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Original Research Article

Withaferin a Inhibits EGF and ERK Signaling to Induce Programmed Cell Death in Colon Cancer Cells

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Abstract

Withaferin A (WA), a bioactive compound derived from the Ashwagandha plant, has been recognized for its anticancer properties. We tested various concentrations of WA, Fluvastatin (FLU), and their combinations (ranging from 0 to 160 µM) for their potential regulatory effects on colon cancer cells (Caco-2 cell line) and non-tumorigenic colon cells (NCM-460). Our results showed that at lower concentrations (around10 µM), WA significantly impacted colon cancer cells, showing minimal toxicity to normal cells (NCM-460). However, at higher concentrations, FLU selectively inhibited cancer cell proliferation, with noticeable toxicity to both cancerous and normal cells. Additionally, WA treatment led to a time and dose-dependent decrease in the production of IL-1β and TNF-α. Along with these effects, the downregulation of ERK and EGF gene expression was observed across all treatments. Additionally, docking analysis revealed the potential regulatory role of WA and its interaction with aspartate aminotransferase, in comparison to the docking data for FLU, which serves as a standard inhibitor for aspartate aminotransferase. These findings suggest that WA can inhibit cell proliferation signaling by targeting ERK and EGF, thereby promoting programmed cell death (PCD) in treated cells. These data also demonstrate that WA, whether alone or in combination with other compounds, can regulate the production of pro-inflammatory cytokines in treated cells.

Keywords: Colorectal cancer, WA, TNF-α, IL-1β, ERK.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths and the third most diagnosed cancer globally. It affects a large number of individuals, particularly those over the age of 50 [1]. In Egypt, although estimates regarding the prevalence of CRC vary, it is reported to be the seventh most frequent cancer diagnosis, with an incidence rate of 9.8 cases per 100,000 people [2]. CRC is characterized by abnormal epithelial cell growth in the colon and gastrointestinal tract. The most common cause of death in CRC patients is metastasis to the liver, lungs, bones, brain, and spinal cord. Several factors, including genetics, family history, and lifestyle choices, can increase susceptibility to CRC at various ages [3]. Key risk factors include heredity, family history, gene mutations, an altered gastrointestinal microbiome, obesity, smoking, alcohol consumption, poor diet (high-fat, low-fiber), inflammatory bowel disease (IBD), gastrointestinal adenomatous polyps, and diabetes [4]. Colorectal cancer often originates from polyps, which are non-tumor lumps in the large intestine that may eventually transform into cancerous masses due to prolonged inflammation. Various therapeutic strategies have been explored to treat CRC, including radiation therapy, chemotherapy, surgery, targeted therapy, and immunotherapy. However, chemotherapy drugs often have harmful side effects, and cancer cells frequently develop resistance to these treatments [5]. As such, there is a need for more effective treatment options.

Natural plant-based compounds are gaining attention as potential alternative treatments for cancer. Several phytochemicals have shown promising results in cancer therapy due to their safety and efficacy. Currently, a few phytochemicals, such as taxanes (paclitaxel), etoposide, and vinca alkaloids (vincristine and vinblastine), are used clinically and have proven to be effective in cancer treatment [6]. One promising natural compound is Withaferin A (WA), a bioactive ingredient derived from the Ashwagandha plant. WA has demonstrated significant anti-cancer properties in various in vitro and in vivo studies [7, 8]. It works by

inhibiting angiogenesis, metastasis, tumor cell proliferation, and promoting apoptosis and the targeting of cancer stem cells. Additionally, WA has shown radiosensitizing and chemosensitizing properties [9]. However, additional research on its pharmacokinetics, safety, and toxicity is needed to facilitate its clinical development.

Fluvastatin (FLU), a lipid-lowering drug, inhibits the conversion of HMG-CoA to mevalonic acid, which is crucial in various cellular processes such as signaling, cell integrity, and cell cycle regulation [10]. These processes impact cancer cell development, spread, and metastasis. FLU has been shown to prevent breast cancer proliferation and reduce angiogenic and metastatic properties. However, FLU has a low oral bioavailability (~24%) and a short half-life, leading to potential side effects, including hepatotoxicity and neuropathy, when used for cancer treatment [11].

The MAPK/ERK pathway, also referred to as the Ras-Raf-MEK-ERK pathway, is a sequence of proteins within a cell that relays signals from a receptor on the cell's surface to the DNA in the cell's nucleus [12]. The signaling process begins when a molecule binds to the receptor on the cell's surface and concludes with the DNA in the nucleus activating a protein that triggers changes in the cell, such as cell division and autophagic machinery [13]. This pathway includes several proteins, such as mitogen-activated protein kinases (MAPKs), originally known as extracellular signal-regulated kinases (ERKs), which communicate by adding phosphate groups to neighboring proteins, thereby acting as "on" or "off" switches [14]. When a protein in this pathway mutates, it can become locked in the "on" or "off" position, a key event in the development of many cancers. In fact, components of the MAPK/ERK pathway were initially discovered in cancer cells, and drugs that can reverse the "on" or "off" state are being explored as potential cancer treatments [15]. The ERK signaling pathway plays an essential role in cell growth, differentiation, and proliferation. It is involved in various processes, including phosphorylation and nucleocytoplasmic shuttling. Additionally, the epidermal growth factor receptor (EGFR), part of the ErbB family of receptor tyrosine kinases, is linked to cancer development and progression [16]. Cytokines like IL-1β and TNF-α also affect cancer cell proliferation, metastasis, and immune responses, and their regulation presents potential therapeutic opportunities for cancer treatment [17].

This study sought to investigate the pharmacological effects of FLU and WA, both individually and in combination, on colon cancer cells using the Caco-2 cell line, and to compare these effects with their impact on normal colon epithelial cells (NCM-460 cell line).

MATERIAL AND METHODS

Cell lines

Normal human colon mucosal epithelial cells (NCM-460) and colon cancer cells (Caco-2) were sourced from Vacsera, Doki, Egypt. FLU and WA were purchased from TCI Chemicals, Tokyo. NCM-460 and Caco2 were maintained in RPMI 1640 medium (Gibco RPMI 1640), supplemented with 5% heat-inactivated bovine serum albumin (BSA), 4 mM sodium pyruvate, and 4 mM L-glutamine. All cell lines were incubated at 37 °C in a 5% CO2 atmosphere. Images of the cultured cells were captured and analyzed using a Zeiss A-Plan 10X inverted microscope.

Cell viability and cytotoxic concentration 50% (CC_{50})

The cytotoxicity of WA, FLU, and their combination was evaluated using the NCM-460 and Caco2 cell lines. Cells were cultured in 96-well plates in a CO2 incubator at 37°C, with a density of 10×10^3 cells per well. The drugs were added to the cells at various concentrations (ranging from 0 to 160 µM) and incubated overnight. Cell viability and cytotoxicity were assessed using Sigma-Aldrich's MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell growth assay. Following the manufacturer's protocol, the amount of formazan dye formed was measured by absorbance at 570 nm, allowing the determination of cell viability and the calculation of the CC50 value [18].

Lactate dehydrogenase (LDH) production

The amount of LDH released into the liquid media from cultured cells was quantified using an LDH assay kit (Abc-65393). To perform the assay, 100 µL of lysed cells was incubated with 100 µL of LDH reaction mix for 30 minutes at room temperature, and the absorbance at 450 nm (OD450) was measured. The procedure was followed according to the manufacturer's instructions. The average values of the treated cells were then normalized by dividing them by the corresponding values of the control group, and the resulting values were expressed as fold changes.

Proliferation assay

We assessed cell morphology using an inverted microscope. To promote cell growth, duplicate samples were seeded on a 6-well plate at a density of 10×10^4 cells per well. The number of surviving cells was determined by a hemocytometer. Subsequently, the old media was removed, and the cells were trypsinized by adding an appropriate volume of trypsin and incubating for three minutes at 37°C. Afterward, the cells were washed twice with PBS. The trypsinized cells were then resuspended in the required volume of complete RPMI medium, and their morphology was examined under the inverted microscope [19, 20].

Enzyme-linked immunosorbent assay (ELISA)

Human TNFα and IL-1β levels were measured using ELISA kits (Abcam 46032 and Abcam 46042).

Caco-2 cells were cultured overnight in 96-well plates. After treating the cells with 500 µg/mL of the relevant extract, they were incubated at different time points (0, 6, 12, 24, 36, and 48 hours). At each time point, the cells were lysed using 1X cell lysis buffer (Invitrogen, USA). A 100 µl sample of the lysate was added to the ELISA plate, followed by 100 µl of the control solution and 50 µl of the 1X biotinylated antibody. The mixture was incubated for 2 hours at room temperature. After incubation, 100 µl of 1X streptavidin-HRP solution was added to each well and incubated in the dark for 30 minutes. Following this, 100 µl of TMB substrate solution was added to each well and incubated for 15 minutes at room temperature. Finally, 100 µl of stop solution was added to each well, and the absorbance at 450 nm was measured [21, 22].

Gene expression levels were assessed by qRT-PCR using total cellular RNA that had been extracted using TriZol (Invitrogen, USA) and purified using an RNA purification kit. M-MLV reverse transcriptase produced cDNA using 1 g of total RNA (Promega, USA). Using the QuantiTect-SYBR-Green PCR Kit (Qiagen, USA) and the primers listed in Table (1), we measured the expression of ERK and EGF mRNA. The housekeeping gene, GAPDH mRNA expression level, was utilized to standardize real-time PCR findings. The PCR reaction system consisted of 10 µL of SYBR green, 0.2 M of each primer, 0.25 µL of RNase inhibitor, 25 U/µL of nuclease-free water, and 2 µL of synthesized cDNA. 35 cycles of amplification at the following temperatures and timings were conducted after a 5-minute denaturation at 94 °C. The followed by 35 cycles of amplification at the following temperatures and times: 30 seconds of denaturation, 15 seconds of annealing at 60 ℃, and 30 seconds of extension at 72 ℃ [23, 24].

Quantitative real-time PCR

Table 1: Oligonucleotides sequences used for mRNA quantification of indicated genes

Description	Primer sequences 5'-3'
ERK sense	AACATCAGACCTACTGCCAGCGC
ERK antisense	CGCAGGATCTGGTAGAGGAAGT
EGF sense	AGAGTCGGATTCGCCGCCGCA
EGF antisense	GACGGCATGGTGCAGGGATCT
GAPDH-sense	AACATCAGACCTACTGCCAGCGC
GAPDH-antisense	CGCAGGATCTGGTAGAGGAAGT

Flow cytometry analysis

The expression of EGF and ERK transcription factor in Caco2 cells treated with WA and FLU were studied using flow cytometry. As a result, PBS was used to clean the cells, and they were trypsinized for 3 minutes. After adding full RPMI media over the trypsinized cells, they were centrifuged for 5 minutes in 3000 rpm. Pellet was resuspended in PBS for washing and PBS containing 2% formaldehyde for fixation after supernatant was removed. For 3 minutes, the cells were resuspended in PBS containing 0.1% Triton-X-100 for permeabilizing them. The cells were resuspended in PBS mixed with 1% BSA and the dilution antibodies and incubated overnight at 4°C; Rabbit polyclonal anti-ERK (Cell Signalling Technology, USA) (1-1000), and mouse monoclonal anti-EGF (Santa Crouse, USA) (1-1000). The cells were washed and centrifuged before being resuscitated in PBS containing 1% BSA and 1-5000 secondary antibodies include goat anti-rabbit IgG (Alexa Fluor 488, Abcam, USA) and goat anti-mouse IgG (Alexa Fluor 594, Abcam, USA). Finally, the protein levels were measured using the flow cytometry assay (BD Accuri 6 Plus) on a resuspended pellet in 500 µL PBS [25].

Molecular docking

The SwissDock online tool was used to perform docking analysis on molecules, employing the systematic structures of FLU and WA, with the crystal structure of aspartate aminotransferase as the target oncoprotein (available at https://www.rcsb.org/structure/3omv from the RCSB Protein Data Bank). Using the default settings, the ligands (FLU and WA) were docked into the enzyme's binding sites. The docking poses were then exported for further visual inspection. SwissDock automatically calculated the docking scores, binding affinities, and free energy for each pose.

Data analysis

All data processing, graphing, and charting were performed using Excel software. Statistical analysis was conducted using a two-tailed t-test. The mRNA concentration was determined through a twofold delta Ct analysis of the qRT-PCR results, using the following steps: (1) To calculate delta-Ct, subtract the Ct value of GAPDH from the Ct value of the gene of interest. (2) The delta-delta Ct is obtained by subtracting the delta Ct of the experimental group from that of the control group. (3) The fold change is calculated using the formula 2^ (-delta-delta Ct). P-values less than 0.05 were considered statistically significant [26, 27].

RESULTS

WA treatment successfully reduced Caco-2 cell viability without detectable disturbance on NCM-460 cell viability.

To investigate the cytotoxic effects of WA and FLU on colon cancer cells (Caco-2) and non-tumorigenic colon cells (NCM-460), the cells were cultured in 96-well plates and exposed to a broad range of concentrations (0–160 µM) of WA, FLU, or their combinations. Notably, WA significantly inhibited the growth of colon cancer cells at a relatively low concentration (around 10 µM) but showed no detectable toxicity on NCM-460 cells, even at high concentrations. In contrast, FLU treatment significantly inhibited the proliferation of cancer cells but also showed toxic effects on normal NCM-460 cells. Notably, the combination treatment with WA and FLU resulted in a pronounced suppression of cancer cell growth while minimizing the cytotoxic effects on normal cells observed with FLU alone. Additionally, cells treated with WA or FLU showed that WA mainly inhibited colon cancer cell proliferation with a cytotoxic concentration (CC50) of 30 µM, whereas the CC50 for normal cells was much higher, at 160 µM. On the other hand, FLU or its combination with WA resulted in a CC50 of 30 µM for normal cells, but the dose-dependent effect on colon cancer cells was less predictable (Figures 1A and B and Table 2 and 3).

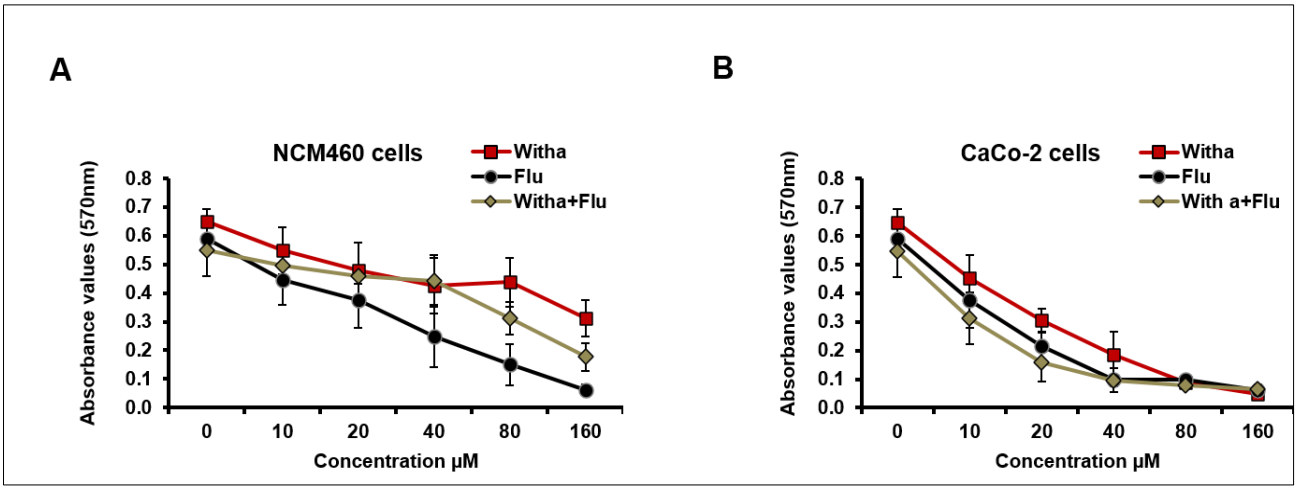

Figure 1: Cell viability rate upon treatment with indicated concentration of each drug

(A and B) The cell viability of NCM-460 and CaCo-2 cells treated with varying concentrations of WA and FLU, either individually or in combination, was assessed by the MTT assay to determine the CC50, with n = 4. Error pars indicate the standard deviation (SD) of three independent experiments.

Table 2: Statistical analysis of NCM-460 cells viability upon treatment by WA and FLU indicated by absorbance values

Treatment	Concentration (µM)	Mean absorbance	Standard deviation (SD)	*p*-value
WA	**0.0**	0.65	0.05	-
	10	0.55	0.08	0.077
	20	0.48	0.10	0.020
	40	0.43	0.10	0.005
	80	0.44	0.09	0.005
	160	0.31	0.06	0.004
FLU	**0.0**	0.59	0.01	-
	10	0.45*	0.09	0.015
	20	0.38**	0.10	0.0042
	40	0.25**	0.11	0.001
	80	0.15**	0.07	0.001
	160	0.06**	0.02	0.001
WA and FLU	**0.0**	0.55	0.06	-
	10	0.50	0.06	0.094
	20	0.46*	0.03	0.019
	40	0.44*	0.09	0.030
	80	0.31*	0.06	0.030
	160	0.18**	0.05	0.001

Table 3: Statistical analysis of Caco-2 cells viability upon treatment by WA and FLU indicated by absorbance values

Treatment	Concentration (µM)	Mean absorbance	Standard deviation (SD)	*p*-value
WA	0.0	0.65	0.05	-
	10	0.45**	0.08	0.006
	20	0.31**	0.04	0.0001
	40	0.19**	0.08	0.0001
	80	0.09**	0.01	0.0001
	160	0.05**	0.01	0.0001
FLU	0.0	0.49	0.01	-
	10	0.38**	0.10	0.0042
	20	0.22**	0.05	0.0001
	40	0.10**	0.04	0.0001
	80	0.10**	0.02	0.0001
	160	0.06**	0.02	0.0001
WA and FLU	0.0	0.55	0.09	-
	10	0.31**	0.09	0.0001
	20	0.16**	0.07	0.0001
	40	0.10**	0.02	0.0001
	80	0.08**	0.01	0.0001
	160	0.07**	0.01	0.0001

WA modified the morphology of cancer cells, affected the number of viable cells, and influenced LDH production.

We examined the morphological alterations and cytotoxic effects of WA, FLU, and their combination on both cancerous and normal cells. As illustrated in Figure 2, treatment of NCM-460 cells with 10 µM FLU resulted in pronounced morphological changes, indicating cytotoxicity. However, co-treatment with WA appeared to mitigate these effects. In contrast, normal colon cells treated with WA alone at the same concentration maintained a morphology comparable to that of the untreated control group. Meanwhile, Caco-2 cancer cells exhibited clear morphological changes following treatment with either drug alone or in combination, as observed under an inverted microscope. Moreover, treatment with 10 µM of either WA or FLU significantly reduced the number of living cancer cells compared to the control cells. Notably, while WA treatment did not lead to significant changes in the number of living normal cells, FLU treatment caused a substantial reduction in the number of normal cells (Figure 3A). Statistical analysis presented in Table 4 highlighted a significant reduction in the number of living cancer cells treated with either WA, FLU, or their combination, while the number of living normal cells significantly decreased in cells pretreated with FLU. Notably, LDH is released from the cytoplasm into the extracellular space as a result of cellular injury, either from internal processes or external factors. Due to its stability in cell culture media, LDH serves as an effective indicator of tissue and cell hurt, toxicity and necrosis. In Caco-2 cells, both treatments significantly increased LDH production, with a nearly 4-fold increase, while their combination led to a more than 6-fold increase compared to control-treated cells. The Triton-100X positive control resulted in an over 8-fold increase in LDH production (Figure 3B). Statistical analysis in Table 5 confirmed the significant increase in LDH production in cancer cells treated with WA, FLU, or their combination. Additionally, LDH production in normal colon cells pretreated with WA and FLU also significantly increased, although it was much lower in WA-treated cells (1.9-fold increase) compared to FLU treatment (6-fold increase). These findings further highlight WA's ability to induce cell death in colon cancer cells while sparing normal colon cells.

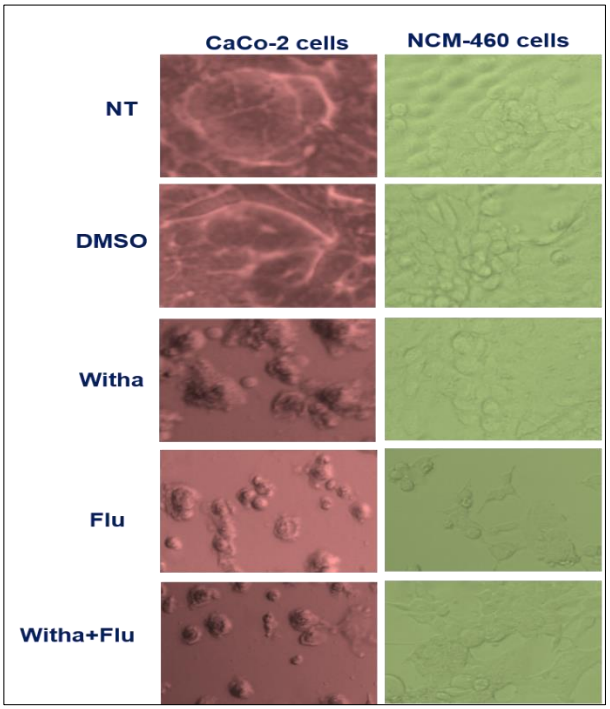

Figure 2: Cell morphology upon treatment with WA, FLU and their combination

Inverted microscopy images of cell morphology and changes in the population of cells upon treatment with either WA or FLU for 24 hours in comparison with the positive control represented by DMSO-treated cells and non-treated (NT) cells.

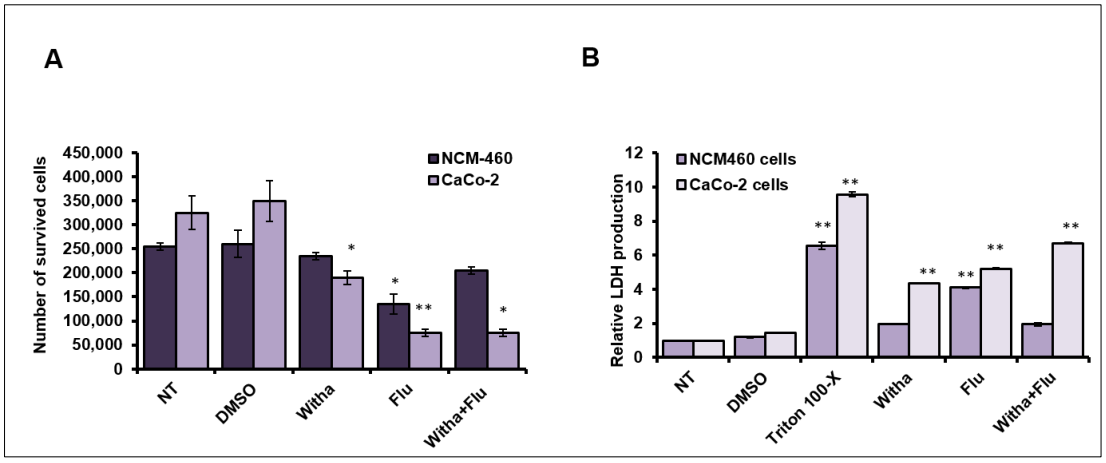

Figure 3: Number of living cells and LDH production in treated cells

(A) The number of surviving cells treated with either WA or FLU on NCM-460 and Caco-2 cells is shown, with error bars representing the SD from two independent experiments. **(B)** The cytotoxic effects of WA and FLU on NCM-460 and Caco-2 cells were assessed by measuring LDH release, which indicates cellular injury. Cells treated with WA and FLU were compared to the positive control (Triton X-100-treated cells) and the negative control (non-treated cells). The SD from four replicates is represented by error bars. Statistical significance was assessed using a two-tailed Student's t-test. Asterisks indicate significance: (**) for $P \leq 0.01$ and (*) for $P \leq 0.05$.

Table 4: Number of survived NCM-460 and Caco-2 cells after treatment

Cell lines	Analysis	Non-treated (NT) cells	DMSO-treated cells	WA	FLU	WA and FLU
NCM-460	mean	255000±7071	260000±28284	235000±7071	135000±21213*	205000*±7071
	P-values	-	0.831	0.106	0.017	0.019
Caco2	mean	325000±35355	250000±35355	190000±14142*	75000±7071**	75000±7071**
	P-values	-	0.588	0.038	0.01	0.0.01

Table 5: Relative LDH production in treated NCM-460 and Caco-2 cells

Cell lines	Analysis	Non-treated (NT) cells	DMSO-treated cells	Triton 100-x	WA	FLU	WA and FLU
NCM460	Mean	0.05±0.01	0.06±0.01	0.32±0.13	0.10±0.04	0.20±0.05	0.10±0.04
	Relative produced LDH	1.0	1.20	6.56**	1.95*	4.10**	1.95
	P-values	-	0.248	0.001	0.05	0.001	0.07
Caco2	Mean	0.04±0.01	0.05±0.01	0.34±0.21	0.15±0.02	0.18±0.05	0.24±0.09
	Relative produced LDH	1.0	1.46*	9.57**	4.36**	5.21**	6.71**
	P-values	-	0.05	0.004	0.001	0.001	0.001

WA and FLU inhibit proliferation transcription factor in treated colon cancer cells

To verify the role of WA and FLU in cancer cell death, we evaluated the expression levels of ERK, an oncogenic transcription factor, and EGF, a marker of proliferation, in Caco-2 cells treated with 10 µM of both drugs or their combination at both RNA and protein levels. As anticipated, the relative expression of both ERK (15%) and EGF (10%) was significantly reduced at the RNA level in cells treated with WA, compared to control cells, which showed ERK (55%) and EGF (35%) expression (Figure 4A and B). Similarly, FLU treatment led to a 50% reduction in the expression of both genes compared to the control-treated cells. Furthermore, the combination of WA and FLU resulted in a more pronounced decrease in ERK and EGF gene expression, with inhibition reaching 90% and 95%, respectively. Statistical analysis, presented in Table 6, indicated that the downregulation of ERK and EGF expression was highly significant in response to WA treatment and its

combination with FLU. Additionally, the concentration of produced EGF over time was measured using an ELISA assay, which demonstrated a marked reduction in EGF production in a time- and dose-dependent manner with WA treatment (Figure 5A). Flow cytometry was also employed to quantify the expression of ERK and EGF proteins at the single-cell level using specific primary antibodies for staining treated cells. The percentage of positive events was correlated with the relative fluorescent intensity of the Alexa Fluor signal from secondary antibodies. Notably, the expression levels of both ERK and EGF proteins were significantly reduced, with levels dropping to approximately 7% and 5%, respectively, compared to the high expression levels seen in control-treated cells (Figure 5B). Remarkably, both drug treatments were able to decrease the expression profiles of ERK and EGF in treated cells, with their expression detected in only about 15% of stained cells. Together, these results suggest that WA inhibits the expression of both ERK and EGF, thereby triggering PCD in the treated cells.

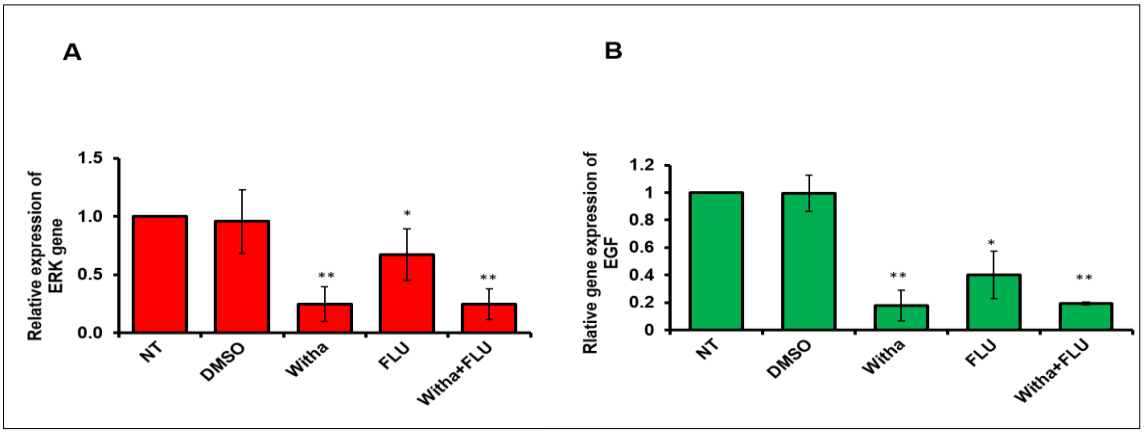

Figure 4: Quantification of ERK and EGF expression in Caco-2 cells treated with WA and FLU

(A and B) The fold change in steady-state mRNA levels of the ERK and EGF genes in Caco2 cells, compared to the negative control (non-treated, NT) cells. GAPDH mRNA levels were used as an internal control, and DMSO-treated cells served as positive control. Error bars represent the SD from two independent experiments. Statistical significance was determined using a student's two-tailed t-test, where (**) indicates a P value ≤ 0.01.

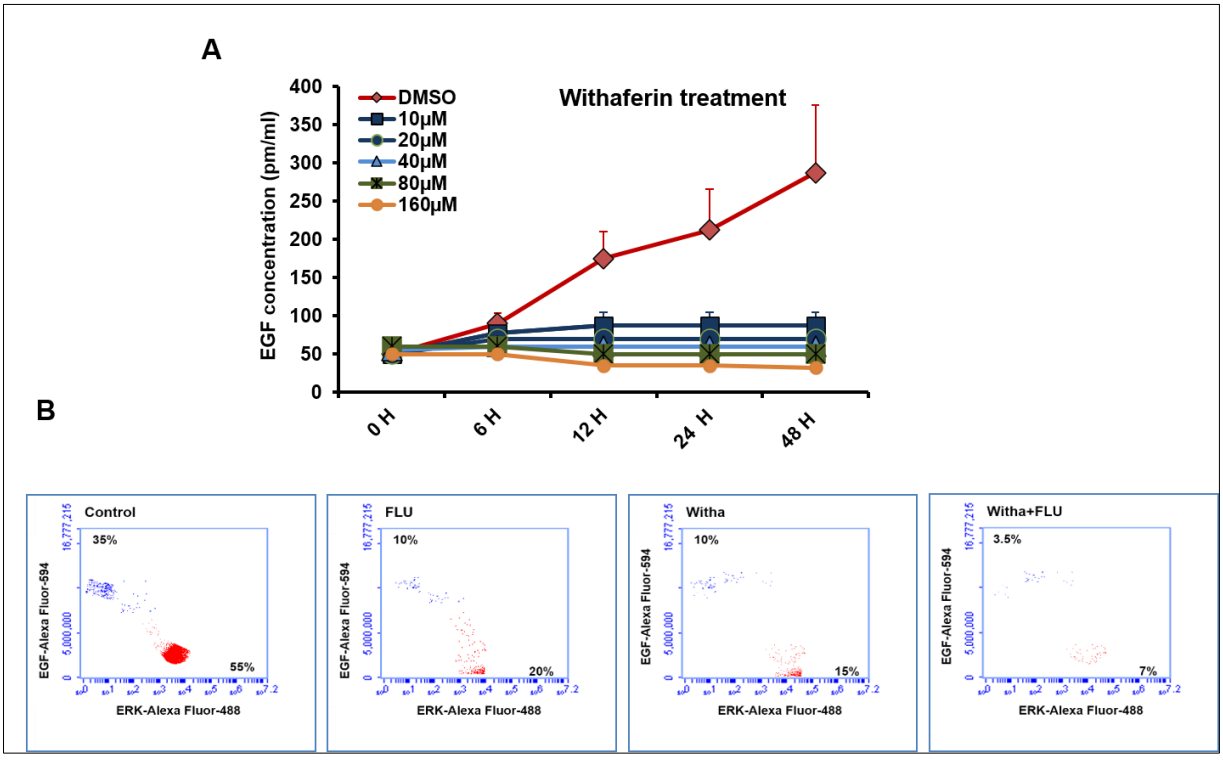

Figure 5: Quantified protein profile of ERK and EGF in WA and FLU treated cells compared to DMSO-treated cells indicated by ELISA assay and flow cytometry

Table 6: Quantification analysis of relative gene expression of ERK and EGF in treated Caco-2 cells with WA and FLU compared to DMSO treated and non-treated (NT) cells

Gene	Treatment	Fold changes	*P*-values	Gene	Treatment	Fold changes	*P*-values
ERK	**NT**	1.000±0.000	-	**EGF**	**NT**	1.000±0.000	-
	DMSO	0.956±0.273	0.84		**DMSO**	0.994±0.131	0.954
	WA	0.244±0.151*	0.019		**WA**	0.177±0.112**	0.009
	FLU	0.672±0.222	0.172		**FLU**	0.400±0.173	0.039
	WA and FLU	0.246±0.133*	0.015		**WA and FLU**	0.194±0.009**	0.001

(A) The concentrations of EGF (in pm/ml) produced over time in the fluid media of Caco-2 cells pretreated with 10 µM WA, compared to control-treated cells. Error bars represent the SD from four replicates. Data are from three independent experiments. (B) A flow cytometric assay quantified the protein expression profiles of ERK (in red dots) and EGF (in blue dots) in drug-treated cells, compared to control-treated (NT) and DMSO-treated cells.

WA induces IL-1β production while reducing TNF-α levels in treated Caco-2 cells.

To evaluate the impact of WA treatment on the production of inflammatory cytokines in treated cells, we measured IL-1β, a cytokine involved in regulating cell death, and TNF-α, a cytokine that induces necrosis, over time in response to various concentrations of WA using an ELISA assay. Notably, WA treatment resulted in a significant, time- and dose-dependent increase in IL-1β production in Caco-2 cells (Figure 6A). Conversely, TNF-α production was significantly decreased over time in cells pretreated with the indicated WA concentrations (Figure 6B). These results underscore the role of WA in modulating the production of the proinflammatory cytokine TNF-α and suggest its potential involvement in inducing PCD by stimulating IL-1β production in colon cancer cells.

Binding based-Cross docking analysis of WA and FLU ligands with aspartate aminotransferase crystal structure

The potential interactions between the aspartate aminotransferase protein, a crucial factor in the translation process of oncogenes, and the WA ligand were evaluated through docking analysis using the SwissDock online tool. This analysis assessed docking scores, binding affinities, and free energy between the ligand and the protein domain. As illustrated in Figure 7, the docking analysis identified several binding sites with high docking scores for the interaction between WA and the aspartate aminotransferase crystal structure, including sites recognized by FLU, the standard aspartate aminotransferase inhibitor. Specifically, two binding sites between WA and the aspartate aminotransferase crystal structure were identified, with binding affinities of 34 and required energies of -11 and -8 (kcal/mol), respectively (Table 7). In comparison, two binding sites within the aspartate aminotransferase crystal structure, interacting with FLU, exhibited binding affinities of 32 and required energies of -8 and -11 (kcal/mol). These findings suggest that WA treatment may interfere with the aspartate aminotransferase protein, potentially disrupting the delivery of aspartic acid during the translation process, which could consequently affect the translation of oncoproteins.

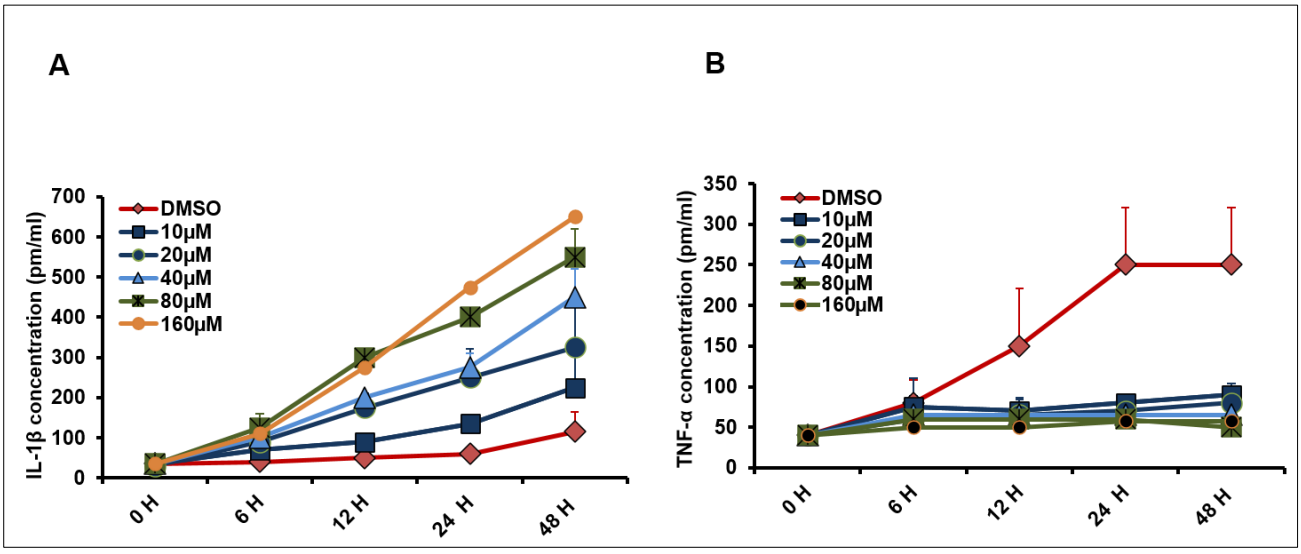

Figure 6: Levels of proinflammatory markers IL-1β and TNF-α in Caco-2 cells treated with WA

(A) The concentration of the cytokine IL-1β (pm/ml) in the culture media of Caco2 cells treated with WA at the specified time points, compared to the positive control (DMSO-treated cells) and the negative control (non-treated, NT cells). **(B)** The concentration of TNF-α (pm/ml) produced by Caco2 cells, showing an increase in a dose- and time-dependent manner after treatment with WA compared to the positive control (DMSO-treated cells) and the negative control (non-treated, NT cells). Error bars represent the SD of four different replicates.

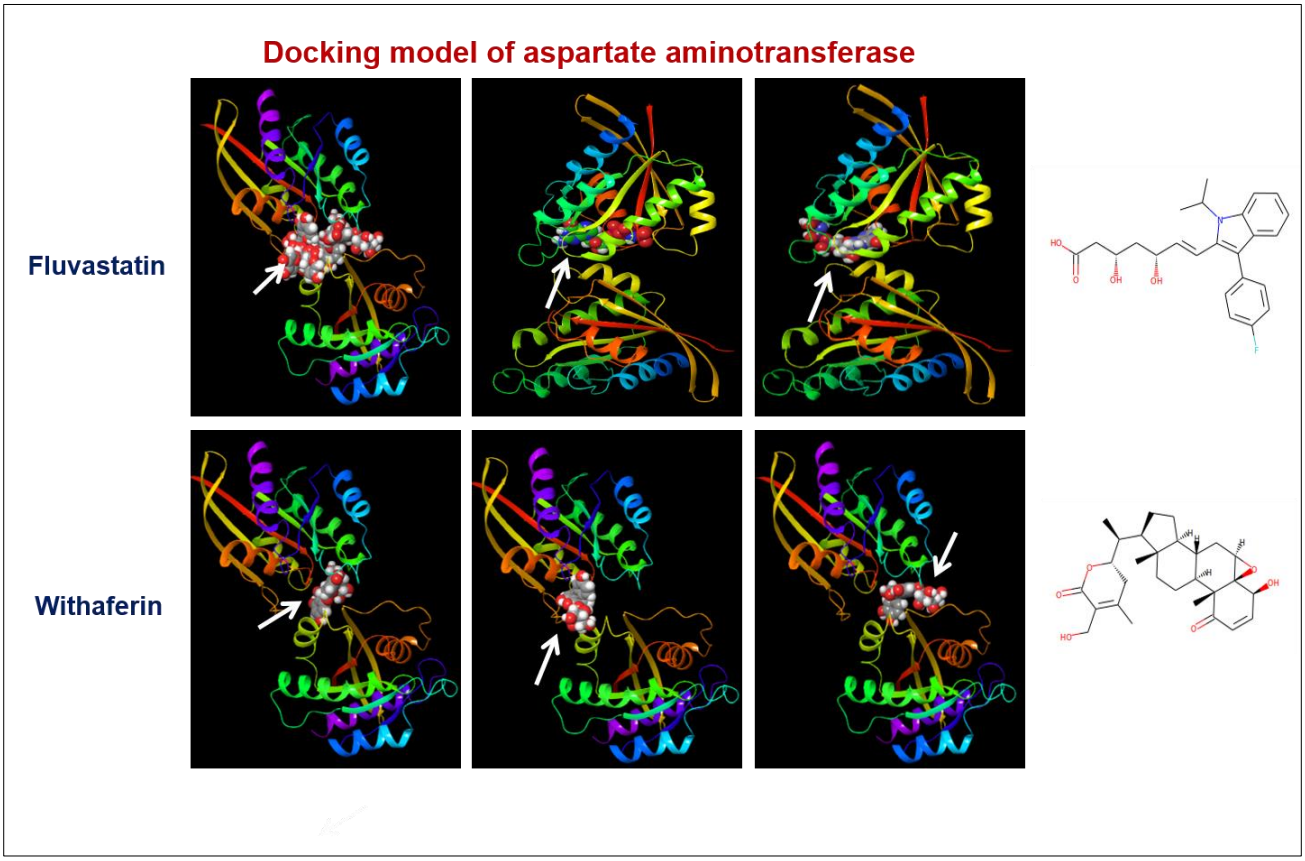

Figure 7: Docking Analysis of WA or FLU Ligands with aspartate aminotransferase crystal structure

The docking analysis reveals the potential binding affinity between WA and the aspartate aminotransferase protein ligand, as predicted by SwissDock software, and compares it with the original binding affinity of FLU, the standard aspartate aminotransferase inhibitor.

Table 7: Binding affinities and required energy in docking analysis of FLU and WA with aspartate aminotransferase

Aspartate aminotransferase domain	Binding affinity	Full Fitness (kcal/mol) From -To	Estimated ΔG (kcal/mol) From -To
Fluvastatin	32	-1110.21 -1250.57	(-10.07) (-8.50)
Withaferin	34	-1050.21 -1230.91	(-11.57) (-8.90)

DISCUSSION

Significant progress has been made in the treatment of CRC with promising medications, such as oxaliplatin, FLU, Sorafenib, and antibodies like bevacizumab and cetuximab. These therapies work by inducing mechanisms of PCD in cancer cells, which helps improve overall patient survival rates [28]. However, traditional chemotherapy's effectiveness is often compromised by the rapid development of drug resistance and various side effects. Recent advancements have shown that combination therapies offer numerous advantages over conventional treatments by reducing side effects and allowing lower medication dosages [29]. Despite the significant improvement in overall survival for CRC patients with combination therapy, toxicity remains a persistent issue, highlighting the need for more targeted treatments that minimize harm to healthy cells.

Natural compounds, particularly those derived from plants, have shown great potential due to their complex pharmacological activities [30]. These compounds have led to the discovery of promising anticancer agents for new drug development. When combined with licensed medications like FLU, natural compounds can selectively target colorectal cancer cells, enhance therapeutic outcomes, and improve patients' quality of life, all while causing fewer adverse effects on healthy cells. This study specifically focuses on the synergistic antitumor effects of WA, a promising natural anticancer compound, when combined with FLU in CRC cells. The goal of this study is to assess how WA impacts colorectal cancer and to elucidate its underlying mechanisms.

Our comprehensive analysis reveals that the combination treatment significantly reduces the viability of CRC cells, demonstrating a strong combination index that not only reduces the required dosage of the drugs but also maintains a safe toxicity profile in normal colon cells. Additionally, the results show that FLU and WA treatment led to inhibition of ERK and blocking of EGF production, which together contribute to induce PCD in the treated cells. This inhibition of specific biological pathways facilitates the efficient destruction of tumor cells via apoptosis. WA, which can be extracted from the root of *Withania frutescens* (a plant native to Europe), has shown similar phytochemical properties to *W. somnifera* [31]. Structural studies have identified key sites within WA, such as the unsaturated A-ring at C3, the epoxide at position five, and C24 in the E ring, which are susceptible to nucleophilic attack. These sites form covalent bonds with cysteine residues in proteins via a Michael addition alkylation reaction, ultimately inhibiting the activity of the target protein [32]. Interestingly, the C27 hydroxy group of WA does not significantly affect its biological activity and can be conjugated with biotin for protein recognition purposes [33].

The study investigated the cytotoxic effects of WA and FLU on colon cancer cells (Coca2) and non-tumorigenic colon cells (NCM-640). The results showed that WA significantly inhibited colon cancer cell proliferation at lower concentrations, with negligible toxicity in normal cells. In contrast, FLU selectively inhibited cancer cell proliferation, though it had detectable toxic effects on normal cells. WA displayed a cytotoxic concentration 50% (CC50) of 10 µM for colon cancer cells, while FLU and the chemotherapy drug 5-Fluorouracil (5-FU) also reduced cell viability in a dose-dependent manner. The IC50 values for 5-FU ranged from 4.9 µM to 14.2 µM in various CRC cell lines [10, 34]. However, in non-malignant colon cells (NCM-460), higher CC50 values of 50 µM and was observed for FUL. These results indicate that WA selectively targets cancer cells while minimizing harm to healthy cells. The combination of FLU and WA significantly influenced cell morphology and survival in both colon cancer and normal cells. WA treatment reduced changes in morphology without affecting normal cells as much as FLU did, suggesting its potential to prevent cancer cell proliferation. The study also revealed that WA reduced the viability of Coca-2 cells in a time- and concentration-dependent manner. Previous studies have documented that WA inhibits the Notch1-mediated pro-survival signaling pathway, causing apoptosis in CRC cells, such as HCT116 [35]. Moreover, WA did not affect the viability of prostate cancer cells or androgen-sensitive normal human fibroblasts, demonstrating its selective tumoricidal effects [36].

The combination of WA and hyperthermia has been shown to induce HeLa cell death via mitochondrial dysfunction and the downregulation of antiapoptotic proteins. Moreover, the combination of WA and other anticancer drugs like cisplatin has demonstrated synergistic effects in inhibiting cell viability, further enhancing the cytotoxic effects of cisplatin WA's ability to regulate inflammation is also significant [37]. It has been shown to reduce the production of proinflammatory cytokines like TNF-α and IL-6 in colon-treated cells, promoting programmed cell death in CRC cells. Furthermore, WA can suppress the expression of inflammatory markers such as COX-2 and IL-6, thereby exerting anti-inflammatory effects [38]. Overall, these findings highlight the potential of WA as a promising therapeutic agent in combination with other anticancer drugs, including FLU, for the treatment of colorectal cancer. WA's ability to selectively target cancer cells, modulate apoptosis, and reduce inflammatory pathways makes it an ideal candidate for enhancing the efficacy and safety of combination therapies in CRC treatment.

CONCLUSION

Our research aimed to investigate the biological functions and anticancer activities of WA. We tested various concentrations of WA, FLU, and their combinations (ranging from 0 to 160 µM) for their potential effects on colon cancer cells (Caco-2 cell line) and non-tumorigenic colon cells (NCM-460). These treatments were compared to untreated and DMSO-treated cells. The cytotoxic effects of WA on Caco-2 cells were assessed by measuring cell viability and lactate dehydrogenase (LDH) levels, which serve as an indicator of cell damage. Additionally, we examined the expression of ERK (an oncogenic transcription factor) and epidermal growth factor (EGF, a marker for proliferation signaling) at both RNA and protein levels in treated Caco2 cells. Flow cytometry was used to quantify ERK and EGF proteins at the single-cell level using specific primary antibodies for staining. The relative protein expression was determined by correlating the percentage of positive events with the fluorescent intensity of secondary antibody signals. Interestingly, at lower concentrations (around 10 µM), WA significantly impacted colon cancer cells, showing minimal toxicity to both cancerous and normal cells (NCM-460). However, at higher concentrations, FLU

selectively inhibited cancer cell proliferation, though it exhibited noticeable toxicity to both cancerous and normal cells. WA, on the other hand, induced toxicity specifically in colon cancer cells with minimal effects on normal cells. Moreover, WA treatment led to a time- and dose-dependent decrease in IL-1β and TNFα production. We also observed the downregulation of ERK and EGF gene expression across all treatments. Docking analysis revealed that WA potentially regulates aspartate aminotransferase, showing interaction data similar to FLU, a standard inhibitor of this enzyme. Our findings suggest that WA can inhibit cell proliferation signaling by targeting ERK and EGF, promoting PCD in treated cells. These results demonstrate that WA, either alone or in combination with other compounds, can regulate pro-inflammatory cytokine production in treated cells.

Author Contribution Statement

T.R., A.S., N.E., and A.E. A.A.E., performed the experiments. A.A. and A.G. helped in supervision and conceptualizing experiments. H.K. designed the research plan, supervised overall research, provided and interpreted the results. H.K. organized and wrote the manuscript.

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Ethical Statement: The current work has been approved by the Ethical Committee of, Faculty of Biotechnology, University of Sadat City.

Availability of Data and Materials: The data support these findings are available from the corresponding author upon reasonable request.

Conflicts of Interest: All authors declare that there are no conflicts of interest.

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