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Comparative study of antitumor effects of bromelain and papain in human cholangiocarcinoma cell lines

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1 **Abstract.** Cholangiocarcinoma (CC) worldwide is the most
2 common biliary malignancy with poor prognostic value and
3 new systemic treatments are desirable. Plant extracts like
4 bromelain and papain, which are cysteine proteases from
5 the fruit pineapple and papaya, are known to have antitumor
6 activities. Therefore, in this study for the first time we
7 investigated the anticancer effect of bromelain and papain
8 in intra- and extrahepatic human CC cell lines. The effect of
9 bromelain, and papain on human CC cell growth, migration,
10 invasion and epithelial plasticity was analyzed using cell
11 proliferation, wound healing, invasion and apoptosis assay,
12 as well as western blotting. Bromelain and papain lead to a
13 decrease in the proliferation, invasion and migration of CC
14 cells. Both plant extracts inhibited NFκB/AMPK signalling as
15 well as their downstream signalling proteins such as p-AKT,
16 p-ERK, p-Stat3. Additionally, MMP9 and other epithelial-
17 mesenchymal-transition markers were partially found to be
18 downregulated. Apoptosis was induced after bromelain and
19 papain treatment. Interestingly, bromelain showed an overall
20 more effective inhibition of CC as compared to papain. siRNA
21 mediated silencing of NFκB on CC cells indicated that brome-
22 lain and papain have cytotoxic effects on human CC cell lines
23 and bromelain and partially papain in comparison impair
24 tumor growth by NFκB/AMPK signalling. Especially brome-
25 lain can evolve as promising, potential therapeutic option that
26 might open new insights for the treatment of human CC.
27

Introduction

Cholangiocarcinoma (CC) is a primary liver tumor arising from the epithelial cells lining the intra- and extrahepatic biliary tract system (1,2). The incidence and mortality rate of CC are increasing worldwide and they represent the second most common primary hepatobiliary cancer (3,4). Surgery is the only curative therapy, but CC is often diagnosed in advanced tumor stage (4,5). Currently, the combination of gemcitabine and cisplatin is the standard chemotherapy for patients undergoing first line palliative treatment (6). Many cytotoxic compounds failed to improve therapy and new anti-tumor treatments are urgently needed to improve survival.

Bromelain is an extract of pineapple (*Ananas comosus*) and contains a mixture of proteases and non-protease components (7). Bromelain is sold in health food stores as a nutritional supplement to promote digestion, wound healing and as an anti-inflammatory agent (8). Oral treatment is well tolerated and there is traditional and anecdotal evidence that bromelain has different properties (7-10). Obviously, bromelain was tested in different experimental and clinical studies and it was demonstrated that this plant food has anticancer and anti-inflammatory activities (7,10-26). However, the precise molecular mechanisms are not fully understood.

Papain is a sulfhydryl protease from *Carica papaya* latex with a powerful digestive action superior to pancreatin, or pancreatic enzymes (27). Papain has high concentration in the fruit, stem, leaves, fruit skin and seeds. The compound has toxic effects on plant-eating bugs, supported wound healing and the juice of ripe papaya shows anti-oxidant effect (28-30). Papain is a known ingredient of herbal medicine in different countries, but there is very limited information on the molecular targets and anticancer effects. Ground-breaking studies for both compounds as anticancer therapies for CC are lacking so far. Using a model of human CC cell lines, we investigated for the first time the antitumor activity of both bromelain and papain on CC.

Materials and methods

Cell culture. Human CC cell lines (TFK-1, SZ-1) were generously provided by Nisar Malek (31). Cell lines were cultured in RPMI-1640 + Glutamax (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Biocrom, Berlin, Germany)

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Abbreviations: CC, cholangiocarcinoma; EMT, epithelial-mesenchymal transition

Key words: antitumor therapy, bromelain, cholangiocarcinoma, papain

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1 and 100 U/ml penicillin/streptomycin (Invitrogen) at 37°C in
2 5% CO₂.

3
4 *Compound preparation and in vitro treatment.* Bromelain
5 (Sigma-Aldrich, Germany - 25 mg, 100 mM) stock solutions
6 were prepared by dissolving them in dimethyl sulfoxide, DMSO
7 (Applichem, Darmstadt, Germany). Papain (Sigma-Aldrich,
8 100 mM) stock solutions were prepared by dissolving them in
9 distilled water. Cells were treated with DMSO or bromelain
10 (150 and 200 μM) or papain (25, 40 and 100 μM) in different
11 concentrations (100 mM) and were analyzed after 24, 48 and
12 96 h.

13
14 *Proliferation assay.* In order to measure the effect of brome-
15 lain and papain on cell proliferation, cells were plated at a
16 concentration of 2,000 cells/ml in a 96-well plate overnight.
17 Cells were treated with DMSO, different concentrations of
18 bromelain (150 and 200 μM) or papain (25, 40 and 100 μM) for
19 different time-points (1-4 days). At the respective time-points,
20 10 μl WST-1 reagent (Roche Diagnostics, Mannheim,
21 Germany) was added to each well and incubated for 2 h at
22 37°C. The absorbance was detected at a wavelength of 492 nm
23 with reference wavelength of 650 nm.

24
25 *Invasion assay.* Cells (2.5x10⁵ cells/2 ml) were seeded in
26 serum-free media into each well of the 6-well BD BioCoat™
27 Matrigel™ Invasion Chamber (BD Biosciences, Bedford,
28 UK). The cells in the inserts were simultaneously treated
29 with bromelain (150 and 200 μM) or papain (25, 40 and
30 100 μM) and the DMSO control. The inserts were placed
31 into the BD Falcon TC Companion Plate containing 10%
32 FCS and incubated for 48 h in a humidified tissue culture
33 incubator, at 37°C, 5% CO₂ atmosphere. Then the invading
34 cells were fixed with 100% methanol and stained with 1%
35 toluidine blue in 1% borax. Cells were then counted under a
36 microscope (Leica DM 5000 B, Leica, Wetzlar, Germany).
37 The calculation of the invading cells were done according to
38 the BD protocol where:

$$\text{Invasion index} = \frac{\% \text{ invasion test cell}}{\% \text{ invasion control cell}}$$

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43 *Migration assay.* Cells were seeded in a 6-well plate and left to
44 reach 80% confluency. Initially, cells were starved for 24 h in
45 media containing 2% FCS. Then SZ-1 and TFK-1 were further
46 incubated for 48 h in the starvation media containing either
47 the control with DMSO, different concentrations of bromelain
48 (150 and 200 μM) or papain (25, 40 and 100 μM). Afterwards
49 a scratch was done using a white tip for each treatment.
50 Then cells were washed with PBS and photographed using
51 Leica DMI 6000 B microscope (Leica, Wetzlar, Germany).
52 Cells were incubated for an additional 24 h after which the
53 images were taken of the wounded area. The migrating cells
54 were calculated according to the following formula:

$$\text{Migration index} = \frac{\text{Width of the wound at 0 h} - \text{width of the wound at 24 h}}{\text{Width of the wound at time 0 h}} \times 100$$

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59 *Protein extraction and western blotting.* SZ-1 and TFK-1 cells
60 cultured with bromelain or papain treatment for immuno-

61 blots were collected and rinsed with cold phosphate-buffered
62 saline (PBS). Then harvested cells were lysed in lysis buffer
63 containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM
64 EGTA, 1% Triton X-100 and protease and phosphatase inhib-
65 itor (Protease Inhibitor Cocktail Tablets, Roche, Mannheim,
66 Germany). The concentration of extracted protein was
67 determined using DC protein assay kit (Bio-Rad, München,
68 Germany) according to the manufacturer's instructions. The
69 absorption was measured at 650-750 nm using a microplate
70 reader (Titertek-Berthold, Pforzheim, Germany). For immune
71 blotting the cell lysates were loaded at a protein concentra-
72 tion of 30 μg per well. Gel electrophoresis (12% acrylamide
73 gels) was performed (Bio-Rad, München, Germany). The
74 membranes were blocked using 5% dried milk (Applichem)
75 for 30 min at room temperature, then they were probed with
76 primary antibodies against E-cadherin (1:1,000, Cell Signaling
77 Technology, 24E10), N-cadherin (2:10,000; Millipore,
78 EPR1792Y), actin (2:10,000; Sigma, AC-74), Akt (1:1,000;
79 Cell Signaling Technology, 4691), p-Akt (Ser473) (1:1,000;
80 Cell Signaling Technology, 9271s), STAT3 (1:1,000; Cell
81 Signaling Technology, 9132), p-Stat3 (Tyr705) (1:1,000; Cell
82 Signaling Technology, 9131s), Rac-1 (1:000; Cytoskeleton, Inc.,
83 ARC03-A), PARP (1:1,000; Cell Signaling Technology, 9542),
84 p44/p42 MAPK (Erk1/2) (1:1,000; Cell Signaling Technology,
85 4685s), phospho-p44/p42 MAPK (Erk1/2) (1:1,000; Cell
86 Signaling Technology, 9101), AMPKα (D5A2) (1:500, Cell
87 Signaling Technology, 5831S), phospho-AMPKα (1:500, Cell
88 Signaling Technology, 2535S), NFκB p65 (1:1,000; Abcam,
89 ab16502).

90
91 *Small interfering RNA (siRNA) knockdown of NFκB.* SZ-1 and
92 TFK-1 cells were plated at a concentration of 1x10⁴ cells/ml in
93 a 6-well plate. After 24 h, cells were transfected with control
94 siRNA-A (Santa Cruz Biotechnology, CA, USA) 5, 10 and
95 20 nM NFκB siRNA (Santa Cruz Biotechnology, sc-29410)
96 using hiperfect transfection reagent (Qiagen, Germany) for
97 48 h. Trial experiments were performed with different concen-
98 trations of siRNA oligonucleotides at different time-points.
99 The transfection efficiency was judged based on western blot
100 analysis of NFκB.

101
102 *Apoptosis.* To determine the apoptosis, SZ-1 and TFK-1
103 cells were seeded (1x10⁴/ml) in 6-well plate and were further
104 treated under the same conditions described for WST-1 assay.
105 After the respective treatments, floating cells were collected
106 and adherent cells were trypsinized, washed twice with
107 ice-cold PBS. The cells were then resuspended in 1 ml of
108 1X binding buffer and were stained with Annexin V-FITC
109 and PI according to the manufacturer's instructions using
110 Annexin V Apoptosis Detection Kit II (BD Biosciences,
111 San Diego, CA, USA). The signal was detected using
112 LSRFortessa flow cytometer (Becton-Dickinson, USA) and
113 analyzed using FlowJo Version 8.7 software (Tree Star Inc.,
114 Ashland, OR, USA).

115
116 *Statistical analysis.* All the experiments were repeated
117 3 times. The results were analyzed using software Graphpad
118 prism version 5.0 (GraphPad Software, San Diego, CA, USA)
119 and SPSS Version 11.0 (SPSS, Chicago, IL, USA). The tests
120 include one way ANNOVA analysis of variance and Student's

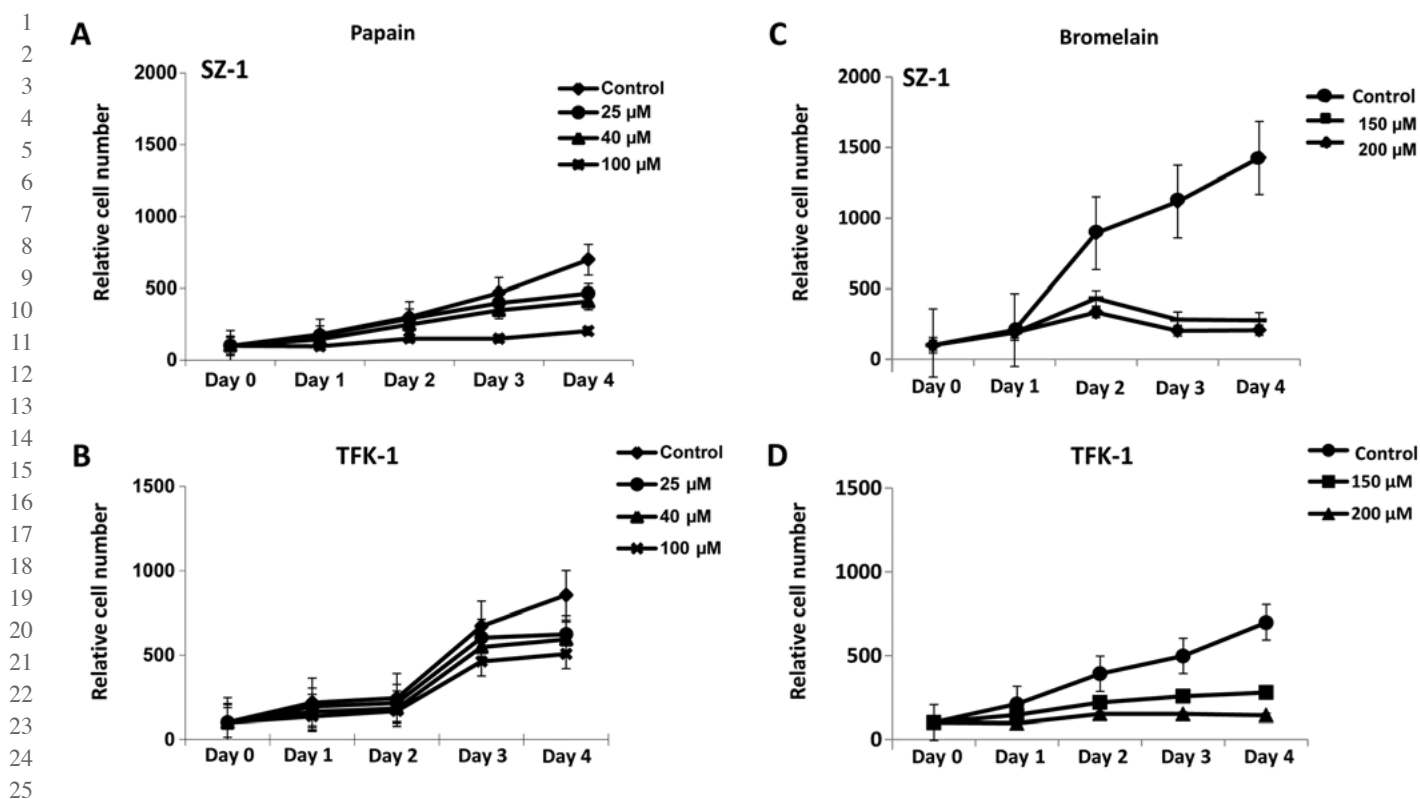


Figure 1. Effect of bromelain and papain on CC cell proliferation. SZ-1 (A and C) and TFK-1 (B and D) cells were treated by bromelain (150 and 200 μ M), papain (25, 40 and 100 μ M), and their controls (DMSO, H₂O) as indicated. Cell proliferation was measured by WST assay. Both plant extracts inhibited cell proliferation in a dose- and time-dependent manner compared to their control.

t-test along with Bonferroni post test and paired and unpaired t-tests. Differences were considered as statistically significant when the P-value was <0.05, <0.005 and <0.001; and not significant (ns).

Results

Bromelain and papain attenuates the viability of CC cells.

To analyze the anti-proliferative potential of bromelain and papain on TFK-1 and SZ-1 cells we determined first the IC₅₀ value for papain by constructing a dose-response growth curve and examined the effect of different concentrations of papain on CC cells. Taking into account the calculated IC₅₀, papain was used in three concentrations: 25, 40 and 100 μ M (Fig. 1A and B). For bromelain therapy the previously published IC₅₀ values were taken into account and dosage of 150 and 200 μ M were used (25,26) (Fig. 1C and D). As shown (Fig. 1) both bromelain and papain treatment effectively decreases the proliferation of viable CC cells for 24, 48, 72 and 96 h. The highest anti-proliferative effect for both bromelain and papain was observed at 96 h under highest dosages (200 and 100 μ M for bromelain and papain, respectively).

Bromelain and papain treatment inhibits migration and invasion of CC cells.

We next examined the effect of bromelain (150 and 200 μ M) and papain (25, 40 and 100 μ M) on cell motility by wound healing assays (Fig. 2). All treatments and experiments were conducted as described in Materials and methods. Papain treatment (25, 40 and 100 μ M) showed considerable inhibition of wound healing especially in TFK-1

cells in comparison to SZ-1 cells (Fig. 2A-C). The highest inhibition effect was observed by papain in TFK-1 cells under 100 μ M treatment. Effective ($p < 0.05$) inhibition of wound healing was observed under 150 and 200 μ M bromelain in both CC cells as compared to the DMSO controls (Fig. 2D-F). Next, we tested cell invasion using Matrigel-coated Transwell chambers under DMSO and bromelain (150 and 200 μ M) and papain (25, 40 and 100 μ M) (Fig. 3). As shown (Fig. 3B, D and F), bromelain inhibited significantly cell invasion in a dose-dependent manner. Approximately 90% decrease in the number of invading cells was observed compared to the control group. Papain also showed similar effective inhibition of invasion in both CC cells compared to the control (Fig. 3A, C and E).

Bromelain and papain treatment induces apoptosis in CC cells.

In order to elucidate that the anti-proliferative effect of both compounds (bromelain and papain) the induction of apoptosis, Annexin V/PI staining was performed and the percentage of apoptotic cells was determined by FACS analysis (Fig. 4). The results clearly showed that bromelain treatment induces relatively high rate of apoptosis on TFK-1 and SZ-1 cells in a time- and dose-dependent manner (Fig. 4C and D). The maximum induction was observed for bromelain for both CC cell lines at 96 h under 200- μ M treatment reaching 65-70% (Fig. 4C and D). Whereas for papain a similar pattern was observed, but the rate of induction was slightly lower compared to bromelain being ~45-50% for both CC cell lines under 100 μ M of papain treatment at 96 h (Fig. 4A and B). We also showed a time-dependent increase of the protein poly

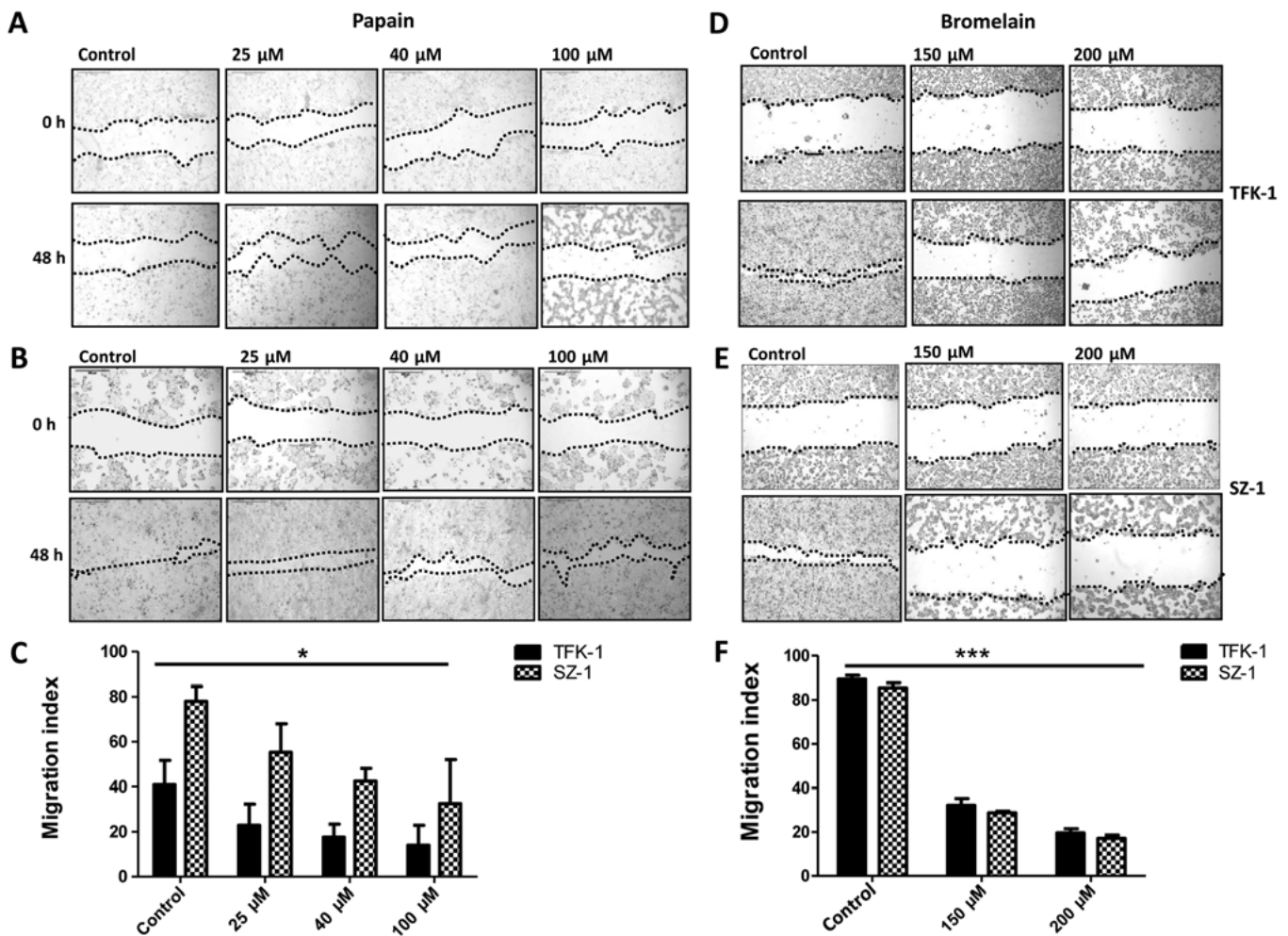


Figure 2. Bromelain and papain inhibits migration of CC cells. SZ-1 (B and E) and TFK-1 (A and D) cells were cultured with bromelain (150 and 200 μ M), papain (25, 40 and 100 μ M), or their controls (DMSO, H₂O). A scratch was made at (time 0 h) in both SZ1 and TFK1 and maintained for 24 h in conditioned medium with bromelain, papain, or their controls (DMSO, H₂O). The dotted lines represent the edges of the wound. Images were taken under a light microscope (x10 magnification). The migration index was calculated as described in Materials and methods and plotted in bar graphs (C and F). Data are expressed as mean \pm SD of triplicates. Differences were considered as statistically significant at *P<0.05 and ***P<0.001.

(adenosine diphosphate ribose) polymerase (PARP) cleavage after bromelain and papain treatment for SZ-1 and TFK-1 cells (Fig. 4). The results are in accord with the findings of the cell proliferation assay. Thus, our results show that bromelain and papain both induced considerable amount of apoptosis on CC cells.

Bromelain effectively inhibits NF κ B/AMPK pathway and common downstream effectors in CC cells in contrast to papain. NF κ B/AMPK signaling has been implicated as an important pathway involved in the carcinogenesis of several tumor diseases (32). In order to gain more insight into the anticancer mechanism exerted by both bromelain and papain in CC cells, we checked the status of AMPK and NF κ B both before and after treatment by immunoblotting. In both cell lines (TFK-1, SZ-1) bromelain (150 and 250 μ M) effectively downregulated NF κ B and other common downstream proteins such as phospho-AKT, phospho-ERK and phospho-STAT3, but the full forms remained unchanged in both cell lines for 48 and 96 h (Fig. 5). An effective upregulation of phospho-AMPK was observed under bromelain treatment

for both time-points in a time- and dose-dependent manner (Fig. 5C and D). Thus, clearly indicating effective inhibition of the NF κ B/AMPK pathway in CC cells by bromelain. In contrast, papain treatment (25, 40 and 100 μ M) did not show effective downregulation of NF κ B in either CC cell line (Fig. 5A and B). For SZ-1 cells under papain treatment full and phosphorylated forms of ERK, STAT3 remained unchanged for all tested time-points (Fig. 5B). In contrast, AKT full form remained unchanged but the phosphorylated form showed slight downregulation in SZ-1 under 100 μ M of papain treatment specifically in 96 h (Fig. 5B). In TFK-1 cells papain treatment effectively downregulated phospho-STAT3 in all dosages (25, 40 and 100 μ M) specifically after 96 h (Fig. 5A). Full forms of other effector proteins like AKT, ERK, STAT3 remained stable under papain treatment but the phosphorylated forms were slightly downregulated mostly under 100 μ M of treatment at 96 h (Fig. 5A and B). Interestingly, NF κ B and AMPK levels remained mostly unaltered under papain treatment for both CC cell lines for all time-points compared to the respective controls. Moreover, phosphorylated AMPK showed considerable upregulation in SZ-1 cells under 100 μ M of

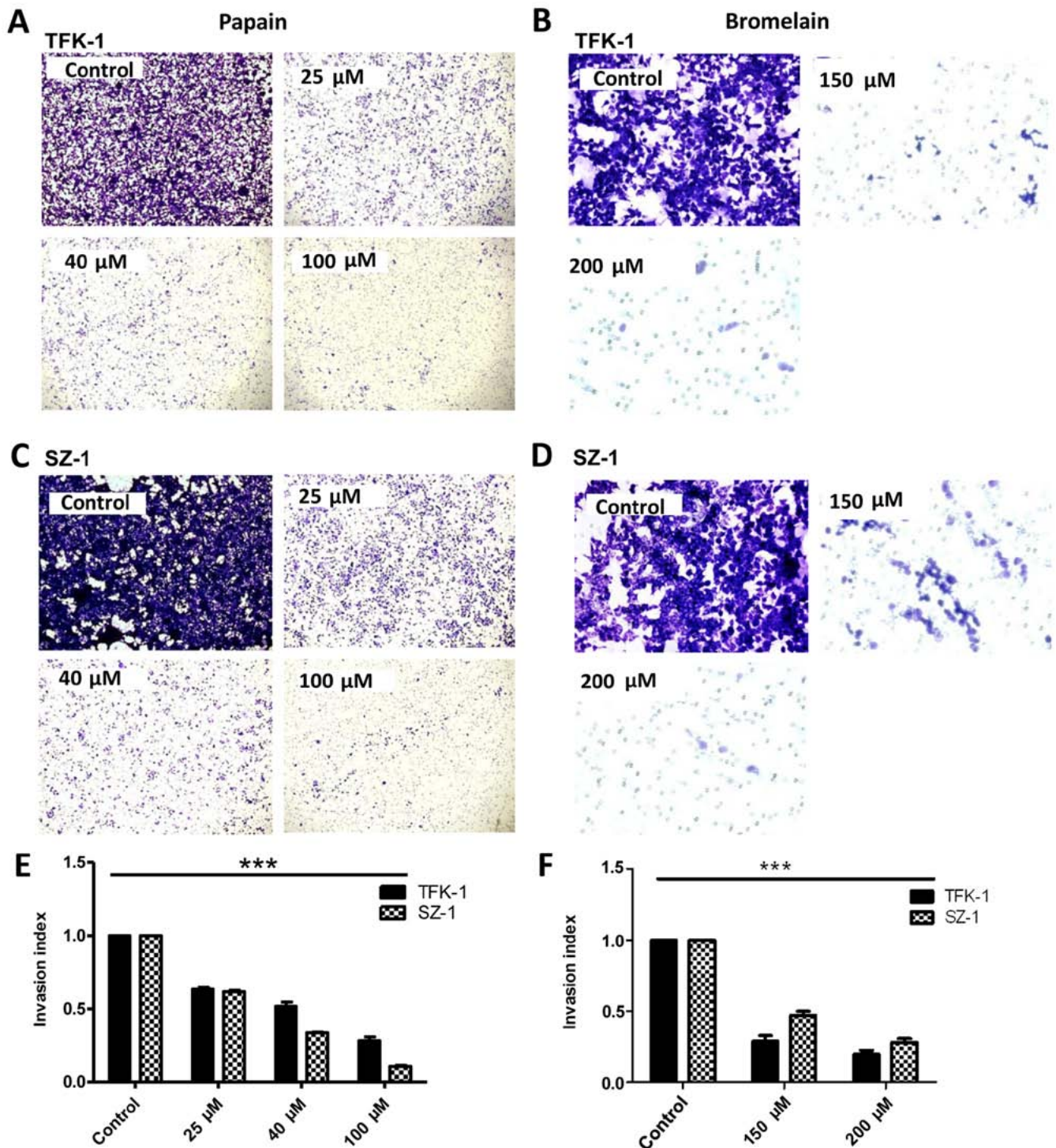


Figure 3. Bromelain and papain inhibits invasion of CC cells. SZ-1 (C-F) and TFK-1 (A, B, E and F) cells were treated for 48 h with bromelain (150 and 200 μM), papain (25, 40 and 100 μM), or their controls (DMSO, H₂O). The number of cells that invaded through the membrane was determined by light microscope (x20 magnification) counterstained and invasion index was calculated as described in Materials and methods and plotted in bar graphs. Differences were statistically significant at ***P<0.001. Data are expressed as mean \pm SD of triplicates.

papain treatment for both time-points (48 and 96 h) (Fig. 5B). In TFK-1 cells slight upregulation of phosphorylated AMPK was observed in a dose-dependent manner of papain treatment in comparison to the control (Fig. 5A). Thus, papain treatment partially influences, but does not inhibit NF κ B/AMPK signaling in contrast to bromelain in CC cells.

We further reconfirmed the importance of NF κ B status for bromelain anticancer activity in CC cells by NF κ B siRNA.

NF κ B silencing in both TFK-1 and SZ-1 cells showed effective inhibition of all the common downstream proteins, such as AKT and ERK, as shown in Fig. 6A and B. Importantly, 200 μM of bromelain treatment achieved similar results of effective inhibition of all downstream effector proteins as that with NF κ B siRNA for both tested CC cells. However, 100 μM of papain treatment did not show effective inhibition of any of the above mentioned common downstream effector proteins

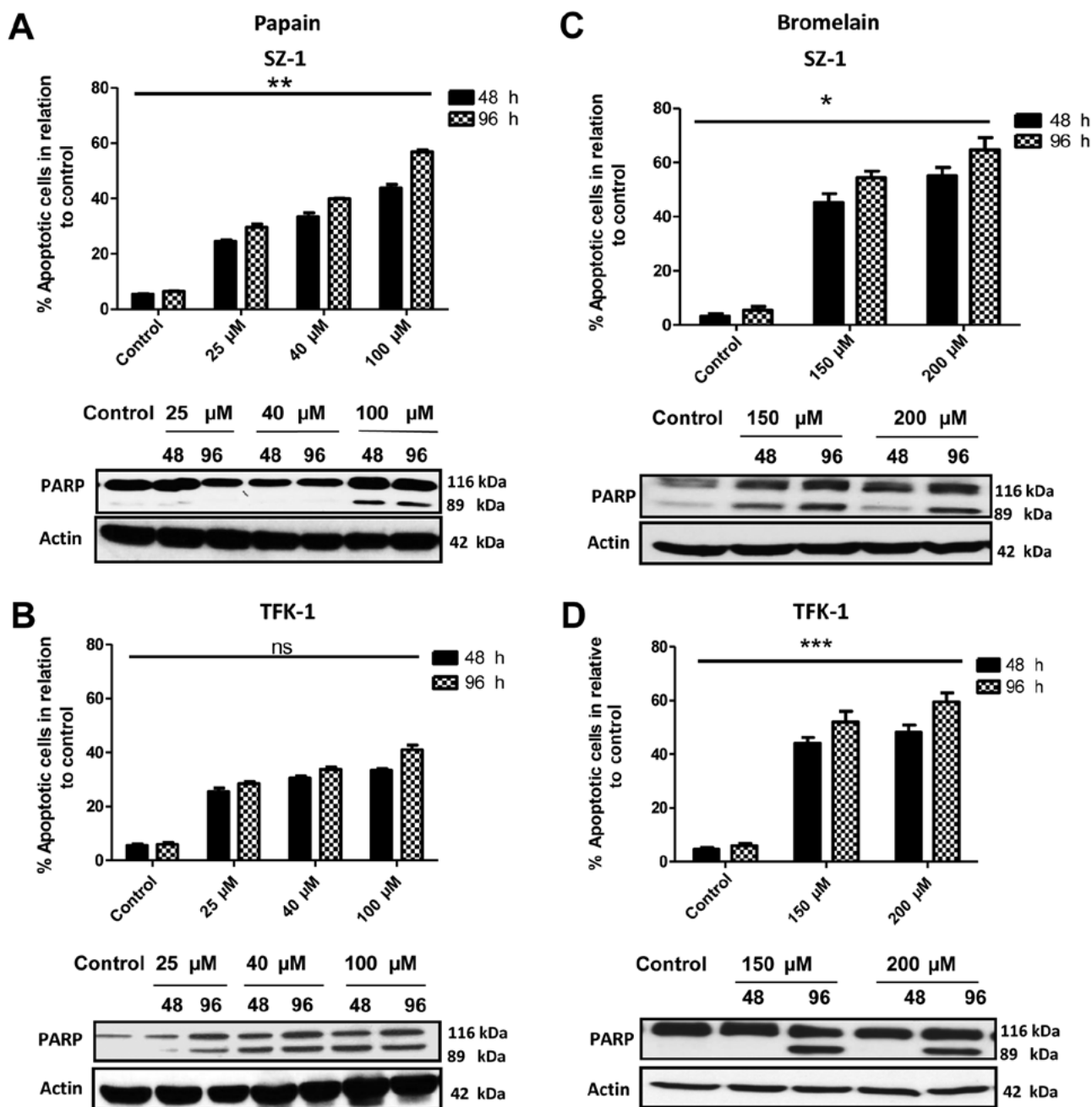


Figure 4. Apoptosis is induced by bromelain and papain. Annexin V staining detected the percentage of apoptotic cells after 48- and 96-h exposure to indicated concentrations of bromelain, papain or their controls of SZ-1 (A and C) and TFK-1 (B and D) cells. Differences were considered as statistically significant at * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$; not significant (ns). Data are expressed as mean \pm SD of triplicates. Western blot analysis of PARP cleavage in treated SZ-1 (A and C) and TFK-1 (B and D) cells. The upper bands represent the full-length PARP (116 kDa); the lower bands represent the cleaved PARP product (89 kDa). Actin was used as a loading control.

in contrast to NF κ B silencing compared to the controls for both CC cell lines (Fig. 6). Based on these results bromelain effectively impairs CC carcinogenesis via inhibition of NF κ B/AMPK signaling, but not papain.

Bromelain significantly impairs EMT in comparison to papain in CC cells. EMT is considered a key process driving invasion and metastasis. Therefore, we examined whether bromelain or papain can attenuate EMT. CC cells were treated with either bromelain (150 and 250 μ M), papain (25, 40 and 100 μ M) or DMSO for 48 and 96 h. The expression of EMT

markers (E-cadherin and N-cadherin) was evaluated by western blotting (Fig. 7). Bromelain treatment showed dose- and time-dependent increase in expression of the epithelial marker E-cadherin (Fig. 7B and D) and downregulation of the mesenchymal marker N-cadherin both in TFK-1 and SZ-1 cells (Fig. 7B and D). Notably, the expression of both E- and N-cadherin remained relatively unaltered under papain treatments (25, 40 and 100 μ M) in both CC cell lines for 48 and 96 h (Fig. 7A and C). Our immunoblots clearly show the effective inhibition of EMT by bromelain in contrast to papain, which shows no influence on EMT in human CC cells.

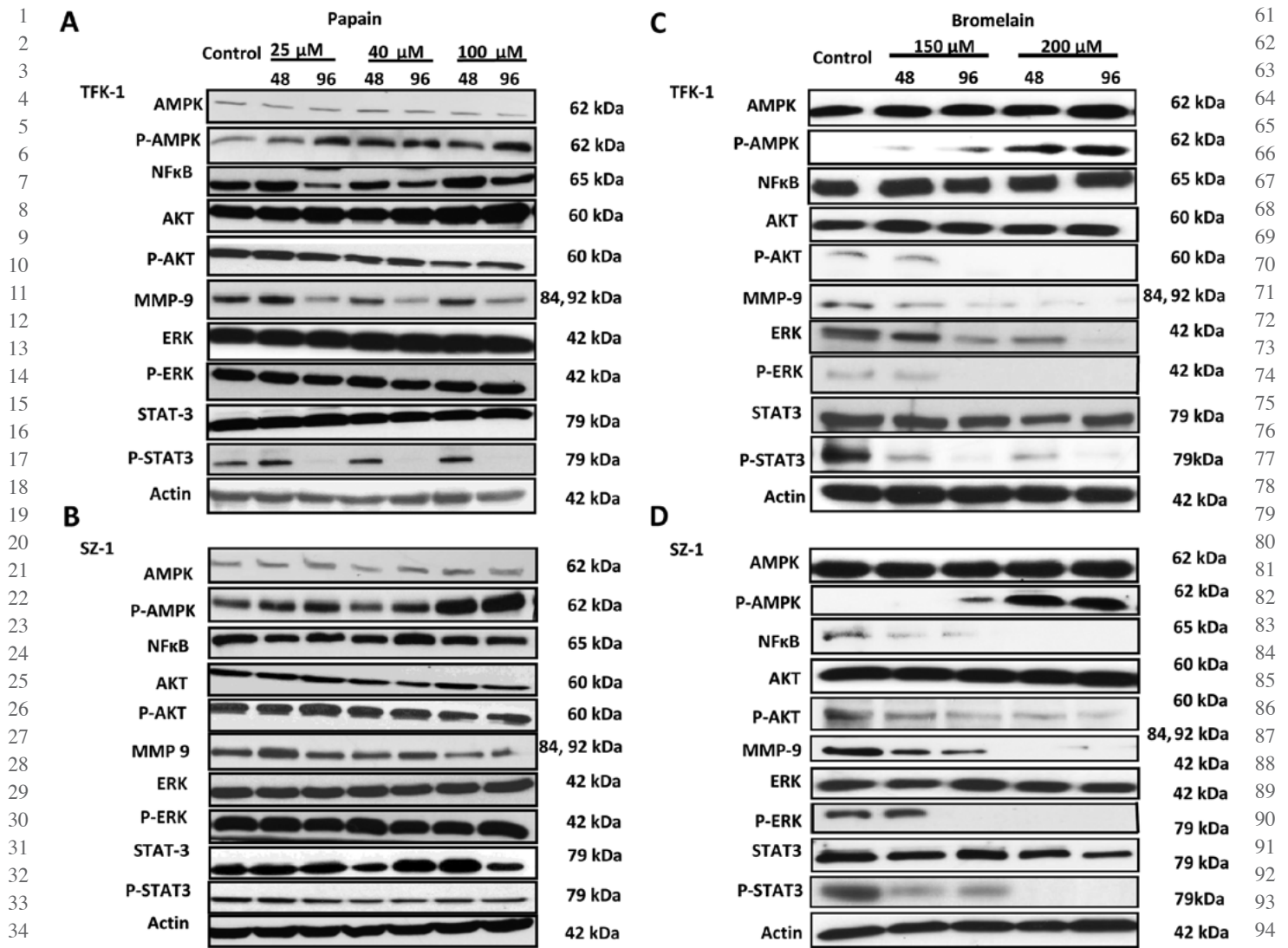


Figure 5. Impact of bromelain and papain on NF κ B/AMPK signaling and their downstream targets. SZ-1 and TFK-1 cells were treated either with bromelain (C and D), papain (A and B) or their controls (DMSO, H₂O) with indicated concentrations (25, 40, 100, 150 and 200 μ M) for 48 and 96 h and cell lysates were analyzed by western blot analysis with antibodies against phosphorylated AMPK, AMPK, NF κ B, phosphorylated AKT, AKT, phosphorylated ERK, ERK, phosphorylated STAT3 and STAT3. Actin was used as loading control. Note, papain application did not fully inhibit NF κ B/AMPK signaling compared to the plant extract bromelain.

Discussion

Plant derived extracts such as bromelain and papain have been used for herbal medicine and are easy to acquire. However, the exact molecular modes of actions and therapeutic effects are not fully understood. So far, no trials have been conducted to test the efficacy of bromelain and papain in human CC. We hypothesized that both compounds might impair CC carcinogenesis and investigated their efficacy in inhibiting growth and proliferation of human CC cell lines (TFK-1, SZ-1). In this study, we found that bromelain and papain have potent anti-tumor activity against CC. We observed four major findings: i) anticancer effect of bromelain was more effective compared to papain, ii) significant restriction of CC cells was mainly achieved via inhibition of NF κ B/AMPK pathway iii) therapy induced apoptosis, iv) EMT was only partially influenced. Importantly, in our experiments we used two human CC cell lines from different origins: TFK-1 cultured from an extrahe-

patic primary tumor and SZ-1 cultured from an intrahepatic primary tumor (31).

Bromelain was tested *in vitro* and *in vivo* cancer models and it was demonstrated in a skin papilloma model that bromelain application reduced tumor formation by apoptotic cell death (33). It was also reported that bromelain influences metastasis, local tumor growth, cell growth and migration (16-19). Furthermore, it is known that bromelain can down-regulate NF κ B and Cox-regression in papillomas and skin cancer (34). For papain it is known that anti-angiogenic effects are accessible (35). In addition, papain has anti-inflammatory, anti-infectious and anti-diabetic characteristics (36). We found that both plant extracts significantly suppressed CC growth, influencing cell migration and invasion. Since bromelain was also previously tested for anticancer ability in other tumor models as described above we selected two widely used and established dosages (150 and 200 μ M) (25,26). Our experiments on CC cells also reconfirmed these two pre-established

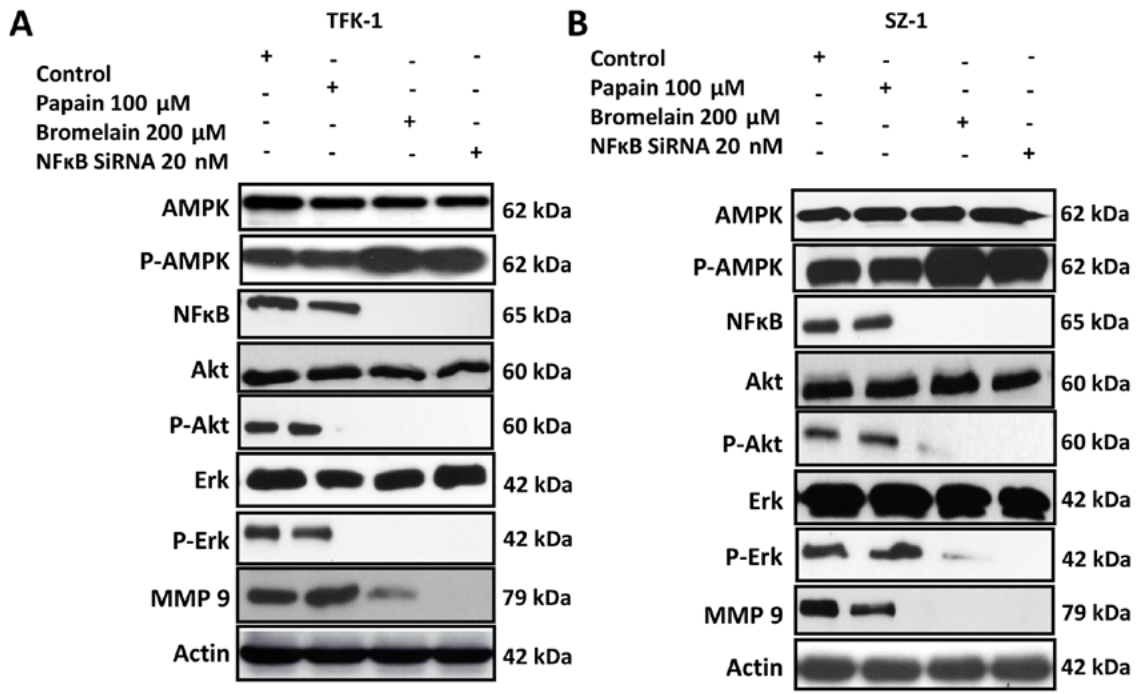


Figure 6. Effects of NFκB siRNA treatment on CC cells. Comparison of bromelain, papain, DMSO, H₂O treatments as well as silencing of NFκB in TFK-1 (A) and SZ-1 (B) cells. Cells were transfected with 20 nM NFκB siRNA or with control siRNA (20 nM). Cell lysates were analyzed by western blot analysis with antibodies against phosphorylated AMPK, AMPK, NFκB, phosphorylated AKT, AKT, phosphorylated ERK, ERK and MMP9. Actin was used as loading control. Note, only bromelain influences significantly the NFκB/AMPK signaling compared to papain.

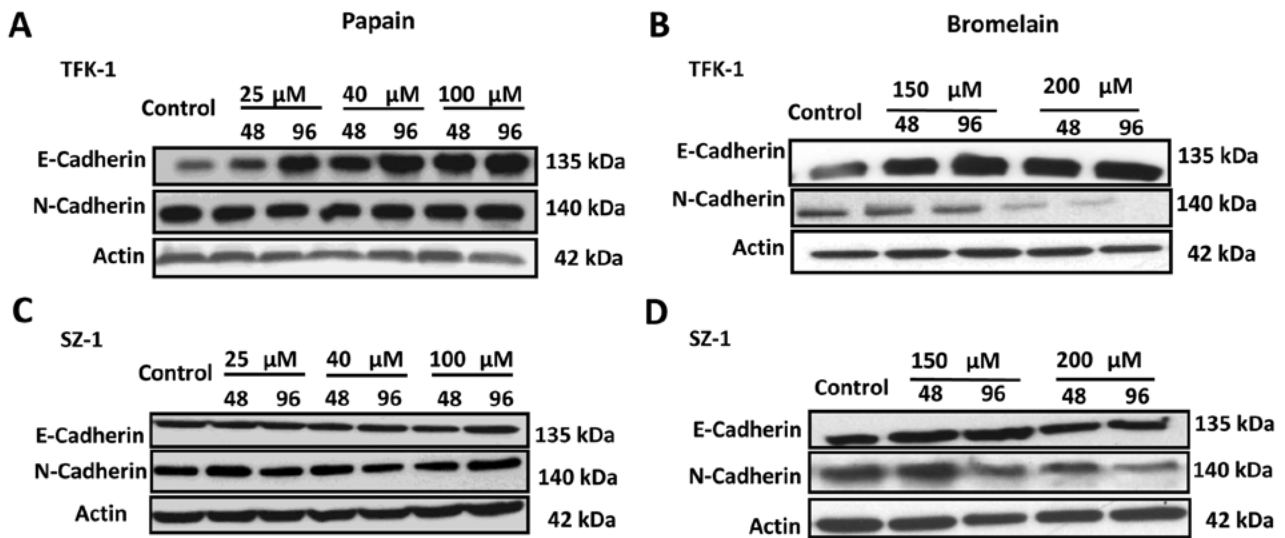


Figure 7. Changes of EMT expression in bromelain and papain treated CC cells. SZ-1 and TFK-1 cells were treated either with bromelain (B and D), papain (A and C) or their controls (DMSO, H₂O) with indicated concentrations (25, 40, 100, 150 and 200 μM) for 48 and 96 h and cell lysates were analyzed by western blot analysis with antibodies against E-cadherin and N-cadherin. Actin was used as loading control. Note, mainly bromelain has an influence on EMT compared to papain therapy.

dosages as the most effective ones for the inhibition studies in CC cells. Interestingly, papain has not been tested as an anticancer drug, therefore, in our study we for the first time performed IC₅₀ experiments and selected three dosages that were found to be suitable for CC cell inhibition. Notably, the dosages used for papain were comparatively lower than to bromelain mainly because we picked up three dosages for papain that covered a range from low to high (25-100 μM).

Notably, 100 μM of papain treatment failed to show effective downregulation of the common downstream proteins. This difference could possibly be due to the existence of some feedback loop with other cancer pathways in CC which in turn can upregulate or activate the common downstreams. Thus, reactivating the CC carcinogenesis process which was temporarily restricted by papain treatment. Papain treatment ceases or stops the proliferation of CC cells temporarily, but

fails to effectively inhibit any cancer signaling cascade or their crosstalk involved in CC carcinogenesis. On the contrary, bromelain not only inhibits proliferation of CC cells but also effectively downregulates the common downstream proteins leading to complete inhibition of CC carcinogenesis mainly via the NFκB/AMPK signaling pathway.

Our results clearly showed that the degree of effectiveness of a plant extract greatly depends on the source, as papain even in higher dosages did not show effective inhibition of AMPK NFκB signaling. The cytotoxic effect of bromelain and papain seemed to be caused by apoptotic cell death. We tried to further elucidate the mechanisms by which the cysteine proteases affect CC cells and to understand the differences between the compounds. Therefore, we investigated the expression status of NFκB and the phosphorylation status of AMPK on protein level. Bromelain treatment caused a significant decrease of NFκB and increase in AMPK phosphorylation. In contrast, papain did not alter NFκB/AMPK signalling significantly. The NFκB/AMPK signalling pathway is amongst others important for the immune system (37). Studies have demonstrated that AMPK signalling downregulates the function of NFκB (38).

We tested the status of NFκB and AMPK under treatment with bromelain and papain on CC cells by selective knock-down using siRNA and compared the results with bromelain/papain single treatment on CC cells. We also further analyzed some important selective downstream effector proteins such as AKT, ERK and STAT3, known to be crucial for inhibition studies. Under treatment upregulation of phosphorylated AMPK was observed, thus, leading to the inhibition or effective downregulation of NFκB and the common downstream proteins phospho-AKT, ERK and STAT3. Moreover, studies indicated MMP9 as one of the key transcription factors for NFκB-mediated inflammation and tumor invasion. Hence, under bromelain treatment effective NFκB inhibition also led to significant downregulation of MMP9 expression. Furthermore, siRNA mediated silencing of NFκB also reconfirmed our hypothesis that both plant extracts might exert their antitumor effects on CC cells via NFκB/AMPK signalling. Importantly, the difference in the overall treatment outcome between these two plant extracts can also be attributed to the inability of papain to effectively inhibit the NFκB/AMPK signalling in CC cells as compared to bromelain. Altogether, it can be highlighted that NFκB/AMPK signalling could be a probable mechanistic approach for antitumor activity of these two plant extracts (especially bromelain) in CC cells and the effectiveness of the compound as an anticancer agent depends on the ability to inhibit this pathway, bromelain and papain may also influence EMT, which is associated with carcinogenesis and metastasis of cancer (39). However, bromelain efficacy was different between the investigated CC cell lines. Some widely studied EMT markers such as N-cadherin and E-cadherin were strongly influenced in TFK-1 as compared to SZ-1 cells under treatment by both plant extracts. Under papain therapy EMT changes were less pronounced compared to bromelain, confirming the importance of NFκB/AMPK signalling and suggesting again that bromelain treatment is more effective as an anti-proliferative compound in CC.

Our data demonstrate that treatment by bromelain is superior to papain as a potential therapy for human CC and that the inhibition effect is mainly caused by impairment of NFκB/

AMPK signalling. Future studies with bromelain either as a single agent or in combination with other drugs are needed in order to investigate in detail the exact mechanistic approach of such plant extracts for CC treatment.

References

- Gatto M and Alvaro D: New insights on cholangiocarcinoma. *World J Gastrointest Oncol* 2: 136-145, 2010.
- Welzel TM, McGlynn KA, Hsing AW, O'Brien TR and Pfeiffer RM: Impact of classification of hilar cholangiocarcinomas (Klatskin tumors) on the incidence of intra- and extrahepatic cholangiocarcinoma in the United States. *J Natl Cancer Inst* 98: 873-875, 2006.
- von Hahn T, Ciesek S, Wegener G, Plentz RR, Weismüller TJ, Wedemeyer H, Manns MP, Greten TF and Malek NP: Epidemiological trends in incidence and mortality of hepatobiliary cancers in Germany. *Scand J Gastroenterol* 46: 1092-1098, 2011.
- Shaib Y and El-Serag HB: The epidemiology of cholangiocarcinoma. *Semin Liver Dis* 24: 115-125, 2004.
- Tyson GL and El-Serag HB: Risk factors for cholangiocarcinoma. *Hepatology* 54: 173-184, 2011.
- Valle J, Wasan H, Palmer DH, Cunningham D, Anthony A, Maraveyas A, Madhusudan S, Iveson T, Hughes S, Pereira SP, *et al*; ABC-02 Trial Investigators: Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N Engl J Med* 362: 1273-1281, 2010.
- Chobotova K, Vernallis AB and Majid FA: Bromelain's activity and potential as an anti-cancer agent: Current evidence and perspectives. *Cancer Lett* 290: 148-156, 2010.
- Gruenewald J, Brendler T and Jaenicke C (eds): *PDR for Herbal Medicines*. 4th edition. Thomson Healthcare, Montvale, NJ, 2007.
- Castell JV, Friedrich G, Kuhn CS and Poppe GE: Intestinal absorption of undegraded proteins in men: Presence of bromelain in plasma after oral intake. *Am J Physiol* 273: G139-G146, 1997.
- Taussig SJ and Batkin S: Bromelain, the enzyme complex of pineapple (*Ananas comosus*) and its clinical application. An update. *J Ethnopharmacol* 22: 191-203, 1988.
- Maurer HR: Bromelain: Biochemistry, pharmacology and medical use. *Cell Mol Life Sci* 58: 1234-1245, 2001.
- Eckert K, Grabowska E, Stange R, Schneider U, Eschmann K and Maurer HR: Effects of oral bromelain administration on the impaired immunocytotoxicity of mononuclear cells from mammary tumor patients. *Oncol Rep* 6: 1191-1199, 1999.
- Brien S, Lewith G, Walker AF, Middleton R, Prescott P and Bundy R: Bromelain as an adjunctive treatment for moderate-to-severe osteoarthritis of the knee: A randomized placebo-controlled pilot study. *QJM* 99: 841-850, 2006.
- Guo R, Canter PH and Ernst E: Herbal medicines for the treatment of rhinosinusitis: A systematic review. *Otolaryngol Head Neck Surg* 135: 496-506, 2006.
- Baumhackl U, Kappos L, Radue EW, Freitag P, Guseo A, Daumer M and Mertin J: A randomized, double-blind, placebo-controlled study of oral hydrolytic enzymes in relapsing multiple sclerosis. *Mult Scler* 11: 166-168, 2005.
- Báez R, Lopes MT, Salas CE and Hernández M: In vivo antitumor activity of stem pineapple (*Ananas comosus*) bromelain. *Planta Med* 73: 1377-1383, 2007.
- Beuth J and Braun JM: Modulation of murine tumor growth and colonization by bromelain, an extract of the pineapple plant (*Ananas comosum* L.). *In Vivo* 19: 483-485, 2005.
- Guimarães-Ferreira CA, Rodrigues EG, Mortara RA, Cabral H, Serrano FA, Ribeiro-dos-Santos R and Travassos LR: Antitumor effects in vitro and in vivo and mechanisms of protection against melanoma B16F10-Nex2 cells by fastuosain, a cysteine proteinase from *Bromelia fastuosa*. *Neoplasia* 9: 723-733, 2007.
- Taussig SJ, Szekerczes J and Batkin S: Inhibition of tumour growth in vitro by bromelain, an extract of the pineapple plant (*Ananas comosus*). *Planta Med* 51: 538-539, 1985.
- Tysnes BB, Maurer HR, Porwol T, Probst B, Bjerkvig R and Hoover F: Bromelain reversibly inhibits invasive properties of glioma cells. *Neoplasia* 3: 469-479, 2001.
- Bhui K, Tyagi S, Srivastava AK, Singh M, Roy P, Singh R and Shukla Y: Bromelain inhibits nuclear factor kappa-B translocation, driving human epidermoid carcinoma A431 and melanoma A375 cells through G(2)/M arrest to apoptosis. *Mol Carcinog* 51: 231-243, 2012.

- 1 22. Romano B, Fasolino I, Pagano E, Capasso R, Pace S, De Rosa G, Milic N, Orlando P, Izzo AA and Borrelli F: The chemopreventive action of bromelain, from pineapple stem (*Ananas comosus* L.), on colon carcinogenesis is related to antiproliferative and proapoptotic effects. *Mol Nutr Food Res* 58: 457-465, 2014.
- 2 23. Hale LP, Greer PK, Trinh CT and Gottfried MR: Treatment with oral bromelain decreases colonic inflammation in the IL-10-deficient murine model of inflammatory bowel disease. *Clin Immunol* 116: 135-142, 2005.
- 3 24. Amini A, Masoumi-Moghaddam S, Ehteda A and Morris DL: Bromelain and N-acetylcysteine inhibit proliferation and survival of gastrointestinal cancer cells in vitro: Significance of combination therapy. *J Exp Clin Cancer Res* 33: 92, 2014.
- 4 25. Pillai K, Ehteda A, Akhter J, Chua TC and Morris DL: Anticancer effect of bromelain with and without cisplatin or 5-FU on malignant peritoneal mesothelioma cells. *Anticancer Drugs* 25: 150-160, 2014.
- 5 26. Amini A, Ehteda A, Masoumi Moghaddam S, Akhter J, Pillai K and Morris DL: Cytotoxic effects of bromelain in human gastrointestinal carcinoma cell lines (MKN45, KATO-III, HT29-5F12, and HT29-5M21). *Onco Targets Ther* 6: 403-409, 2013.
- 6 27. da Silva CR, Oliveira MBN, Motta ES, de Almeida GS, Varanda LL, de Pádula M, Leitão AC and Caldeira-de-Araújo A: Gebxic and cytotoxic safety evaluation of papain (*Carica papaya* L.) using in vitro assays. *J Biomed Biotechnol* 2010: 197898, 2010.
- 7 28. Webman EJ, Edlin G and Mower HF: Free radical scavenging activity of papaya juice. *Int J Radiat Biol* 55: 347-351, 1989.
- 8 29. Mehdipour S, Yasa N, Dehghan G, Khorasani R, Mohammadirad A, Rahimi R and Abdollahi M: Antioxidant potentials of Iranian *Carica papaya* juice in vitro and in vivo are comparable to α -tocopherol. *Phytother Res* 20: 591-594, 2006.
- 9 30. Oloyede OI: Chemical profile of unripe pulp of *Carica papaya*. *Pak J Nutr* 6: 379-381, 2005.
- 10 31. Zender S, Nিকেleit I, Wuestefeld T, Sørensen I, Dauch D, Bozko P, El-Khatib M, Geffers R, Bektas H, Manns MP, *et al*: A critical role for notch signaling in the formation of cholangiocellular carcinomas. *Cancer Cell* 23: 784-795, 2013.
- 11 32. Karin M, Cao Y, Greten FR and Li ZW: NF-kappaB in cancer: From innocent bystander to major culprit. *Nat Rev Cancer* 2: 301-310, 2002.
- 12 33. Kalra N, Bhui K, Roy P, Srivastava S, George J, Prasad S and Shukla Y: Regulation of p53, nuclear factor kappaB and cyclooxygenase-2 expression by bromelain through targeting mitogen-activated protein kinase pathway in mouse skin. *Toxicol Appl Pharmacol* 226: 30-37, 2008.
- 13 34. Bhui K, Prasad S, George J and Shukla Y: Bromelain inhibits COX-2 expression by blocking the activation of MAPK regulated NF-kappa B against skin tumor-initiation triggering mitochondrial death pathway. *Cancer Lett* 282: 167-176, 2009.
- 14 35. Mohr T and Desser L: Plant proteolytic enzyme papain abrogates angiogenic activation of human umbilical vein endothelial cells (HUVEC) in vitro. *BMC Complement Altern Med* 13: 231, 2013.
- 15 36. Gill LS: Ethnomedical uses of plants in Nigeria. Uniben Press, Benin, Nigeria, 1992.
- 16 37. Salminen A, Hyttinen JM and Kaarniranta K: AMP-activated protein kinase inhibits NF- κ B signaling and inflammation: Impact on healthspan and lifespan. *J Mol Med Berl* 89: 667-676, 2011.
- 17 38. Guo Y, Zhang Y, Hong K, Luo F, Gu Q, Lu N and Bai A: AMPK inhibition blocks ROS-NF κ B signaling and attenuates endotoxemia-induced liver injury. *PLoS One*: 9: e86881, 2014.
- 18 39. Franco-Chuaire ML, Magda Carolina SC and Chuaire-Noack L: Epithelial-mesenchymal transition (EMT): Principles and clinical impact in cancer therapy. *Invest Clin* 54: 186-205, 2013.
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