



## Research Article

# Theobromine suppresses NF- $\kappa$ B signaling and promotes apoptosis in lung cancer and osteosarcoma cells

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### Abstract

**Objectives:** Theobromine has been reported to exhibit anti-inflammatory and antioxidant effects. However, its molecular impact on cancer-associated signaling pathways remains poorly understood. This research was undertaken to evaluate the pro-apoptotic signaling effects of Theobromine on A549 lung cancer and Saos-2 osteosarcoma cells, focusing on modulation of the NF- $\kappa$ B signaling pathway.

**Methods:** A549 and Saos-2 cells were exposed to Theobromine and the reference NF- $\kappa$ B inhibitor sulfasalazine at their IC<sub>50</sub> concentrations. Cell viability and IC<sub>50</sub> values were determined using the WST-1 assay. Apoptosis was quantified by Annexin V-FITC/PI staining, and total and phosphorylated NF- $\kappa$ B and IKK protein levels were quantified by flow cytometry.

**Results:** Data were analyzed using one-way analysis of variance (ANOVA). In A549 lung cancer cells, Theobromine significantly induced apoptosis ( $p < 0.001$  for late apoptotic cells). A significant reduction in both total and phosphorylated NF- $\kappa$ B and IKK protein levels was observed compared with controls ( $p < 0.05$  for p-NF- $\kappa$ B and IKK,  $p < 0.001$  for p-IKK). In Saos-2 osteosarcoma cells, similarly increased apoptotic cell populations were observed ( $p < 0.001$  for early and late apoptotic cells). The treatment also resulted in reduced total and phosphorylated NF- $\kappa$ B and IKK levels ( $p < 0.05$  for NF- $\kappa$ B,  $p < 0.001$  for p-NF- $\kappa$ B,  $p < 0.01$  for IKK, and  $p < 0.0001$  for p-IKK).

**Conclusion:** This study provides the first evidence that Theobromine suppresses the NF- $\kappa$ B/IKK signaling pathway and promotes apoptosis in A549 and Saos-2 cancer cells. These findings suggest that Theobromine may act as a safe, naturally derived NF- $\kappa$ B modulator with potential applications as an adjuvant or chemopreventive agent in cancer therapy.

**Keywords:** Lung cancer, osteosarcoma, theobromine

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The cancer types expected to see the largest increases in global cases include breast, lung, and colorectal cancer. More than 34 million cancer cases are projected to be diagnosed annually by 2070 [1, 2]. Lung cancer, in particular, represents the most common cause of cancer-related deaths globally. Its aggressive clinical behavior is largely attributed to early dissemination and the formation of metastases. Although there are methods used to treat the disease, its prognosis is alarming due to the low 5-year survival rate [3]. Bone metastases are frequently encountered among lung

cancer metastases. Bone metastases are associated with serious complications, including pain, fractures, and impaired quality of life. The dynamic interaction between tumor cells and the bone microenvironment contributes to both tumor progression and resistance to treatment [4].

Chronic inflammation is one of the hallmarks of cancer. The NF- $\kappa$ B signaling pathway exhibits a central role in inducing the inflammatory cascade and linking it to tumor development. Under normal conditions, NF- $\kappa$ B binds to its inhibitory partners, the I $\kappa$ B proteins, which block its DNA-binding activity. When

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cells are exposed to external stimuli, the I $\kappa$ B kinase (IKK) is activated. As a result, NF- $\kappa$ B is released and transferred to the nucleus, thereby initiating the transcription of target genes [5–7]. Theobromine, a methylxanthine derivative found abundantly in cocoa products, has long been examined for its bronchodilatory and diuretic effects [8, 9]. Recent evidence, however, points to its anti-inflammatory and free radical scavenging effects [10]. Despite these indications, the role of Theobromine in cancer-associated inflammation, particularly in lung cancer and osteosarcoma models, remains unexplored.

This study sought to clarify whether Theobromine modulates the NF- $\kappa$ B signaling cascade in A549 and Saos-2 cancer cell lines. Given the prominent role of NF- $\kappa$ B in regulating cell survival, proliferation, and tumor progression, we aimed to determine whether Theobromine can modulate this pathway under basal conditions. To address this, Theobromine and the reference NF- $\kappa$ B inhibitor sulfasalazine were applied at their IC<sub>50</sub> concentrations, and their impact on cell viability and apoptotic cell death was evaluated. In addition, total and phosphorylated NF- $\kappa$ B (p65) and IKK levels were quantified by flow cytometry to assess the activity of Theobromine on pathway activation. Through this integrated analysis, the study seeks to clarify whether Theobromine exerts intrinsic regulatory effects on NF- $\kappa$ B signaling and to provide mechanistic insight into its potential as a natural modulator of this key oncogenic pathway.

## Materials and Methods

### Cell culture

A549 and Saos-2 cells were cultivated in DMEM (Capricorn Scientific, Germany) with 10% FBS (Invitrogen, Carlsbad) and 1% pen/strep at 37°C and 5% CO<sub>2</sub>.

Theobromine (500  $\mu$ M) and sulfasalazine (10 mM) were first dissolved in dimethyl sulfoxide (DMSO) and diluted to 5–100  $\mu$ M and 0.2–4 mM, respectively.

### Water-soluble tetrazolium salt (WST-1) cytotoxicity assay

Each cell line was plated in 96-well plates at a density of  $2 \times 10^4$  cells/well and incubated for 24 hours. After incubation, Theobromine (0.5, 10, 25, 50, 100  $\mu$ M) and sulfasalazine (0, 0.2, 0.5, 2, 3, 4 mM) were applied at varying concentrations for 24 and 48 hours. Then, 10  $\mu$ L of WST-1 solution was added to each well and incubated for 4 hours. The absorbance was read at 450 nm using a Thermo Varioskan Microplate Reader. All experiments were performed in at least three independent replicates ( $n=3$ ), and the results are presented as mean  $\pm$  standard deviation (SD). IC<sub>50</sub> values were calculated using nonlinear regression analysis by fitting the data to a sigmoidal dose–response (variable slope) curve using GraphPad Prism software (version 9.1.0).

### Annexin V binding assay for apoptosis detection

To distinguish apoptotic, necrotic, and live cell populations, we carried out flow cytometry analysis. A549 lung cancer

cells and Saos-2 osteosarcoma cells were seeded into 6-well plates. IC<sub>50</sub> values of Theobromine and Sulfasalazine were applied and incubated for 24 h. The cells adhered to the plate were detached using Trypsin. The cells were collected. Then, 5  $\mu$ L of Annexin V-FITC and propidium iodide (PI) were added. After incubating for 15 minutes, the cells were analyzed using an ACEA NovoCyte flow cytometry device (ACEA Biosciences Inc., San Diego, CA, USA). For each sample, at least 10,000 events were acquired. Compensation was applied using single-stained controls to correct for spectral overlap between fluorochromes.

Cells were gated based on forward scatter (FSC) and side scatter (SSC) parameters to exclude debris. Apoptotic populations were determined using Annexin V-FITC and propidium iodide (PI) staining, where viable cells were defined as Annexin V<sup>-</sup>/PI<sup>-</sup> (lower left quadrant), early apoptotic cells as Annexin V<sup>+</sup>/PI<sup>-</sup> (lower right quadrant), late apoptotic cells as Annexin V<sup>+</sup>/PI<sup>+</sup> (upper right quadrant), and necrotic cells as Annexin V<sup>-</sup>/PI<sup>+</sup> (upper left quadrant).

### Measurement of protein levels by flow cytometry

After treatment with Theobromine and Sulfasalazine, the levels of total NF- $\kappa$ B, total IKK, phosphorylated NF- $\kappa$ B, and phosphorylated IKK were determined. The cells were exposed to FCM fixation buffer for 15 minutes and FCM permeabilization buffer for 5 minutes. After washing, primary antibodies for Anti-RELA (St. John's/STJ94468), Anti-Phospho-RELA-Thr345 (St. John's/STJ90353), Anti-IKK Alpha/Beta (St. John's/STJ93667), and Anti-Phospho-IKK Alpha/Beta-Ser 176/177 (St. John's/STJ90301) were used. Then, secondary antibody (Advanta/R-05071) was applied for 1 hour. The cells that received the treatment were analyzed using the NovoCyte D3000 flow cytometer (Agilent Technologies, Inc.).

### Statistical Analysis

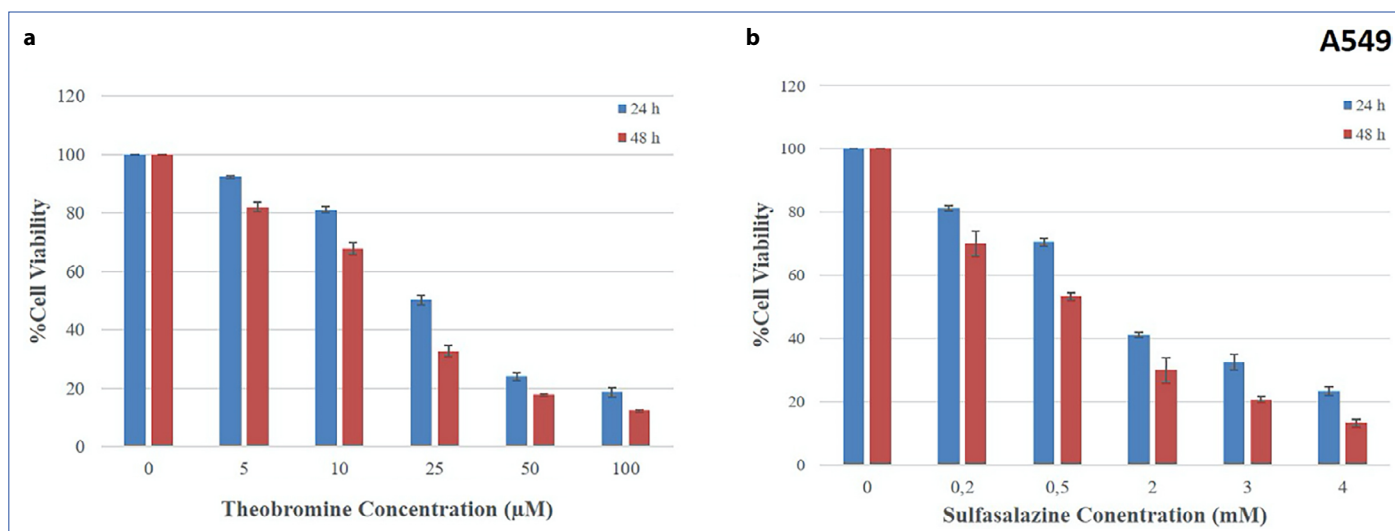
All statistical analyses were performed using GraphPad Prism software (version 9.1.0). Data are presented as mean  $\pm$  standard deviation (SD). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

## Results

Theobromine exhibited stronger cytotoxicity in A549 and Saos-2 cancer cells.

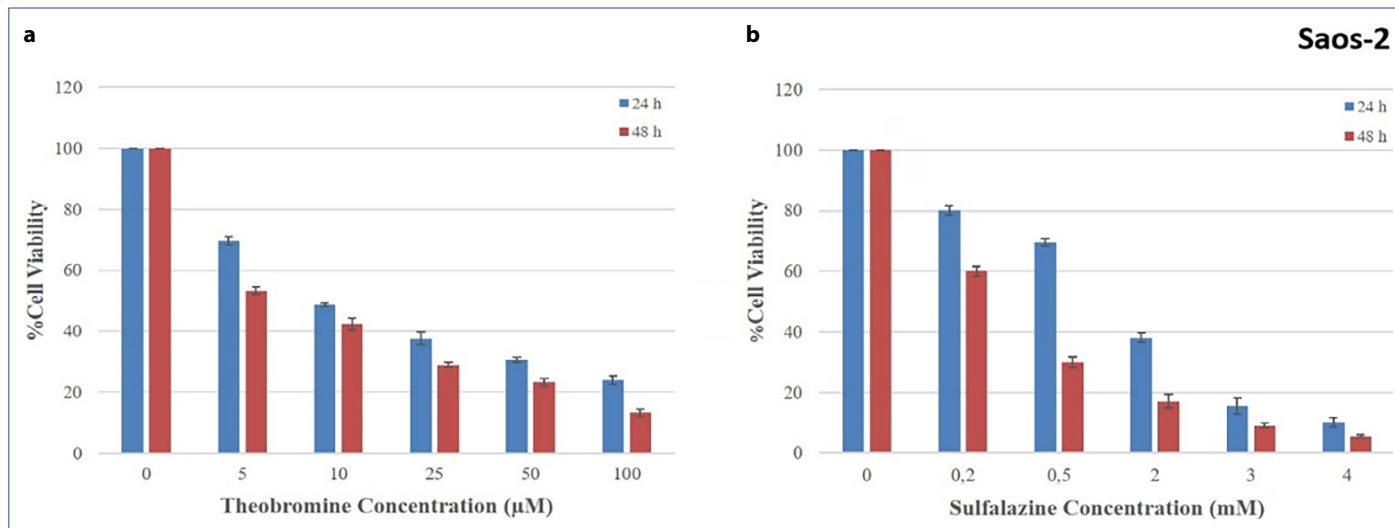
In A549 cells, Theobromine exerted a dose-dependent inhibitory effect, decreasing cell viability from 100% to 18.64% at 100  $\mu$ M after 24 h and to 12.3% after 48 h. Sulfasalazine, on the other hand, produced weaker cytotoxicity, lowering viability from 100% to 23.4% at 4 mM after 24 h and to 13% after 48 h. The IC<sub>50</sub> values were found to be  $25.97 \pm 3.5$   $\mu$ M and  $1.249 \pm 0.2$   $\mu$ M for Theobromine and Sulfasalazine, respectively (Fig. 1a, b).

In Saos-2 cells, Theobromine treatment led to a progressive decline in cell viability from 100% at 0  $\mu$ M to 24.04% at 100



**Figure 1.** Dose- and time-dependent cytotoxic effects of Theobromine and Sulfasalazine on A549 cells. (a) Cell viability of A549 cells following 24 h and 48 h treatment with increasing concentrations of Theobromine (5–100 µM). (b) Cell viability of A549 cells following 24 h and 48 h treatment with increasing concentrations of Sulfasalazine (0.2–4 mM). Data are presented as mean±SD (n=3).

SD: Standard deviation.



**Figure 2.** Dose- and time-dependent cytotoxic effects of Theobromine and Sulfasalazine on Saos-2 cells. (a) Cell viability of Saos-2 cells exposure to Theobromine (5–100 µM). (b) Cell viability of Saos-2 cells following 24 h and 48 h exposure to Sulfasalazine (0.2–4 mM). Data are presented as mean±SD (n=3).

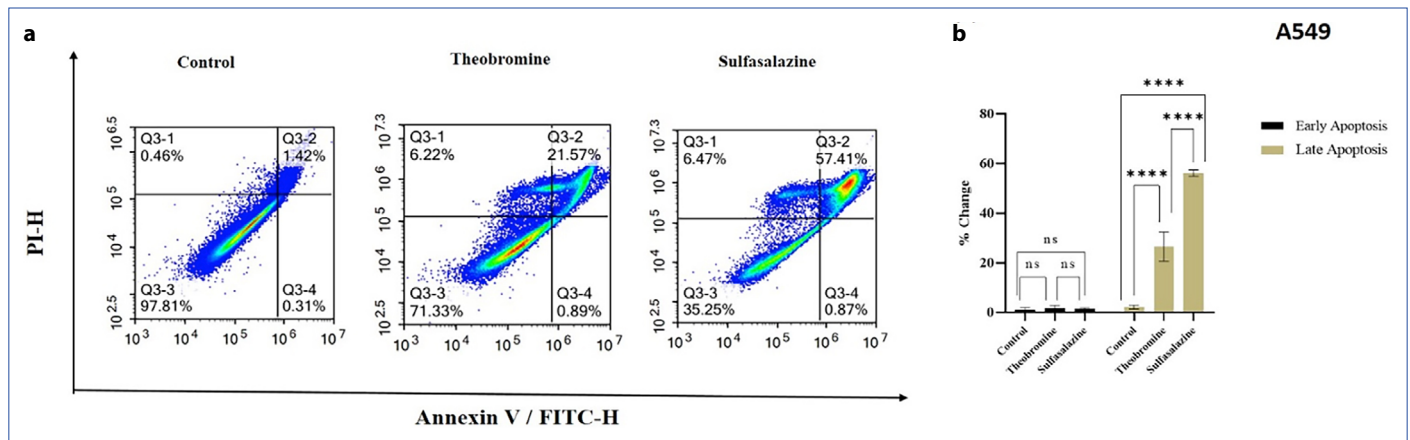
µM after 24 h, with mean viability values of 69.5%, 48.7%, 37.7%, and 30.6% at 5, 10, 25, and 50 µM, respectively. Prolonged incubation for 48 h further potentiated its cytotoxic activity, decreasing viability to 13%. The calculated  $IC_{50}$  value was  $12.64 \pm 2.5$  µM, indicating strong antiproliferative efficacy. Sulfasalazine exhibited a similar concentration-dependent pattern, reducing viability from 100% to 10.2% at 4 mM after 24 h and to 5.6% after 48 h, with an estimated  $IC_{50}$  of  $0.8862 \pm 0.001$  mM (Fig. 2a, b).

Overall, these findings demonstrate that Theobromine exerts a pronounced cytotoxic effect in both Saos-2 and A549 cells. The dose-dependent inhibition of cell viability indicates that Theobromine effectively suppresses cancer

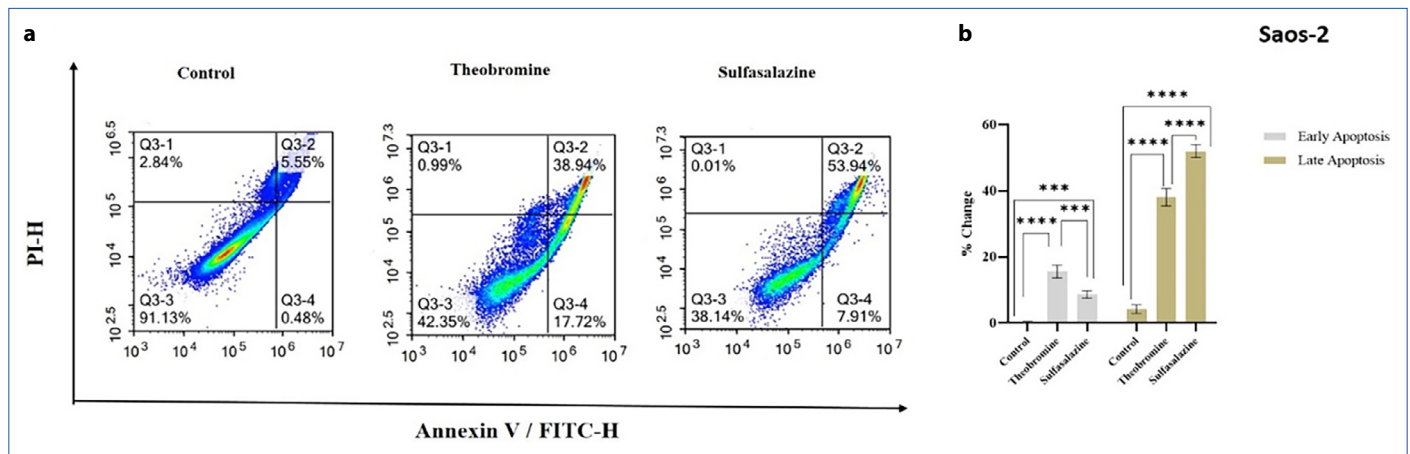
cell proliferation, supporting its potential as a natural compound with anticancer properties.

### Apoptotic cell death induced by theobromine in A549 and Saos-2 cells

In A549 cells, the control group exhibited a predominantly viable population ( $97.81 \pm 0.45\%$ ), while early and late apoptotic fractions were  $1.10 \pm 0.85\%$  and  $2.14 \pm 0.82\%$ , respectively. Treatment with Theobromine moderately elevated late apoptosis ( $26.67 \pm 6.02\%$ ), suggesting partial activation of apoptotic signaling ( $p < 0.0001$  vs control). Sulfasalazine induced a pronounced apoptotic response in terms of late apoptosis ( $56.00 \pm 1.00\%$ ) compared with both the control and Theo-



**Figure 3.** Annexin V-FITC/PI analysis of A549 cells (a) Representative flow cytometry dot plots. (b) Quantification of apoptotic cell populations. Data are presented as mean $\pm$ SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test \*\*\*\*:  $p < 0.0001$ ; ns, not significant.



**Figure 4.** Annexin V-FITC/PI analysis of Saos-2 cells. (a) Representative flow cytometry dot plots. (b) Quantification of apoptotic cell populations. Data are presented as mean $\pm$ SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. \*\*\*:  $p < 0.001$ ; \*\*\*\*:  $p < 0.0001$ .

bromine-treated groups ( $p < 0.0001$  vs control). The overall apoptotic rate induced by Sulfasalazine was approximately 2.5-fold higher than that caused by Theobromine, confirming its strong pro-apoptotic potential in A549 cells (Fig. 3a, b).

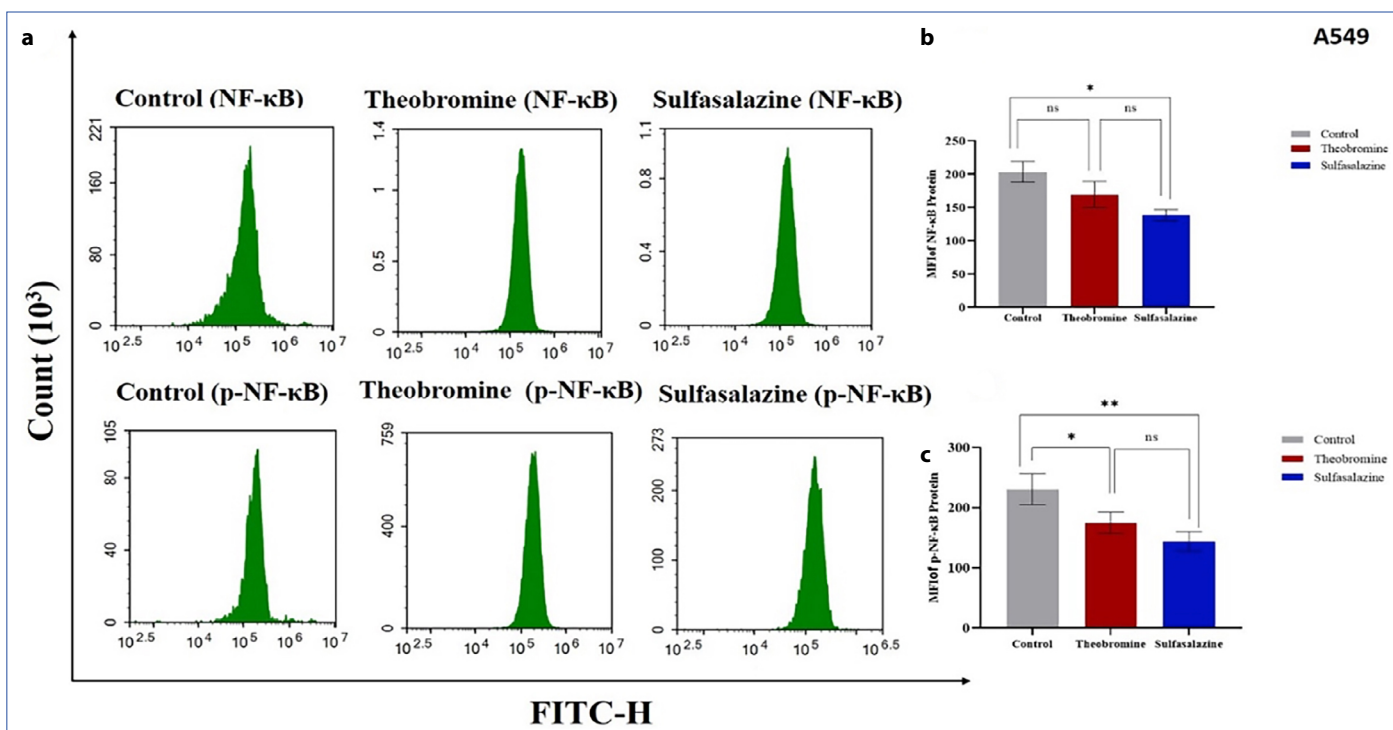
In Saos-2 cells, the control group exhibited minimal apoptosis, with  $0.36 \pm 0.22\%$  of cells in early and  $4.18 \pm 1.28\%$  in late apoptotic phases. Treatment with Theobromine significantly increased both early ( $15.67 \pm 1.15\%$ ) and late ( $37.67 \pm 1.53\%$ ) apoptotic cell populations compared with the control group ( $p < 0.0001$ ). Sulfasalazine enhanced apoptosis, inducing  $8.64 \pm 1.04\%$  early and  $51.67 \pm 2.08\%$  late apoptotic cells. These results suggest that Theobromine elicits a stronger apoptotic response (Fig. 4a, b).

Overall, the Annexin V-FITC/PI analysis revealed that both Theobromine and Sulfasalazine reduced cell viability primarily by promoting apoptosis in a concentration- and cell type-dependent manner. While both compounds were capable of triggering programmed cell death, the extent of apoptotic induction varied between the two cancer models, reflecting

differences in cellular sensitivity and underlying molecular characteristics. These results collectively indicate that apoptosis plays a central role in the cytotoxic effects observed following treatment with Theobromine and Sulfasalazine.

### Theobromine reduces NF- $\kappa$ B and IKK protein levels in A549 and Saos-2 cells

To assess how Theobromine modulates the NF- $\kappa$ B/IKK signaling pathway in A549 lung cancer and Saos-2 osteosarcoma cells, the expression levels of the relevant proteins were measured by flow cytometry. The mean fluorescence intensity (MFI) values were quantitatively analyzed. Analysis of both the total and phosphorylated forms of NF- $\kappa$ B and IKK proteins provides insight into pathway activity at different levels. Total protein levels reflect the overall amount and expression status of the relevant molecules in the cell, while the phosphorylated forms (p-NF- $\kappa$ B and p-IKK) represent the active conformations of these proteins, which initiate or maintain signaling. Therefore, the combined evaluation



**Figure 5.** Flow cytometric analysis of total NF- $\kappa$ B and p-NF- $\kappa$ B expression in A549 cells. (a) Representative histograms showing fluorescence intensity distribution. (b) Quantification of mean fluorescence intensity (MFI) for total NF- $\kappa$ B. (c) Quantification of MFI for p-NF- $\kappa$ B. Data are presented as mean $\pm$ SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; ns: Not significant.

of total and phosphorylated forms comprehensively reveals not only Theobromine's effects on protein abundance but also its regulatory effects.

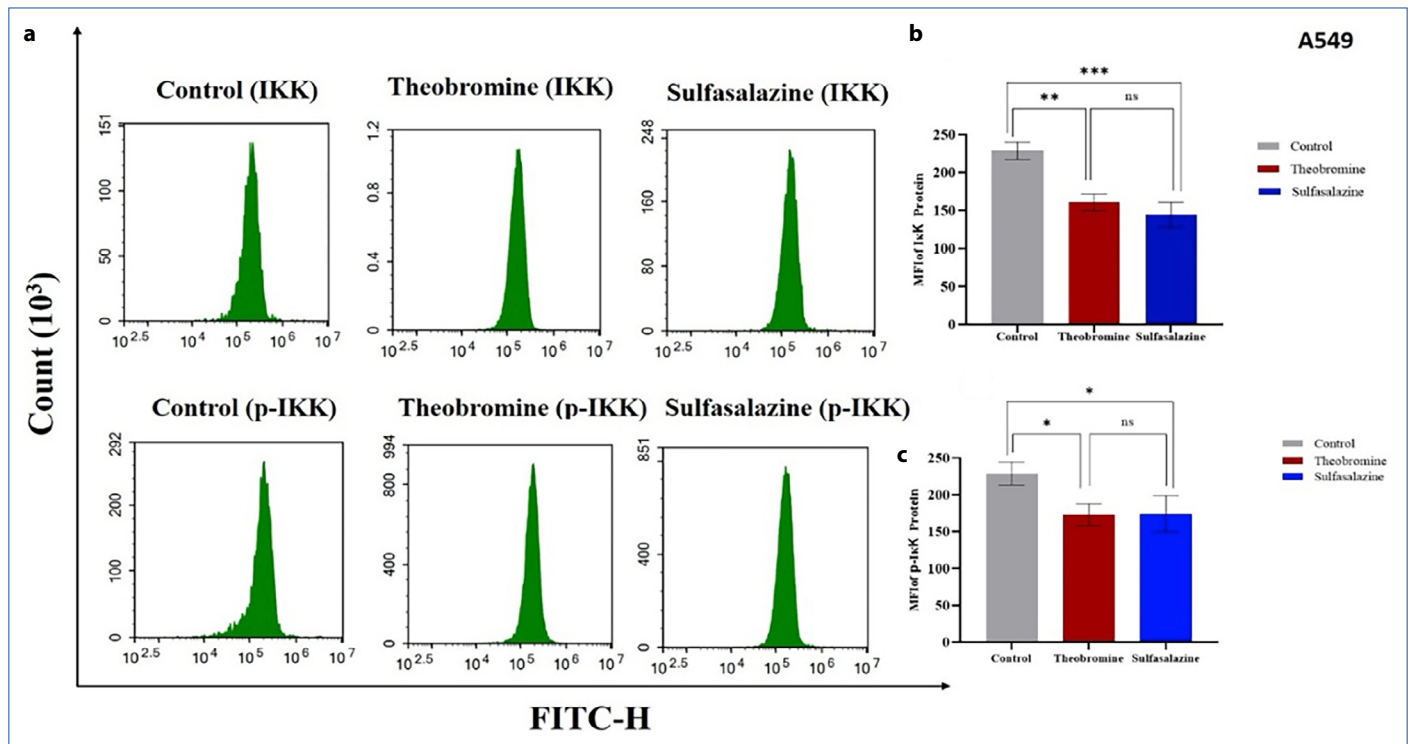
For A549 cells, total NF- $\kappa$ B levels in the control group were  $203.3 \pm 10.3$ , whereas treatment with Theobromine reduced this value to  $169.5 \pm 12.2$ . Sulfasalazine markedly suppressed total NF- $\kappa$ B to  $138.6 \pm 7.9$  ( $p < 0.05$ ), confirming its strong inhibitory effect (Fig. 5a, b). Phosphorylated NF- $\kappa$ B (p-NF- $\kappa$ B) levels exhibited a more pronounced response. Control cells showed an expression level of  $230.5 \pm 14.5$ , which significantly decreased to  $174.9 \pm 10.7$  following Theobromine treatment ( $p < 0.05$ ). Sulfasalazine further reduced p-NF- $\kappa$ B to  $143.9 \pm 9.1$  ( $p < 0.01$ ) (Fig. 5a, c). The difference between Theobromine and Sulfasalazine was not statistically significant ( $p > 0.05$ ), indicating that Theobromine suppresses NF- $\kappa$ B activation to a degree comparable to pharmacological inhibition. Total IKK expression was  $229.1 \pm 11.8$ , while Theobromine treatment lowered this level to  $160.8 \pm 11.2$  ( $p < 0.01$ ). Sulfasalazine elicited an even greater reduction ( $144.4 \pm 13.7$ ,  $p < 0.001$ ) (Fig. 6a, b). Consistent with these findings, phosphorylated IKK (p-IKK) levels were significantly reduced following treatment. The control group displayed  $228.3 \pm 15.0$ , which decreased to  $173.1 \pm 15.9$  after Theobromine exposure ( $p < 0.05$ ). Sulfasalazine produced a similar reduction, yielding  $174.4 \pm 25.0$  ( $p < 0.05$ ) (Fig. 6a, c).

In Saos-2 osteosarcoma cells, total NF- $\kappa$ B protein levels were  $276.9 \pm 9.8$  in the control group, but decreased to  $218.4 \pm 6.7$  with Theobromine treatment ( $p < 0.05$ ). Sulfasalazine treat-

ment produced a more significant decrease to  $189.4 \pm 6.0$  ( $p < 0.01$ ) (Fig. 7a, b). p-NF- $\kappa$ B levels were  $272.5 \pm 1.9$  in the control group, decreasing to  $165.1 \pm 7.7$  with Theobromine ( $p < 0.001$ ) and  $149.7 \pm 6.0$  with sulfasalazine ( $p < 0.001$ ). The difference between Theobromine and sulfasalazine was not statistically significant ( $p > 0.05$ ) (Fig. 7a, c). Total IKK values were  $213.3 \pm 17.0$  in the control group and decreased to  $156.7 \pm 8.2$  with Theobromine treatment ( $p < 0.05$ ). Sulfasalazine provided a more potent suppression to  $138.1 \pm 4.5$  ( $p < 0.01$ ). No significant difference was observed between the two treatments in terms of total IKK ( $p > 0.05$ ) (Fig. 8a, b). p-IKK levels were  $302.9 \pm 7.4$  in the control group, which decreased to  $179.1 \pm 5.3$  with Theobromine ( $p < 0.0001$ ). Sulfasalazine administration showed a significant decrease to  $150.9 \pm 7.4$  compared to both the control and Theobromine groups ( $p < 0.0001$  and  $p < 0.05$ ) (Fig. 8a, c).

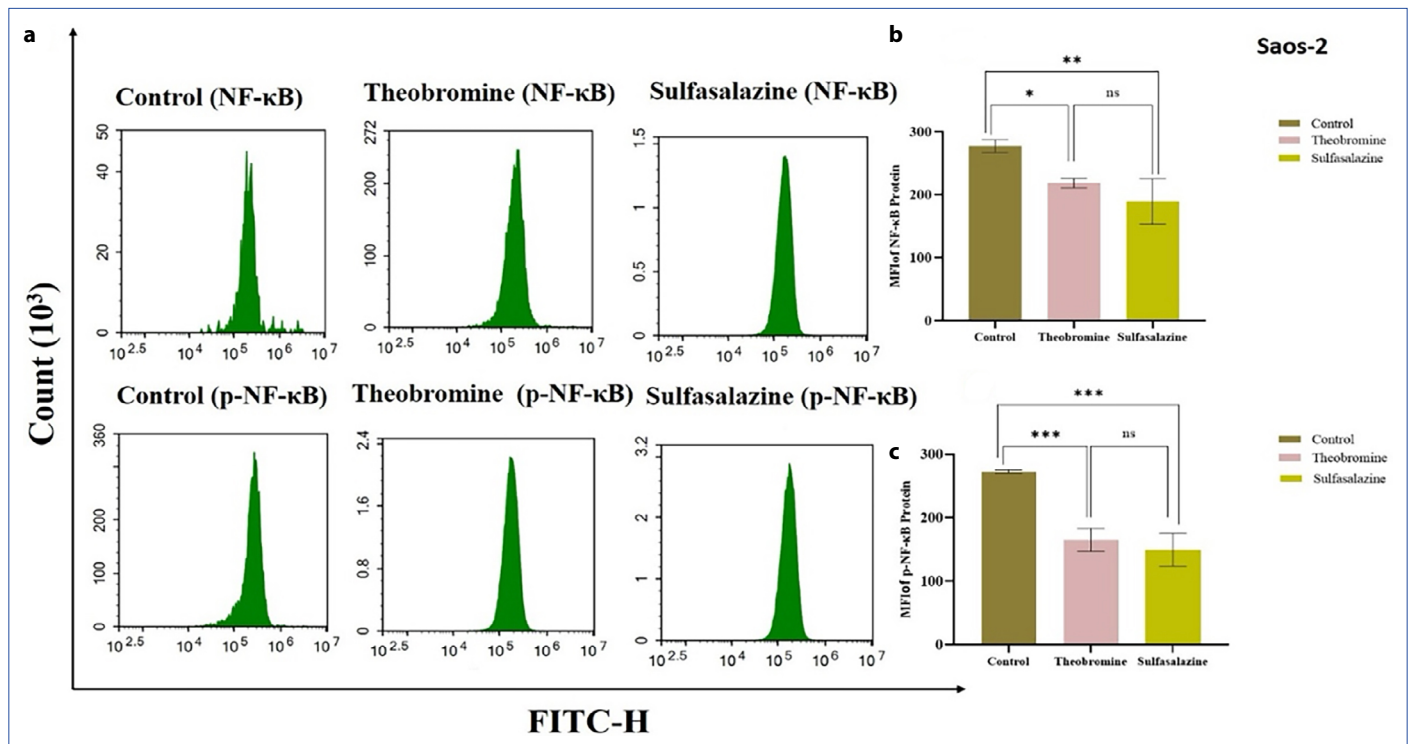
## Discussion

In this study, Theobromine, a natural methylxanthine derivative, was shown to have significant cytotoxic and proapoptotic effects in A549 lung cancer and Saos-2 osteosarcoma cells. The findings indicate that Theobromine exerts these effects through inhibition of the NF- $\kappa$ B signaling pathway. The NF- $\kappa$ B signaling pathway is a key regulator of cell proliferation, inflammation, and survival. Therefore, Theobromine's inhibitory effect on these pathways supports its potential anticancer activity.



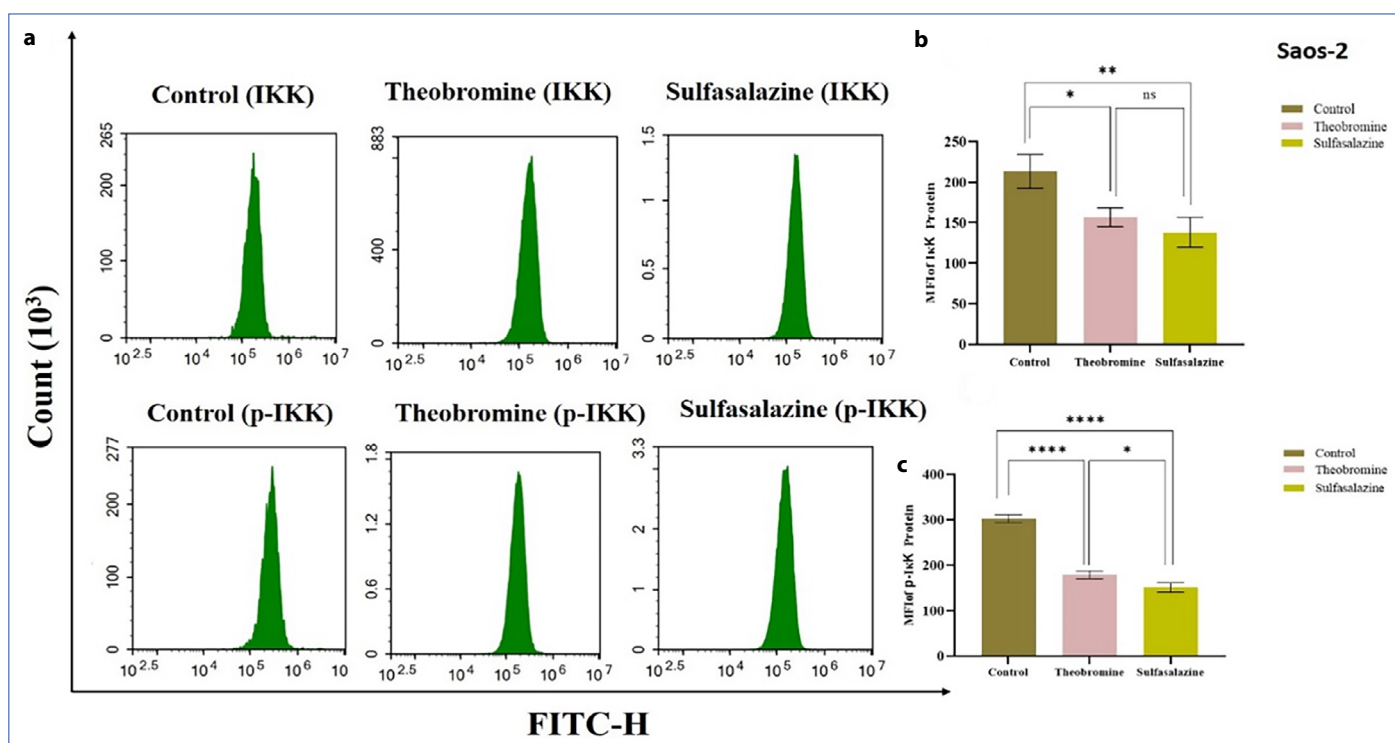
**Figure 6.** Flow cytometric analysis of total IKK and p-IKK expression in A549 cells. (a) Representative histograms showing fluorescence intensity distribution. (b) Quantification of mean fluorescence intensity (MFI) for total IKK. (c) Quantification of MFI for p-IKK. Data are presented as mean±SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

\*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001; ns: Not significant.



**Figure 7.** Flow cytometric analysis of total NF-κB and p-NF-κB expression in Saos-2 cells. (a) Representative histograms showing fluorescence intensity distribution. (b) Quantification of mean fluorescence intensity (MFI) for total NF-κB. (c) Quantification of MFI for p-NF-κB. Data are presented as mean±SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

\*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001; ns: Not significant.



**Figure 8.** Flow cytometric analysis of total IKK and p-IKK expression in Saos-2 cells. (a) Representative histograms showing fluorescence intensity distribution. (b) Quantification of mean fluorescence intensity (MFI) for total IKK. (c) Quantification of MFI for p-IKK. Data are presented as mean±SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

\*: p<0.05; \*\*: p<0.01; \*\*\*\*: p<0.0001; ns: Not significant.

Theobromine was observed to decrease cell viability in a dose-dependent manner in A549 lung cancer and Saos-2 osteocarcinoma cells. This dose- and time-dependent decrease in cell viability is consistent with previous studies reporting the antiproliferative properties of Theobromine in A549 cells [9]. Furthermore, Annexin V/PI analyses showed that Theobromine induces cell death via apoptosis. This result is consistent with the literature reporting that methylxanthine derivatives can promote apoptosis by activating mitochondrial pathways and stimulating caspase enzymes [11, 12].

The significant decrease in total and phosphorylated NF- $\kappa$ B and IKK levels proves that Theobromine has a strong suppressive effect not only on gene expression but also on signal activation. However, the number of studies examining the effects of Theobromine on cellular signaling pathways is quite limited. Gu et al. [13] demonstrated that Theobromine reduces oxidative stress and inflammatory responses by suppressing the NF- $\kappa$ B signaling pathway in IL-1 $\beta$ -stimulated human chondrocytes and also prevents type II collagen degradation. In contrast, Lee et al. [14] showed that Theobromine increased the production of IL-6, TNF- $\alpha$ , and NO by activating MAPK and NF- $\kappa$ B pathways in macrophages. Taken together, these two studies suggest that Theobromine may have a dual effect, depending on the cell type and physiological context. In normal or immune cells, Theobromine appears to enhance defense mechanisms by stimulating NF- $\kappa$ B activation, while under inflammatory conditions, it suppresses this pathway,

reducing oxidative stress and cytokine production. This discrepancy suggests that Theobromine's effects are influenced by factors such as the cellular microenvironment, the type of stimulus, and metabolic state. Therefore, Theobromine may act as a protective and immunomodulatory agent in some cell types and as an anti-inflammatory agent in others.

These dual regulatory properties of Theobromine in various cell systems make it particularly important to elucidate its potential mechanisms in tumor cells. Therefore, our study investigated the effects of Theobromine on the NF- $\kappa$ B signaling pathway. The number of studies examining the effects of Theobromine on cancer-related signaling pathways is quite limited. In the study conducted by Oz-Bedir et al. [15], Theobromine was shown to suppress the NRF2 signaling pathway in invasive bladder cancer cells while activating this pathway in non-invasive cells. Similarly, Shojaei-Zarghani et al. [16] reported that Theobromine inhibited tumor and precarcinoma lesion formation by suppressing the Akt/GSK3 $\beta$ / $\beta$ -catenin signaling pathway in a 1,2-dimethylhydrazine-induced rat colorectal cancer model. Another study by the same research group demonstrated that Theobromine, administered alone or in combination with theanine, suppressed the Akt/mTOR and JAK2/STAT3 oncogenic signaling pathways and increased expression of the Smad2 tumor suppressor protein. These findings suggest that Theobromine has broad-spectrum anticancer potential, capable of targeting not only inflammatory processes but also multiple cellular signaling networks

involved in cancer development [17]. To date, no study has specifically investigated the effects of Theobromine on the NF- $\kappa$ B signaling pathway in cancer cells. In the present study, Theobromine treatment markedly reduced total and phosphorylated NF- $\kappa$ B and IKK protein levels in A549 and Saos-2 cells, indicating that it effectively suppresses NF- $\kappa$ B pathway activation and promotes apoptotic cell death. Theobromine's ability to downregulate both total and phosphorylated forms of NF- $\kappa$ B and IKK indicates that it acts at multiple levels of this signaling cascade. This dual inhibition suggests that Theobromine may interfere with upstream kinases involved in IKK phosphorylation or disrupt the release and nuclear translocation of NF- $\kappa$ B. Interestingly, although sulfasalazine is a well-established pharmacological NF- $\kappa$ B inhibitor, Theobromine demonstrated a comparable inhibitory effect at significantly lower concentrations. This finding highlights the potential of Theobromine as a natural, low-toxicity NF- $\kappa$ B modulator with possible therapeutic relevance. The fact that Theobromine is a dietary compound found in cocoa and tea adds translational value to these results, suggesting its potential use as an adjuvant or chemopreventive agent in cancer management. Nevertheless, while the *in vitro* results strongly indicate Theobromine's inhibitory effect on NF- $\kappa$ B signaling, further investigations are warranted. Future studies should focus on elucidating its effects in inflammatory or cytokine-stimulated cancer models and determining its efficacy *in vivo* using xenograft or orthotopic tumor models. Additionally, exploring downstream molecular targets and cross-talk with other oncogenic pathways, such as PI3K/Akt or JAK/STAT, could provide deeper mechanistic insight into Theobromine's multifaceted anticancer action.

Although Theobromine is considered a naturally occurring compound with relatively low toxicity, its pharmacokinetic properties and bioavailability should be carefully considered when evaluating its translational potential. Importantly, the concentrations used in *in vitro* studies are often higher than those achievable through dietary intake, suggesting that the observed effects may not be directly translatable under normal physiological conditions. Therefore, while Theobromine demonstrates promising anticancer effects at the cellular level, further investigations are required to determine whether these effects can be reproduced *in vivo*. Future studies should focus on pharmacokinetic profiling, dose optimization, and potential formulation strategies to enhance bioavailability. Additionally, evaluating Theobromine in combination therapies or advanced delivery systems may provide more clinically relevant outcomes.

Although our findings demonstrate apoptosis induction through Annexin V/PI analysis and modulation of NF- $\kappa$ B/IKK signaling, the present study is limited by the absence of additional apoptosis-related protein analyses. Key markers such as caspase-3 activation, PARP cleavage, and Bcl-2 family proteins were not evaluated. Inclusion of these markers would provide deeper mechanistic insight into the apoptotic pathways involved. Therefore, future studies

should incorporate these molecular targets to better define whether apoptosis is mediated through intrinsic, extrinsic, or combined pathways.

Taken together, our findings provide new mechanistic evidence that Theobromine inhibits NF- $\kappa$ B signaling and promotes apoptosis in cancer cells. These results contribute to a growing body of evidence supporting Theobromine as a promising natural compound with anti-inflammatory and anticancer properties, offering potential for future development as a safe and effective adjunct in cancer therapy.

## Conclusion

In summary, this study provides novel evidence that Theobromine exerts significant cytotoxic and pro-apoptotic effects in A549 lung cancer and Saos-2 osteosarcoma cells by suppressing the NF- $\kappa$ B signaling pathway. Theobromine decreased both total and phosphorylated protein levels of NF- $\kappa$ B and IKK, demonstrating inhibition at multiple regulatory points within this cascade. Its efficacy was comparable to that of sulfasalazine, despite being a natural compound. These results suggest that Theobromine may serve as a promising, low-toxicity modulator of NF- $\kappa$ B signaling with potential application as an adjuvant or chemopreventive agent in cancer therapy. Despite the promising *in vitro* findings, the present study has certain limitations, primarily due to its restriction to cell culture models. Future studies should focus on validating these results *in vivo* using appropriate animal models to better assess the pharmacokinetics, bioavailability, and systemic effects of Theobromine and sulfasalazine. In addition, further investigations are needed to evaluate their therapeutic efficacy, safety profile, and potential synergistic interactions in more complex biological systems, including 3D tumor models and patient-derived organoids. Such studies would provide more comprehensive insights into their translational potential and possible clinical applicability in cancer therapy.

## Disclosures

**Ethics Committee Approval:** This study was conducted exclusively using established commercial cell lines under standard *in vitro* laboratory conditions.

**Informed Consent:** No human participants, human biological samples, identifiable personal data, or live experimental animals were involved in the study.

**Conflict of Interest Statement:** None declared.

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**Use of AI for Writing Assistance:** None declared.

**Authorship Contributions:** Concept – E.T., T.O.S.; Design – E.T., T.O.S.; Supervision – E.T.; Materials – T.O.S.; Data collection and/or processing – E.T.; Analysis and/or interpretation – E.T.; Literature review – E.T., T.O.S.; Writing – E.T.; Critical review – E.T.

**Peer-review:** Externally peer-reviewed.

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