Rockville MD) under standard conditions with 10% fetal bovine serum and 5% CO_2 at 37 °C. The cell line authenticity was frequently confirmed by analyzing various genetic and epigenetic markers every 6–8 months.

3.2 Test and Control Agents Formulation

Curcumin, quercetin, epicatechin gallate, and berberine (>90%) were obtained from Sigma-Aldrich, St Louis, USA. The purity of the test agents was as follows: curcumin (>65%), quercetin (>95%), berberine (>95%), and epicatechin gallate (98%). Doxirubicin hydrochloride (purity >95%) was procured from Fisher Scientific, Pittsburgh, PA 15275. Stocks were prepared by dissolving these test agents in dimethyl sulphoxide (DMSO) at 10 mM concentration, and small aliquots were stored at 22 °C until use. Fresh test agent-containing culture medium was replaced every second day during the course of treatment. Control cell lines without test agent treatment were grown with each set of treatments for the same duration.

3.3 MTT Viability Assay

The effects of test agents on cell viability were determined in an MTT assay, which is

based upon 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide uptake. Cells (5000/well) were seeded in 96-well plates in medium containing 10% FBS. After 24 hours, the medium was changed to test medium specific for each experiment. After 72 h of treatment, MTT (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 mg/mL and incubated for 2 h at 37 °C followed by the addition of solubilization/ stop solution and incubated for additional 1 h at 37 °C. Absorbance was measured at 570 nm using a spectrophotometer. Experiments were performed in triplicate in 3 independent experiments.

3.4 Colony Proliferation Growth Measurement

Colony formation in soft agar was assessed with curcumin, quercetin or a combination of both at indicated concentrations. Briefly, 2 ml of mixture of serum supplemented media and 1 % agar containing indicated concentrations of curcumin or quercetin or a combination of both were added in a 35 mm culture dish and allowed to solidify (base agar) respectively. Next, on top of the base layer was added a mixture of serum supplemented media and 0.7 % agar (total 2 mL) containing 10,000 A375 cells in the presence of curcumin, quercetin or a combination of both and was allowed to solidify (top agar). Subsequently, the dishes were kept in tissue culture incubator maintained at 37°C and 5 % CO_2 for 14 days to allow for colony growth. All assays were performed in triplicates. The colony assay was terminated at day 14, plates were stained and colonies counted on ChemiDoc XRS instrument (Bio-Rad, Hercules, CA).

3.5 Wnt3a Expression and Effect of Curcumin and Quercetin

Mouse L cells were transfected with a Wnt-3A expression vector and stable clones were selected in medium containing G418. These L cells are available from ATCC that produce and secrete Wnt3A protein (LWnt3A cell, ATCC#CRL-2647). In these cells, Wnt3A is expressed from the PGK promoter.

3.6 Caspase 3/7 Assay

Caspase 3/7 assay was performed using Promega Caspase-Glo 3/7 Assay Systems according to manufacturer's instructions. In brief, 5000 cells were plated per well with test compounds for 18 h. Caspase-Glo 3/7 reagent mix was added and incubated for 60 min at room temperature. The resulting luminescence was detected in EnVision Multi Detection System.

3.7 Isolation of Whole Cell Extract

Cells were trypsinized and whole cell protein was obtained by lysing the cells on ice for 20 min in 700 µl of lysis buffer (0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 0.5 M PMSF 50 µg/ml aprotinin, 10 µg/ml leupeptin, 50 µg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). The lysates were then sonicated for 20 s and spun at 15 000 x g for 10 min, and the supernatant was saved. Protein determinations were made using the method of Bradford.

3.8 Western Blot Analysis

Western blot analysis to measure of ß-catenin, and Wnt signaling pathway players, DVL2, Axin2, cyclin D1, and cox2 was performed on the whole cell extract. A375 cells were treated with curcumin and quercetin (3.1 to 50 µM). Cells were harvested and total cell extract was prepared. Protein (30-50 µg) was mixed 1:1 with sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris– HCl, pH 6.8) loaded onto a 10% SDS–PAGE gel and run at 120 V for 2 h. Cell proteins were transferred to nitrocellulose (ECL; Amersham, Arlington Heights, IL) for 3 h at 250 mA. Equal loading of the protein groups on the blots was evaluated using Bradford assay as well as ß-actin band. The nitrocellulose membrane was then blocked with 5% milk in TBST overnight, washed 4 times, and then incubated with the primary Ab (anti ß -catenin, dishevelled 2 (DVL2), Axin2, cyclin D1, and cox2, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. The blots were washed four times with TBST and incubated for 1 h with HRP-conjugated anti-IgG AB (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were developed using ECL Western blot substrate (Millipore) according to the manufacturer's instructions.

In a separate experiment, A375 cells were treated with curcumin or quercetin at concentrations ranging from 3.1 to 50 µM, cells harvested, and total cell extract prepared. Western blotting was performed as described above to measure proteins involved in apoptosis.

3.9 Statistical and Isobologram Analysis

Statistical analysis where indicated were carried out using ANOVA using Prism 6 software. Synergism, additivity, and antagonism were assessed by isobologram analysis with a fixed-ratio experimental design using the Chou-Talalay method (Chou & Talalay, 1984). Analysis of the results were done using the Calcusyn software (Biosoft, Cambridge, United

Kingdom) to determine Combination Index (CI). Average CI at the IC_{30}, IC_{50}, and IC_{70} <1 indicates synergism, CI= 1 indicates additivity, and CI > 1 indicates antagonism, respectively.

4. Results

4.1 Curcumin and quercetin inhibit proliferation of A549 and HCT116 cells

We initially evaluated 4 natural products in cell proliferation assay, MTT (Figure 1). These are known to have several pharmacological activities, including anti-inflammatory and anticancer properties. In colon cancer cell line, HCT116, epicatechin, known to have antioxidant properties (Abdulkhaleq *et al.*, 2017), did not show antiproliferative activity (Figure 2). Epicatechin also failed to show antiproliferative activity in lung cancer cell line, A549 (Figure 2C). Curcumin and quercetin did show antiproliferative activities in these two cell lines in a concentration-dependent manner with an IC_{50} of 8.5 and 11.4 μM in A549 and 2.9 and 9.8 μM in HCT116 cell lines, respectively. Therefore, all further studies were carried out with curcumin and quercetin.

4.2 Co-treatment with curcumin and quercetin shows greater inhibition of cell proliferation in A549. HCT116, A375, and MCF-7 cells

Follow-up experiments were performed to evaluate antiproliferative activities of four cancer cell lines, A549, HCT116, A375, and MCF-7 cells, treated either individually or in combination with curcumin and quercetin. As shown in Figure 3A, treatment of A549 cells with quercetin and curcumin inhibited A549 cell proliferation. Greater inhibition of cell proliferation was observed after co-treatment with curcumin and quercetin. For combination studies, curcumin concentration was fixed at 2 μM and increasing concentration of quercetin was used. In A549 cell line, curcumin and quercetin shiwed IC_{50} showed of 4.8 and 11.8 μM, respectively (Table-1). Co-treatment of A549 lung cancer cell line with fixed concentration of curcumin (2 μM) and varying concentrations of quercetin (2, 4, and 8 μM) improved IC_{50} to 2.0, 0.7, and <0.5 μM, respectively (Figure 3A, Table-1), showing gradual and more than additive potency with increasing concentration of quercetin. A reference agent, doxorubicin, was also used as a positive control, which showed an IC_{50} of 0.76 μM (Table-1). Similarly, in HCT116 cells, co-treatment with curcumin and quercetin showed greater inhibition of cell proliferation with gradual increases in quercetin concentration (Figure 3B, Table-1).

The anticancer activity of curcumin and quercetin was also evaluated in two other cancer lines, melanoma (A375) and breast cancer (MCF7). Quercetin showed mild antiproliferative activity in melanoma cell lines with an IC_{50} of 50 μM (Figure 3 C), whereas curcumin had an IC_{50} of 8.5 μM. Robust antiproliferative activity was observed in A375 cells when co-treated with curcumin and quercetin, curcumin at 2 μM concentration and quercetin at increasing concentrations (2, 4, 8 μM). A robust antiproliferative activity was observed with a sharp drop in the IC_{50} to 4.8, 3.0 and 1.5 μM from 50 μM for quercetin and 10 μM for curcumin (Figure 3C, Table-1). The reference agent, doxorubicin, showed an IC_{50} of 1.15 μM. Similarly, in MCF7 cells, the IC_{50} for curcumin and quercetin were 18 μM and 45 μM (Figure 3D, Table-1), respectively, but when combined, it showed greater potency in inhibiting cell proliferation with gradual reductions in IC_{50} of 16, 7.5, 3.5μM, respectively (Figure 3C, Table-1). For all further studies on mechanism of antiproliferative activities of curcumin and quercetin, A375 cells were used.

4.3 Inhibition of cell proliferation following co-treatment with curcumin and quercetin occurs by synergistic mechanism

The greater than expected antiproliferative effects observed in this study following co-treatment with curcumin and quercetin in four cancer lines suggested the possibility of synergistic effects. This necessitated the analysis of co-treatment results by isobologram (Chou & Talalay, 1984; Del Curatolo *et al.*, 2018). Figure 4 shows isobologram in two cell lines, A549 and A375 cells. The results derived from the isobologram are shown in Table-2. The combination indexes in both cell lines were found to be <1, suggesting that the co-treatment with curcumin and quercetin has synergistic effect on inhibiting proliferation of these two cell lines. We also analyzed results of co-treatments with curcumin and quercetin in HCT116 and MCF7 cells and the results were similar to A549 and A375 cells in terms of synergism (not shown).

4.4 Curcumin and quercetin induces apoptosis via caspase 3/7 activation in A375 cells

We next evaluated if curcumin and quercetin show cell apoptotic activity through the activation of caspase 3/7. Caspase 3/7 activity was determined in A375 cell treated with quercetin and curcumin either alone or in combination. As shown in Figure 5A, curcumin at 12 μM concentration showed robust activation, although a concentration below 6 μM showed mild activation. Similar observations were noted when the cells were treated with quercetin where no

activity was seen up to 6 μM, but a linear increase in activity at concentrations above 6 μM was evident (Figure 5A). In a separate study when A375 cells were co-treated with curcumin and quercetin (Figure 5B), we observed that the increasing concentration of quercetin (2 μM to 8 μM) added to a fixed concentration of 2 μM curcumin showed a clear increase in potency as evidenced by decrease in the IC_{50} (Figure 5B), again suggesting that co-treatment with curcumin and quercetin lower IC_{50} and show greater potency.

4.5 Curcumin and quercetin inhibit colony growth proliferation of melanoma cancer cell line

Using the same melanoma cell line A375, we evaluated antiproliferative activities of curcumin and quercetin alone or in combination in a separate assay, colony growth proliferation. As shown in Figure 6, curcumin showed more than 80% inhibition of proliferation at a concentration between 3.1 μM and 6.2 μM whereas quercetin showed about 80% inhibition at a concentration between 25 μM and 50μM. When curcumin and quercetin were combined (Figure 7), the inhibition curve shifted to left, suggesting a greater inhibitory effect on cell proliferation. These results, together with MTT assay (Figures 3) confirm the increased potency of curcumin and quercetin combination in inhibiting the proliferation of cancer cells.

4.6 Wnt3a promotes A375 cell growth

In order to investigate the role of Wnt/β-catenin signaling in curcumin and quercetin-mediated antiproliferative activity, we first carried out experiment ascertained if Wnt is involved in the growth of melanoma cell line. For this, we used a cell line that produces recombinant Wnt3a protein and one that does not produce Wnt3a protein and performed A375 cell proliferation assay in spent media from two these two types of L cells. The results showed that in normal growth media A375 cells proliferate normally, but when the media was replaced with the L cell spent media, there was hardly any growth (Figure 8). However, adding spend media fromWn3a producing L cells showed growth of A375 cells (Figure 8), suggesting that Wnt3a protein induces proliferation of A375 cells.

4.7 Curcumin and quercetin inhibit Wnt signaling pathway in melanoma cancer cell line A375

Having confirmed that Wnt protein is involved in the proliferation of A375 cells, we investigated the canonical Wnt/β-catenin signaling pathway mediated antiproliferative activities of curcumin and quercetin. A375 cells were treated with increasing concentrations of curcumin and quercetin (3.1 μM to 50 μM), cells were lysed and protein separated by Western blotting to measure canonical Wnt/β-catenin signaling pathway proteins. The Wnt/β-catenin pathway is highly regulated through the participation of a number of players (MacDonald *et al.*, 2009). We investigated curcumin and quercetin-mediated regulation of some of the key players in this pathway. We focused on dishevelled 2 (Dvl2) protein and axin, and downstream target genes of Wnt signaling, cyclin D1 and cyclooxygenase 2 (COX2). Dvl2 is required to recruit axin to the Frizzled/LRP complex in a signal dependent manner (Clevers, 2006). As shown in Figure 9, both curcumin and quercetin reduced Dvl2 and axin2 in a concentration-dependent manner concomitant with decreases in β-catenin protein. Robust concentration-dependent decreases in cyclin D1 and Cox2 were also noted. These results suggest that both curcumin and quercetin influence players in the Wnt/β-catenin signaling pathway.

4.8 Curcumin and quercetin induce apoptotic pathway in melanoma cancer cell line A375

Since we observed that caspase 3/7 was induced in cells treated with curcumin and quercetin either alone or combined, we looked into curcumin and quercetin effect on players in the apoptotic pathway, both upstream and downstream of caspase 3/7. ERK2/ERK1 are two isoforms of extracellular signal-regulated kinase (ERK) that belong to the family of mitogen-activated protein kinases (MAPKs). A sequential phosphorylation cascade that transduces signals from the cell membrane to the nucleus activates these enzymes.

Programmed cell death by apoptosis is controlled by activation of caspases and nucleases leading to the death of the injured cells without affecting neighboring cells. The intrinsic pathway of apoptosis regulates the activity of the Bcl-2 proteins, which in turn releases of proapoptotic factors, such as cytochrome *c*, from the mitochondria into the cytoplasm. This event leads to the activation of caspase-9, followed by the activation of caspase-3/7. As shown in figure 10, Increased phosphorylation of ERK1/2 was observed following curcumin as well as quercetin treatment. The key player in the apoptotic pathway Bcl-2 was found to be reduced, suggesting activation of apoptotic pathway, which was further confirmed by activation of

caspases3/7. This sequence of events leads to cleavage of poly (ADP-ribose) polymerase (PARP). Indeed, PARP cleavage was increased in curcumin or quercetin treated A375 melanoma cell line (Figure 10).

5. Discussion

The aim of the present investigation was to evaluate combination efficacy of two most potent natural products after screening in 4 different cancer cell lines, namely lung cancer cell line A549, colon cancer cell line HCT1116, melanoma cancer cell line A375 and breast cancer cell line MCF7. Based on the IC_{50} we found curcumin and quercetin as the most potent anticancer agents. Earlier studies have reported both curcumin and quercetin to possess anticancer (Chen *et al.*, 1999; Gulati *et al.*, 2006; Jiao *et al.*, 2009; Boly *et al.*, 2011; Vallianou *et al.*, 2015; Nam *et al.*, 2016) and anti-inflammatory (Aggarwal, 2010; Rogerio *et al.*, 2010; Byun *et al.*, 2013) properties. In the present study we focused on combination efficacy. Co-treatment of A549, A375, HCT116 and MCF7 cells with curcumin and quercetin consistently showed greater inhibition of cell proliferation in MTT assays. To examine whether the greater inhibition of cell inhibition occurs as a result of additive or synergistic effect, we carried out analysis using isobologram (Chou & Talalay, 1984; Del Curatolo *et al.*, 2018). The results derived from this analysis clearly suggested that the co-treatment with curcumin and quercetin had synergistic effect in inhibiting cancer cell proliferation. Since curcumin and quercetin have poor bioavailability (Pan *et al.*, 1999; Murota *et al.*, 2002; Vareed *et al.*, 2008; Zhongfa *et al.*, 2012; Tuorkey, 2014), these agents are likely to have greater efficacy in topical applications. A375 cells have been frequently used to study the mechanism of action of curcumin (Chen *et al.*, 2014; Zhang *et al.*, 2015b). Therefore, we chose A375 cells to further investigate the mechanism of action of curcumin and quercetin in cancer cell proliferation.

First, we examined the role of apoptotic pathway in curcumin and quercetin-mediated attenuation of A375 cell proliferation. Caspases, a family of cysteine proteases that are required for cytokine maturation and apoptosis execution (Salvesen & Dixit, 1997) have been suggested to contribute to both apoptotic (Salvesen & Dixit, 1997) and non-apoptotic cellular phenomena (Hashimoto *et al.*, 2011). In mammals, caspase-1, -4, and -5, are referred to as inflammatory caspases. These caspases possess a large prodomain and cleave the procytokines. Another group

of caspases with a large prodomain includes caspase-2, -8, -9, and -10. These are termed as the initiator caspases of apoptosis, and those with short prodomain comprising caspase-3, -6, and -7 are termed as effector caspases of apoptosis (Figure 11). The effector caspases are activated through proteolytic cleavage by the initiator caspases. We showed that caspase 3/7 are activated by curcumin and quercetin when A375 cells were treated either alone or in combination. via Bcl-2 mediated pathway. This was further confirmed by the inhibition of ERK1/2 phosphorylation, a component in the apoptotic pathway. ERK activity has been associated with markers of apoptosis like effector caspase-3 activation, poly(ADP-ribose) polymerase (PARP) cleavage, annexin-V staining, and DNA fragmentation (Wang *et al.*, 2000). Measurements of cleaved PARP (Boulares *et al.*, 1999) confirmed that curcumin and quercetin induce apoptotic pathway in melanoma cancer cell line, A375. Indeed, curcumin has been shown to inhibit TNF-α-induced NF-kB down-regulation (Aggarwal *et al.*, 2006) which in turn down-regulated NF-kB inducible genes like Cox-2. These authors also showed that curcumin down-regulated TNF-α-induced Bcl2 expression, similar to the results obtained in the present studies (Figures 10, 11). However, it should be noted that Aggarwal et al (Aggarwal *et al.*, 2006) carried out studies in U937 (human myeloid leukemia) cells induced with TNF-α. In this study, U937 cells, either left untreated or pretreated with 50 μM curcumin for 2 h and then exposed to TNF-α for different times. Nevertheless, the findings in both studies are consistent in terms of curcumin's ability to induce apoptotic pathway. It was also reported that curcumin represses the TNF-α-induced NF-kB -dependent gene products involved in cell proliferation (Aggarwal *et al.*, 2006). In more recent studies, curcumin was shown to activate apoptotic pathway in A375 cells treated either alone (Zhang *et al.*, 2015b) or in combination with natural borneol (Chen *et al.*, 2014). Thus, the findings in the present study sre in line with the reported effect of curcumin in modulating apoptotic pathway.

Another pathway that regulate cancer cell proliferation is the Wnt/β-catenin pathway (Shtutman *et al.*, 1999) that induces a number of genes, including cyclin D1, cyclooxygenase 2 (COX2), Axin 2, and Dvl2. Cyclin D1, a target of β-catenin pathway, is overexpressed in a variety of tumors and mediates the progress of cells from the G_1 to the S phase (Polsky & Cordon-Cardo, 2003). Likewise, (COX-2) is overexpressed in tumor cells and mediates proliferation (Chun & Surh, 2004). The role of c-myc in proliferation of tumor is well

established (Schmidt, 2004). Indeed, in the present study, both curcumin and quercetin down-regulated cyclin D1, Cox 2, Dvl2, and Axin 2, which were associated with lowering of β-catenin, suggesting that curcumin and quercetin influence Wnt/β-catenin signaling pathway through modulation of players like Dvl2 and Axin 2, which in turn reduces β-catenin, resulting in the suppression of cell proliferating genes like cyclin D1 (Figure 12). In neuroblastoma cell line curcumin was shown to suppress cancer cell proliferation through the inhibition of Wnt/β-catenin signaling (He *et al.*, 2014). Quercetin was also shown to regulate Wnt/β-catenin signaling activity to induce growth suppression of 4T1 murine mammary cancer cells (Kim *et al.*, 2013). However, these authors (Kim *et al.*, 2013) measured only β-catenin and Dickkopf-related protein 1(DKK1) protein. In another study Shan et al (Shan *et al.*, 2009) measured cyclin D1 in quercetin treated human SW480 colon cancer cells and concluded the involvement of Wnt signaling pathway in quercetin-mediated inhibition of human SW480 colon cancer cells. Similarly, He et al (He *et al.*, 2014) showed activation of glycogen synthase kinase -3β (GSK-3β), a negative regulator of the Wnt/β-catenin signaling pathway. GSK-3β is a kinase and the main role of GSK-3β is to facilitate phosphorylation of β-catenin leading to its degradation and resulting suppression of β-catenin translocation to the nucleus (Figure 12). The activation of GSK-3β is reported to be associated with reduced levels of β-catenin (He *et al.*, 2014).

In the present study, we carried out systematic investigation of antiproliferative efficacy following co-treatment with curcumin and quercetin. We measured several players in the Wnt/β-catenin as well as apoptotic pathways to unravel the mechanism of action of these two agents known to have anticancer properties. Very few studies have been carried out on the combination efficacy of curcumin and quercetin, albeit without the underlying mechanism. Efficacy of curcumin and quercetin has also been studied by adding epigallocatechin in this combination. This combination was reported to have protective effects against cancer and cardiovascular disorders (Jagtap *et al.*, 2009). In one study with human gastric cancer MGC-803 cells, Zhang et al (Zhang *et al.*, 2015a) focused on apoptotic pathway and showed that the combined treatment with curcumin and quercetin was more effective in inhibiting the proliferation of MGC-803 cells than the individual treatment. This effect was reported to occur through modulation of AKT and ERK phosphorylation. The cell-based findings on anticancer effect of curcumin and quercetin was also evaluated in animal models of carcinogenesis. In benzo(a)pyrene -induced mouse

model of lung carcinogenesis, the combined efficacy of curcumin and quercetin was examined (Liu *et al.*, 2015), which sowed that benzo(a)pyrene induced lipid peroxides and reactive oxygen species were significantly improved following treatments with curcumin and quercetin. While direct measurement of tumor reduction was not investigated in this study (Liu *et al.*, 2015), the improvement in antioxidant status was suggested to have protective effects. Similarly, in chemically-induced colon and mammary gland carcinogenesis model in rats, curcumin and quercetin combination therapy with high concentration of curcumin and quercetin was effective as chemopreventive agent in the colon cancer model, but weakly effective in mammary cancer model (Pereira *et al.*, 1996). At least one clinical study was carried out on the combination treatment with curcumin and quercetin in patients with familial adenomatous polyposis (Cruz-Correa *et al.*, 2006). Patients were treated with 480 mg of curcumin and 20 mg of quercetin three times daily for 6 months. These authors reported positive results of combination therapy in reducing the number and size of adenomatous polyps. However, because of low number of patients (n=5), these results need to be validated in a larger randomized double-blind, placebo-controlled clinical studies.

Thus, the present investigation provides more in-depth information on curcumin and quercetin-mediated modulation of two main pathway of cancer cell proliferation, namely Wnt/β-catenin signaling and apoptotic pathways. As shown in figure 11, curcumin and quercetin modulate several players in the apoptotic pathways, including Bcl-2, caspase 3/7 and PARP. In terms of modulation of players in the Wnt/β-catenin signaling pathways, we demonstrated that many key proteins are altered in favor of inhibition of cancer cell proliferation. As shown in figure 12 A, Wnt remains bound with heparan sulfate glycosaminoglycan, especially glypican-3, a member of the glypican-related integral membrane heparan sulfate proteoglycans (GPC3) and a marker of hepatocellular carcinoma (Sung *et al.,* 2003). Factors that induce sulfatase 2 (SULF2) enzyme releases Wnt (Lai *et al.,* 2010), facilitating the formation of a complex with LRP5/6 and frizzled protein. Curcumin/Quercetin possibly destabilizes the Wnt-Fz-LRP6 complex via down-regulation of Dvl2 and Axin resulting in the repression of Wnt responsive gene cyclin D1 (Figure 12 B) and downstream players like COX2. In the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin, Adenomatous polyposis coli (APC), GSK3 and casein kinase 1 (CK1). CK1 and GSK3 sequentially phosphorylate β-catenin to facilitate β-catenin ubiquitination

and proteasomal degradation. This prevents β-catenin from reaching the nucleus, and repressing Wnt target genes.

In conclusion, given the limitations of natural products in maintaining efficacious concentration in the circulation because of less than optimal pharmacokinetic properties, we believe that the combination therapy of curcumin and quercetin together with small molecules currently employed for cancer therapy could be a possibility to evaluate efficacy in inhibiting certain type of cancers like colon cancer and skin cancer.

6. Conflict of Interest

Nishtha Srivastava is a student of veterinary science at Manor College of Veterinary Technology, Jenkintown, PA. Rai Ajit K Srivastava is currently employed at Gemphire Therapeutics, Livonia, MI. There is no conflict of interest.

7. Funding of Research

The research carried out here was funded through an internship to Nishtha S. Srivastava by Integrated Pharma Solutions, Philadelphia.

8. AUTHOR CONTRIBUTIONS

Nishtha Srivastava conducted experiments and participated in the interpretation and writing of the manuscript. Rai Ajit Srivastava designed experiment, reviewed research data, interpreted the results and wrote the manuscript.

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Tables

Table 1

IC_{50} of test and the reference agents. Cells were grown as described in the materials and methods section and the data were graphed using Prism GraphPad version 6 to determine the IC_{50}.

Table 2

Isobologram-derived data on the analysis of synergistic effect of curcumin and quercetin combination studies. The combination index (CI) <1 indicates synergism; >1 antagonism; and =1 additive.

Figure Legends:

Figure 1. Srivastava

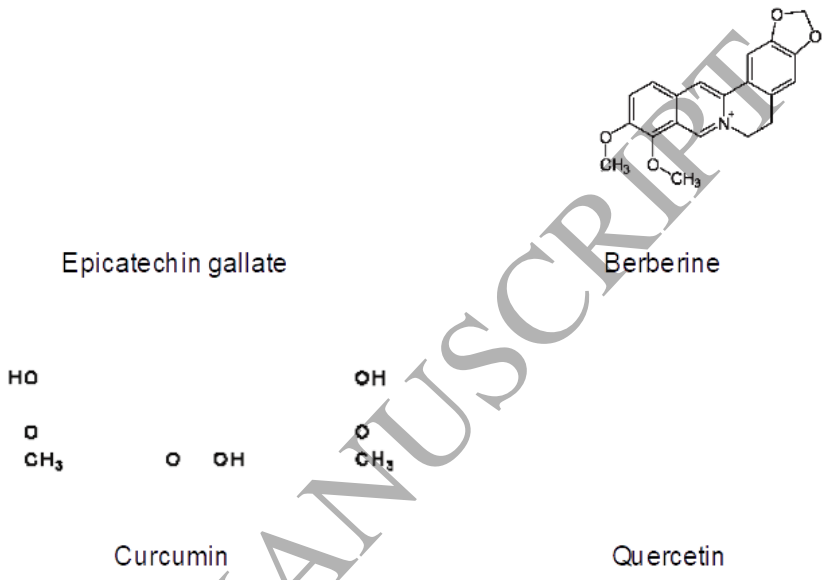
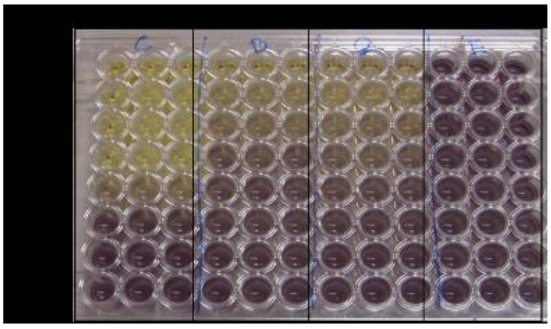


Figure 1

The chemical structure of the test agents used in the present study. Epigallocatechin, berberine, curcumin, quercetin.

Figure 2. Srivastava

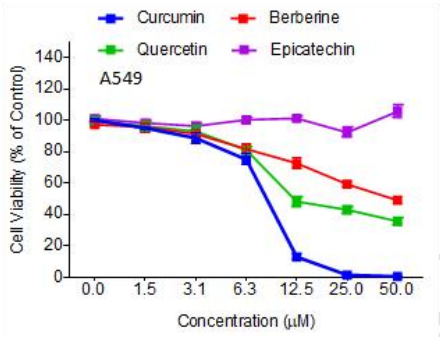
A



B

Test Agent	A549 IC50 (µM)	HCT116 IC50 (µM)
Curcumin	8.5	2.9
Quercetin	11.4	9.8
Berberine	>50	12.5
Epicatechin	Not Active	Not Active

C



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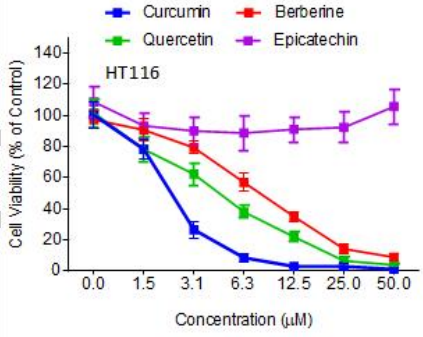


Figure 2

Effect of test agents on anticancer activity in MTT assay. Panel A, 96-well MTT assay showing inhibition of cancer cell proliferation; Panel B, IC_{50} of test agents in two cancer cell lines, HCT116 and A549; Panel C, Concentration curve of inhibition of A549 cell proliferation; Panel D, Concentration curve of inhibition of HCT116 cell proliferation.

Figure 3. Srivastava

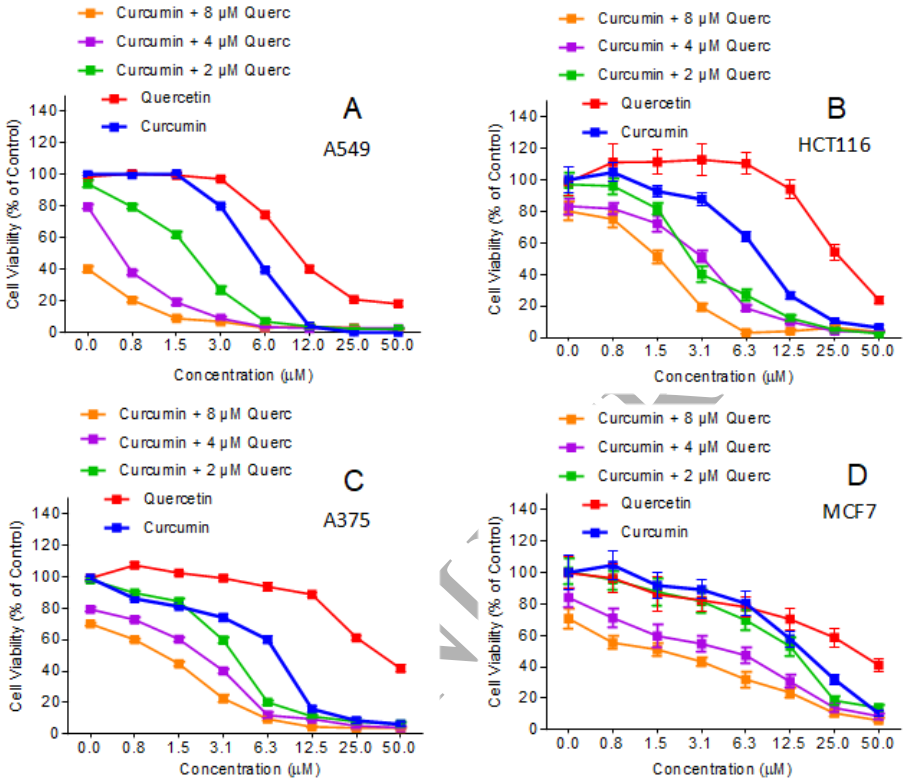


Figure 3

Combination Effect of Curcumin and Quercetin on Inhibition of Cancer Cell Proliferation. Panel A, Effect on proliferation of A549 cells treated with quercetin or curcumin alone or in combination; Panel B, Effect on proliferation of HCT116 cells treated with quercetin or curcumin alone or in combination; Panel C, Effect on proliferation of Melanoma cancer cells (A375) treated with quercetin or curcumin alone or in combination; Panel D, Effect on proliferation of Breast cancer cells (MCF7) treated with quercetin or curcumin alone or in combination.

Figure 4. Srivastava

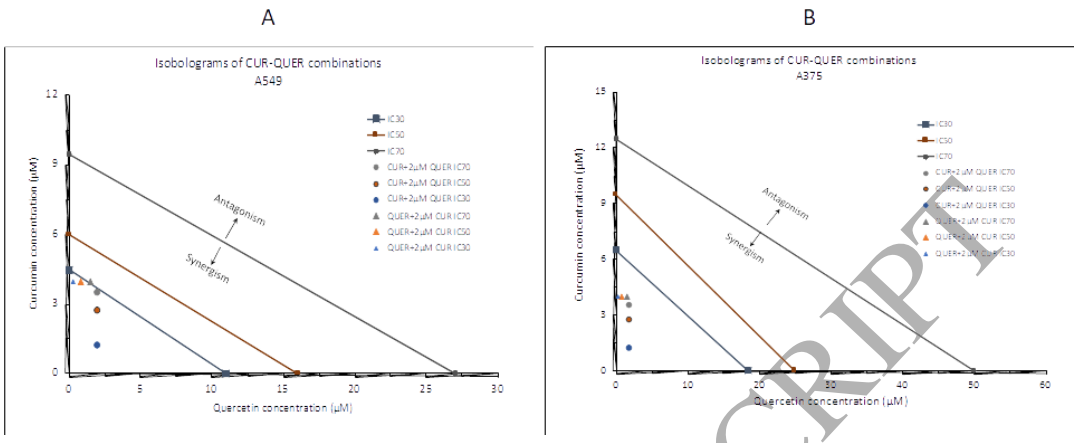


Figure 4.

Isobologram of results from combination studies of curcumin and quercetin using MTT assay. The co-treatments with test agents were carried out as described in the materials and methods section. Panel, isobologram of curcumin and quercetin treatment in A549 cells; Panel B, isobologram of curcumin and quercetin treatment in A375 cells. Additive, synergestic and antagonistic effects derived from isobologram are schematically shown. In both cell lines, curcumin and quercetin combination showed synergism.

Figure 5. Srivastava

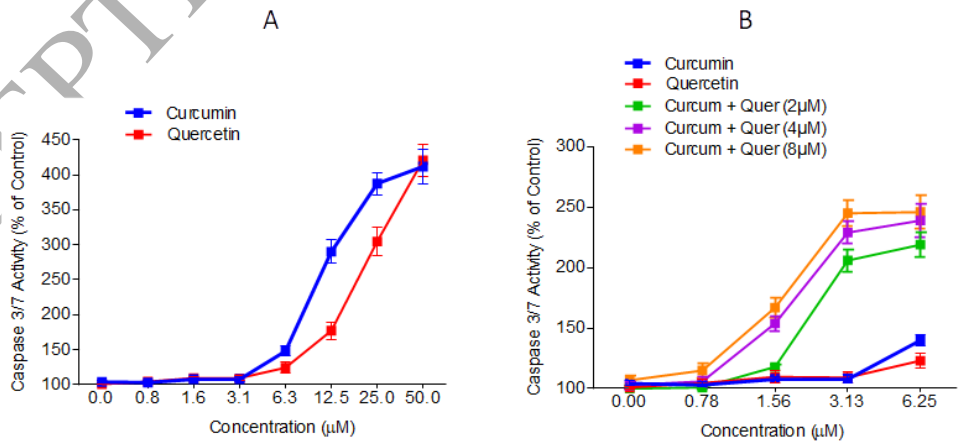


Figure 5

Effect of Curcumin and Quercetin co-treatment on Caspase 3/7 Activity. Panel A, Effect on Caspase 3/7 activity in melanoma cancer cells (A375) treated with quercetin or curcumin; Panel B, Effect on Caspase 3/7 activity in melanoma cancer cells (A375) treated with quercetin or curcumin alone or in combination. 2 μM concentration of curcumin and 3 different concentrations (2, 4, 8 μM) of quercetin were used; Panel C, Bar graph of data shown in Panel C for comparison with bar graph in Panel D; Panel D, Bar graph of combination effect of curcumin and quercetin on caspase 3/7 activity in A375 cells treated with 2 μM each of curcumin and quercetin.

Figure 6. Srivastava

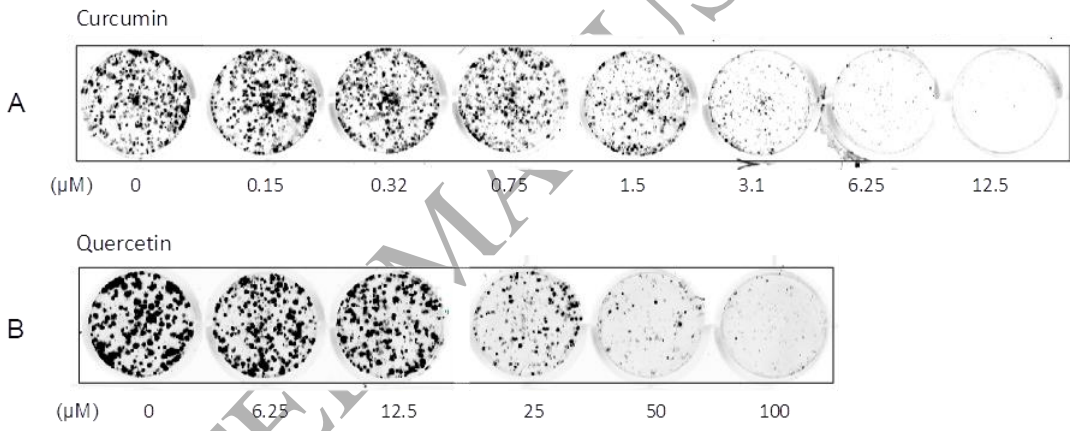


Figure 6

Inhibition of Proliferation of Melanoma Cells (A375) by Curcumin and Quercetin in Colony Growth Proliferation Assay. Panel A, Concentration effect of curcumin on proliferation of A375 cells; Panel B, Concentration effect of quercetin on proliferation of A375 cells.

Figure 7. Srivastava

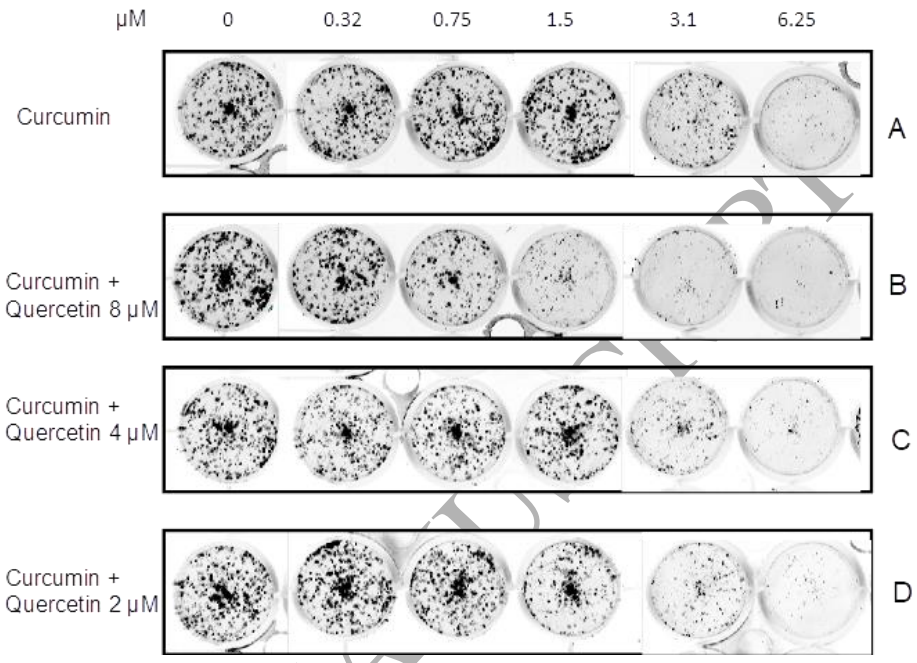


Figure 7

Combination Effect of Curcumin and Quercetin on Inhibition of Proliferation of Melanoma Cells (A375) in Colony Growth Proliferation (CGP) Assay. Panel A, Concentration effect of curcumin (2 μM) alone on inhibition of proliferation of melanoma cells (A375) in CGP Assay; Panel B, Quercetin 8 μM with varying concentration of curcumin; Panel C, Quercetin 4 μM with varying concentration of curcumin; Panel D, Quercetin 2 μM with varying concentration of curcumin.

Figure 8. Srivastava

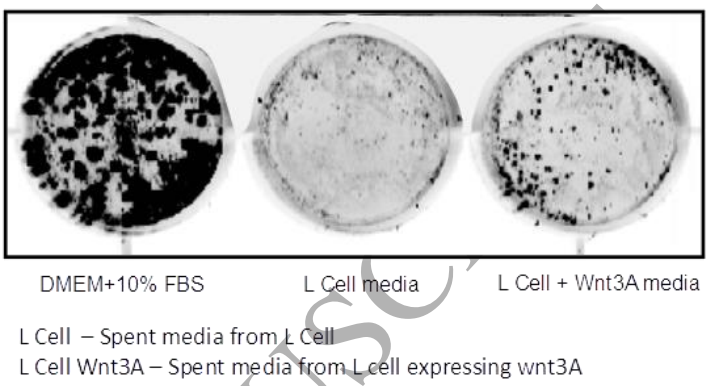


Figure 8

Effect of wnt3A on Growth of Melanoma Cell Line, A375.

Left Panel, Growth of L cells overexpressing Wnt3A protein; Middle Panel, Spent media from L cell without Wnt3A expression did not induce A375 growth; Right Panel, Spent media from L cell overexpressing Wnt3A expression induced A375 growth.

Figure 9. Srivastava

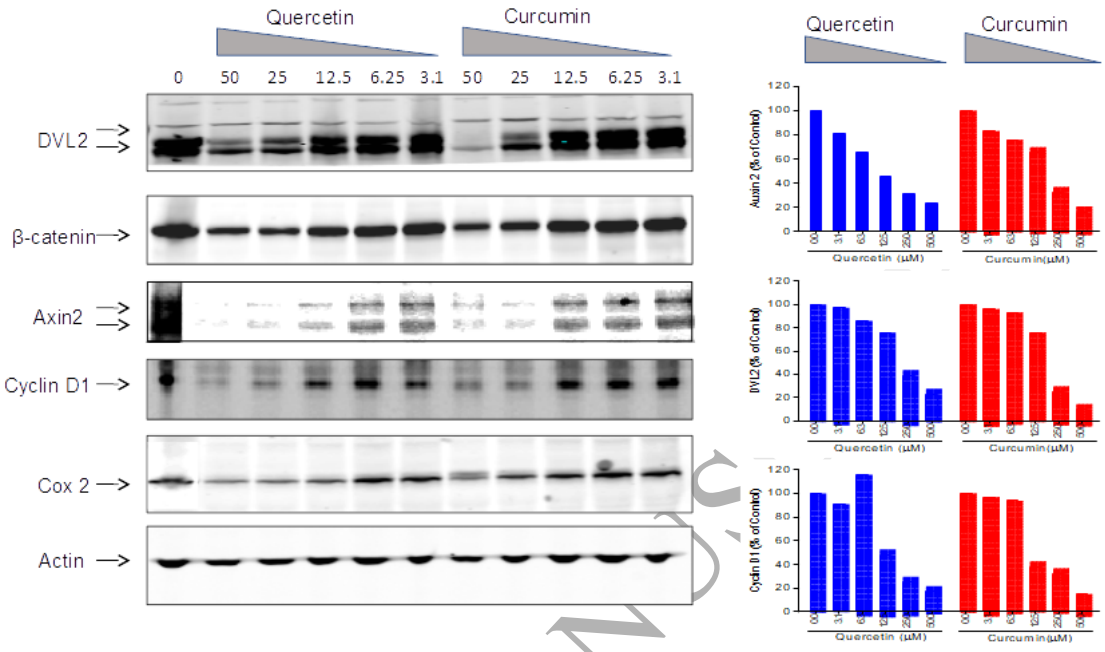


Figure 9

Effect of curcumin and quercetin on wnt signaling pathway proteins.

A375 melanoma cancer cells were treated with indicated concentrations (3.1 to 50 µM) of curcumin and quercetin for 24 hours followed by harvesting the cells and preparing whole cell extract. Total cell protein (30-50 µg) was mixed 1:1 with sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris– HCl, pH 6.8) loaded onto a 10% SDS–PAGE gel, and electrophoresis performed. Separated proteins in the gel were transferred to nitrocellulose membrane and incubated with the primary antibodies of indicated proteins followed by HRP-conjugated anti-IgG antibody and detected by ECL. Equal loading of the protein on the blots was evaluated using Bradford assay as well as ß-actin band. Left panel shows detected protein bands and the right panel shows plotting of the intensity of the scanned proteins shown on left panel.

Figure 10. Srivastava

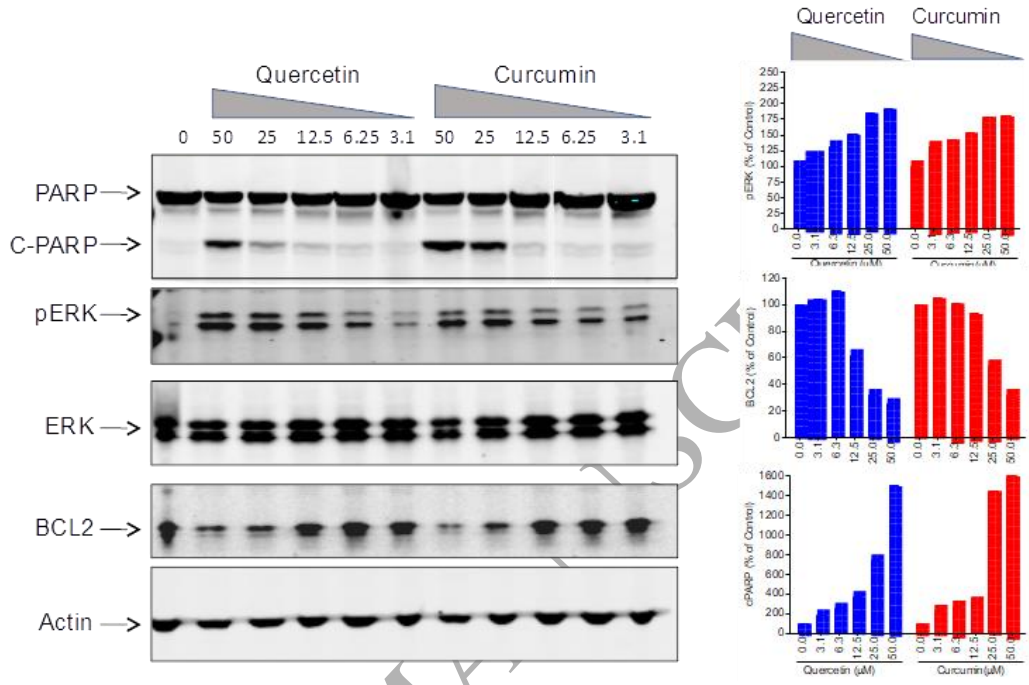


Figure 10

Effect of curcumin and quercetin on apoptotic pathway proteins.

Cells were treated and processed as described in Figure 9. Transferred proteins on the nitrocellulaose membrane were identified using appropriate antibodies against PARP, c-PARP, ERK, pERK, BCL2, and internal standard actin. Left panel shows detected protein bands and the right panel shows plotting of the intensity of the scanned proteins shown on left panel.

Figure 11. Srivastava

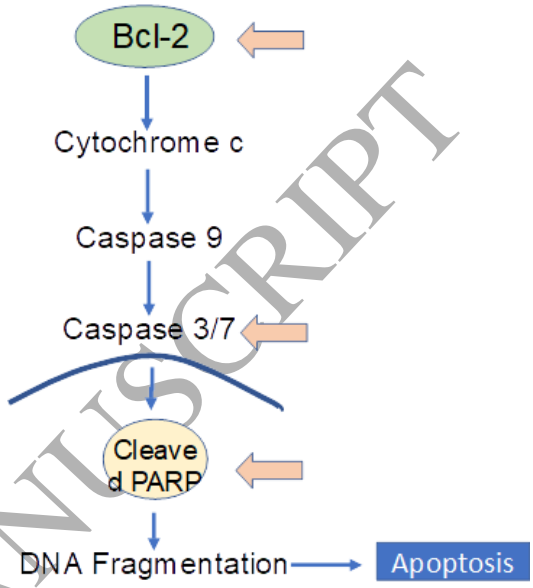


Figure 11.

Curcumin and quercetin activate players in the apoptotic pathway. As shown, curcumin and quercetin inhibit Bcl2 expression, which in turn modulate downstream players in the apoptotic pathway shown by orange arrow. Additionally, quercetin, but not curcumin inhibit ERK1/2 phosphorylation to activate caspase 3/7.

Figure 12. Srivastava

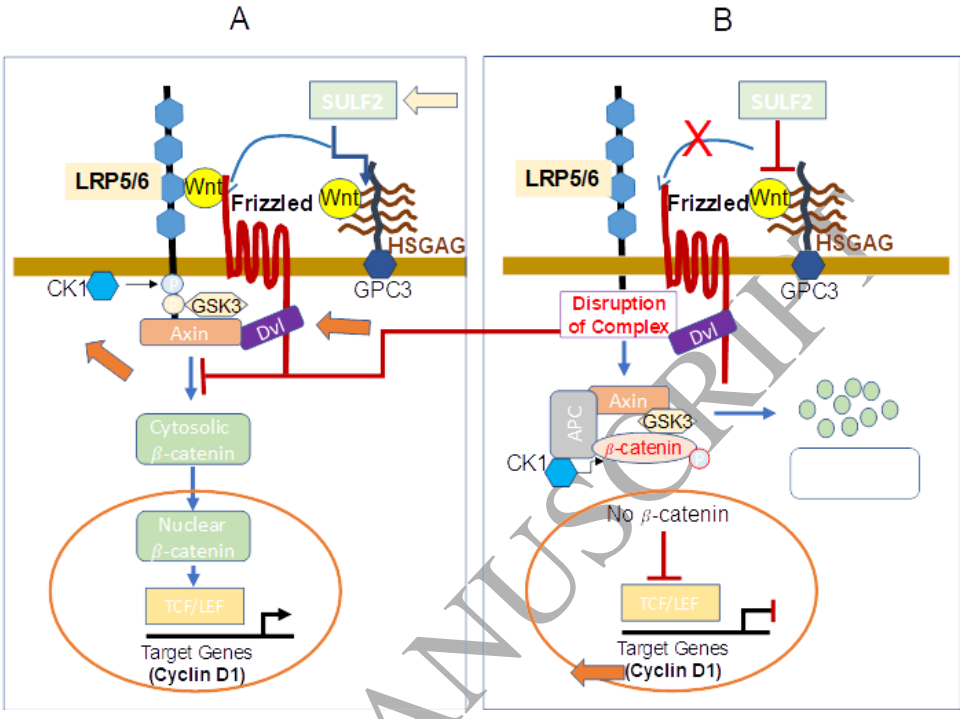
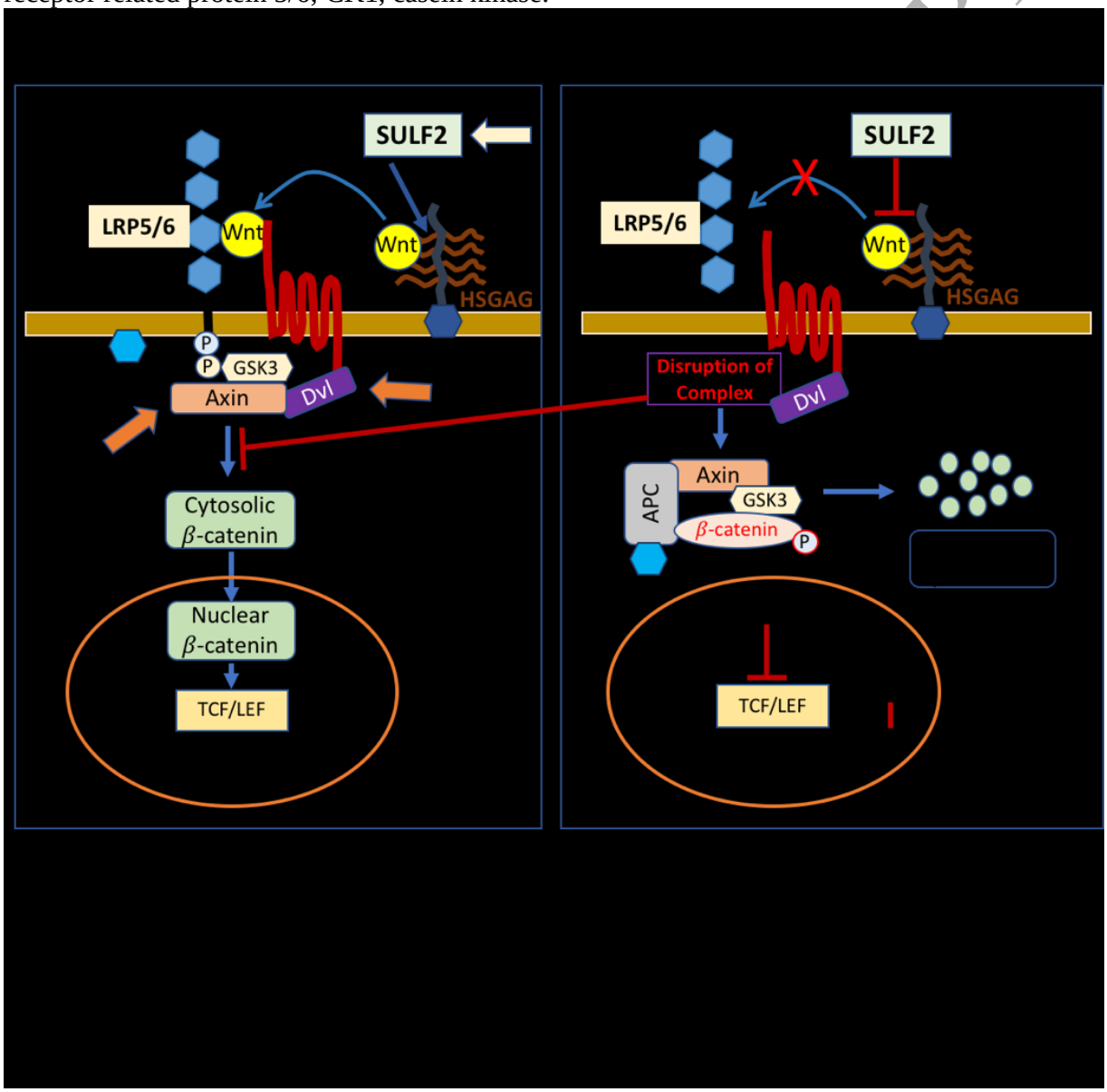


Figure 12.

Curcumin/ Quercetin Mediated Modulation of Wnt/ β-catenin Signaling Pathway. Panel A: Wnt/β-catenin Signaling Pathway and Sites of Curcumin/quercetin Action. As shown, in the presence of Wnt ligand, Wnt pathway gets activated though binding of Wnt to transmembrane Frizzled (Fz) receptor, resulting the formation of a complex (Wnt-Fz-LRP6) with LRP5/6 that triggers recruitment of the scaffolding protein Dishevelled (Dvl) resulting phosphorylation of LRP5/6 and recruitment of Axin to the receptors. These events disrupt Axin-mediated phosphorylation/degradation of β-catenin. As a result, cytoplasmic β-catenin rises and travels to the nucleus to serve as TCF/LEF co-activator and induces Wnt responsive genes. The possible sites of action of curcumin and quercetin are shown by bold orange arrows. Down-regulation of SULF2 enables Wnt to remain bound with the membrane heparin sulphate proteoglycan and turning on β-catenin degradation pathway shown in Panel B. Curcumin/Quercetin possibly also destabilizes the Wnt-Fz-LRP6 complex via down-regulation of Dvl2 and Axin resulting in the repression of Wnt responsive gene cyclin D1 and downstream players like Cox2. Panel B. In the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin, APC, GSK3 and CK1. CK1

and GSK3 sequentially phosphorylate β-catenin to facilitate β-catenin ubiquitination and proteasomal degradation. This prevents β-catenin from reaching the nucleus, and repressing Wnt target genes.

HSGAG, Heparan sulfate glycosaminoglycan; Dvl, deshevelled; TCF/LEF, T-cell factor/lymphoid enhancer factor; GPC3, Glypican-3- a member of the glypican-related integral membrane heparan sulfate proteoglycans; APC, Adenomatous polyposis coli; LRP5/6, LDL receptor related protein 5/6; CK1, casein kinase.



Graphical Abstract

Table-1

Test Agent	**A549 IC_{50} (µM)**	**HCT116 IC_{50} (µM)**	**A375 IC_{50} (µM)**	**MCF7 IC_{50} (µM)**
Curcumin	4.8	9.8	8.5	18
Quercetin	11.8	25	50	45
Curcumin + 2 µM Quercetin	2.0	3.1	4.8	16
Curcumin + 4 µM Quercetin	0.7	3.3	3.0	7.5
Curcumin + 8 µM Quercetin	<0.5	1.5	1.5	3.1
Doxorubicin	0.76	[1]ND	1.15	[1]ND

Table- 2

Single

IC30		IC50		IC70	
CUR (µM)	QUER (µM)	CUR (µM)	QUER (µM)	CUR (µM)	QUER (µM)
0	11	0	16	0	27
4.5	0	6	0	9.5	0

Combination

COMBINATION I (QUER + CUR 2 µM)				COMBINATION II (CUR + QUER 3 µM)			
IC	CUR. (µM)	QUER (µM)	Combination Index (CI)	IC	CUR (µM)	QUER (µM)	Combination Index (CI)
30	2	1.2	0.448	30	0.3	4	0.916
50	2	2.7	0.575	50	0.8	4	0.717
70	2	3.5	0.442	70	1.5	4	0.477

CUR, curcumin; Quer, quercetin
CI=COMBINATION INDEX; CI>1 Antagonism; CI=1 Additive effect; CI<1 synergism