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Urolithin A Targets Both PI3K/p-AKT/mTOR and p-c-RAF/MEK/p-ERK Signaling Pathways in Colorectal Cancer

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ABSTRACT

Urolithin A (UA) emerges as a promising natural anticancer agent and adjunct in treating colorectal cancer (CRC). This study aims to delve into the mechanism of action of UA in CRC cells. In this investigation, two colon cancer cell lines, namely HT29 and SW620, underwent treatment with specific cellular apoptotic inhibitors preceded by UA pre-treatment. The evaluation of UA's mechanism in CRC cells involved flow cytometry and western blotting. The findings revealed that apoptotic inhibitors U0126 and LY294002 mitigated the apoptotic effect of UA across all cell lines. Furthermore, in HT29, elevated levels of p-ERK and p-AMPK were observed. In SW620, the expression levels of phosphorylated AKT and the cellular proliferation regulator mTOR decreased significantly ($P < 0.001$), while the levels of the tumor suppressors, p-c-RAF and p-PTEN, increased. These observations suggest that UA targets the p-c-RAF/MEK/p-ERK signaling pathway in the early stages of CRC, while its mechanism in metastasis is contingent on the PI3K/p-AKT/mTOR pathway, as well. This study presents the initial evidence elucidating UA's anticancer properties by influencing these signaling pathways during both the early stages and metastasis of CRC.

INTRODUCTION

Colorectal cancer (CRC) impacts the lower digestive system, encompassing the small intestine, large intestine, and rectum (Rawla *et al.*, 2019). CRC is a significant health concern globally, and its prevalence can vary across regions, including the Middle East. The Middle East is a diverse region with variations in lifestyle, dietary habits, and healthcare infrastructure, all of which can impact cancer incidence and outcomes (Elwali *et al.*, 2023). The intricate regulatory mechanisms in CRC pathogenesis involve multifaceted levels of communication, cascades, crosstalk, and extensive networking (Wan *et al.*, 2020). The primary causative factor in CRC is believed to be the interaction of cytokines, acting as chemical mediators of inflammation; cytokine receptors, present on the surface of various cell types; secondary messengers, responsible for transmitting signals from the cell surface to the cellular interior; and transcription factors, regulating the expression of numerous genes associated with CRC (Alam *et al.*, 2018).

CRC treatment using natural products has gained attention as researchers explore alternative and complementary approaches to conventional therapies. Natural products, derived from plants, herbs, and other sources, often contain bioactive compounds with potential anti-cancer properties (Liberal *et al.*, 2017; El-Wetidy *et al.*, 2021; Layos *et al.*, 2022). It's important to note that while these natural products show promise, further research and clinical trials are needed to validate their efficacy and safety in CRC treatment. Moreover, natural products should not be considered as a replacement for conventional medical treatments, but rather as complementary strategies or preventive measures. Patients should consult with healthcare professionals before incorporating these products into their cancer treatment plans. Several studies showed that ingestion of hydrolyzed tannins, found in pomegranates, walnuts, raspberries, blueberries, and others, by certain gut microflora results in the production of specific metabolites called Urolithins (Djedjibegovic *et al.*, 2020; Kujawska and Jodynys-Liebert, 2020; Al-Harbi *et al.*, 2021; D'Amico *et al.*, 2021; Chen *et al.*, 2022; Wang *et al.*, 2023). These metabolites had significant biological effects (D'Amico *et al.*, 2021; Chen *et al.*, 2022; Wang *et al.*, 2023). Because of its safety, stability, and different biological activities, the most promising metabolite was Urolithin A (UA). UA is a microbiota-derived metabolite produced by the metabolism of the polyphenols ellagitannins and ellagic acid by the gut microbiota (Kujawska and Jodynys-Liebert, 2020). According to numerous studies, UA exhibits antioxidative, anti-inflammatory, antiproliferative, and proapoptotic properties (Djedjibegovic *et al.*, 2020; Kujawska and Jodynys-Liebert, 2020; Al-Harbi *et al.*, 2021; D'Amico *et al.*, 2021).

UA has been associated with anti-inflammatory effects, potentially inhibiting inflammatory pathways that contribute to cancer development. Chronic inflammation is a known risk factor for CRC, and compounds

with anti-inflammatory properties may modulate these pathways (Grivennikov *et al.*, 2010; Vini *et al.*, 2021). UA is known for its antioxidant properties, which may help neutralize reactive oxygen species (ROS) and reduce oxidative stress. High levels of oxidative stress can contribute to DNA damage and mutations, promoting cancer development (Federico *et al.*, 2007; Djedjibegovic *et al.*, 2020).

UA may interact with various cellular signaling pathways involved in cancer development and progression. Studies on other polyphenols suggest that UA might influence cell cycle regulation, apoptosis, and other CRC-related pathways (González-Sarrías *et al.*, 2010, Al-Harbi *et al.*, 2021). Several studies proposed several signaling pathways to define the anticancer activities of the UA. In lung cancer cells A549 and H460, UA suppressed the Kirsten-Rat Sarcoma Viral (K-RAS) oncogene homolog, activating p53 and inhibiting the epithelial-to-mesenchymal transition (Cheng *et al.*, 2021). The PI3K/AKT/mTOR signaling pathways were modulated in Pancreatic Ductal Adenocarcinoma cells treated with UA, which targeted K-RAS-regulatory kinases (Totiger *et al.*, 2019). However, UA did not affect HPNE, which are normal pancreatic epithelial cells (Totiger *et al.*, 2019).

UA treatment has also been shown to improve mitochondrial activity. Mitochondrial failure is characterized by inadequate ATP synthesis, DNA mutation, ROS production, and ultrastructural changes in organelle shape (San-Millán, 2023). Previous research discovered that UA increased mitophagy and increased *Caenorhabditis* worm life span as well as rodent muscle power (Ryu *et al.*, 2016).

Several studies have proposed diverse signaling pathways to elucidate the anticancer mechanism of UA. A previous study claimed that UA might inhibit the proliferation of the UMUC3 bladder cancer cell lines by deactivating the proliferation cascade PI3K/AKT and downregulation of the Mitogen-Activated Protein Kinase

(MAPK) (Liberal *et al.*, 2017). In the macrophages exhibited to the inflammatory agent, lipopolysaccharide, UA suppressed the expression of the Nuclear Factor kappa-light chain enhancer (NF- κ B) of activated B cells and other proinflammatory genes of the autophagic pathways (Boakye *et al.*, 2018). In the study conducted by Sharma and colleagues (2010), UA was found to deactivate the Wnt pathway, which is necessary for CRC progression. However, it is noteworthy that some colon cancer cells, such as HCT116, exhibited negligible effects in these pathways (Norden and Heiss, 2019).

It's essential to note that the field of UA research, especially in the context of CRC, is evolving. New studies may provide more detailed insights into its molecular mechanisms and therapeutic potential. As a result, the current study sought to evaluate UA's apoptotic impact in the presence of various cellular inhibitors, to determine its precise mode of action in CRC.

MATERIALS AND METHODS

1-Cell Culture And Treatment with UA:

In the current study, two CRC cell lines were used. The CRC adenocarcinoma cells; HT29, and the metastasis CRC cells; SW620, were obtained from (ATCC, MNZ, Virginia, USA). Cells were maintained in complete culture media, as been described before (Napolitano *et al.*, 2015; González-Sarrías *et al.*, 2016)

UA was purchased as a lyophilized powder from (MedChemExpress LLC, NJ, USA). The treatment dosage was 100 μ M to get the maximum apoptotic effect, as shown by El-Wetidy and colleagues (2021).

2-Identification of Signaling Pathway Through Apoptotic Pathway Inhibitors:

The Annexin V/propidium iodide (PI) kit employed for the detection of apoptosis/necrosis levels by flow cytometry (Invitrogen, Thermo Fischer Scientific, OR, USA) was employed to discern the signaling pathway in the presence of apoptotic pathway inhibitors. The inhibitors used were U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene), SP600125 (1,9-Pyrazoloanthrone, Anthrapyrazolone),

LY294002 (2-(4-Morpholinyl)-8-phenyl-1 (4 h)-benzopyran-4-one hydrochloride) (Harikrishnan *et al.*, 2018), and NAC (N-Acetylcysteine) which is known to reduce ROS production and prevent hemin-induced ferroptosis or inhibit various apoptotic pathways (Mlejnek, 2022).

In brief, cells were plated at the confluency of 3×10^5 cells per well for 24 hours pre-treatment with UA (El-Wetidy *et al.*, 2021). On the next day, the cells were treated with 10 μ M of one of the inhibitors and incubated at 5% CO₂ incubator and 37°C. Subsequently, the cells were treated with UA (100 μ M) for 24 hours. Both floating and adhering cells were extracted, pooled, and incubated for 15 minutes on ice in the dark with Annexin V-FITC and PI. The BD FACSCalibur Cell Analyzer (BD Biosciences, CA, USA) was utilized as described previously (Crowley *et al.*, 2016; El-Wetidy *et al.*, 2021).

3-Western Blot Analysis:

On the experimental day, cells were lysed in Radioimmunoprecipitation Assay (RIPA) lysis buffer (Thermo Fischer Scientific, OR, USA) following the manufacturer's instructions. Immunoblotting analysis was performed as previously outlined (Cai *et al.*, 2024). A c-digit blot scanner (LI-COR, Nebraska, USA) facilitated the visualization of the blotted membrane. Estimation of the intensities of the resulting bands was assessed by the ImageJ (National Institutes of Health, USA). Various primary antibodies, including phosphorylated ERK 1/2 and phosphorylated AKT from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), phosphorylated 5'Adenosine Monophosphate-activated Protein Kinase 1 (AMPK1), phosphorylated Phosphatase and Tensin (p-PTEN) homologue, phosphorylated proto-oncogene serine/threonine-protein kinase c (p-c-Raf), and Mammalian Target of Rapamycin (mTOR) from Cell Signaling Technology, Inc. (Danvers, MA, USA), were employed to investigate the signaling pathways.

4-Statistical Analysis: Statistical analysis utilized the Statistical Package for the Social

Sciences (SPSS) software, version 21 (SPSS Inc., Chicago, IL, USA). Significance levels were set at less than 0.05.

RESULTS

To investigate the apoptotic pathway by which UA might induce its inhibitory

impact, we exposed the cells to four death suppressors that are specific to unique apoptotic pathways. In HT29, U0126 and LY294002 substrates protected around 79% and 76% of the cells, respectively, against the pro-apoptotic action of 100 μ M UA ($P < 0.01$) (Fig. 1).

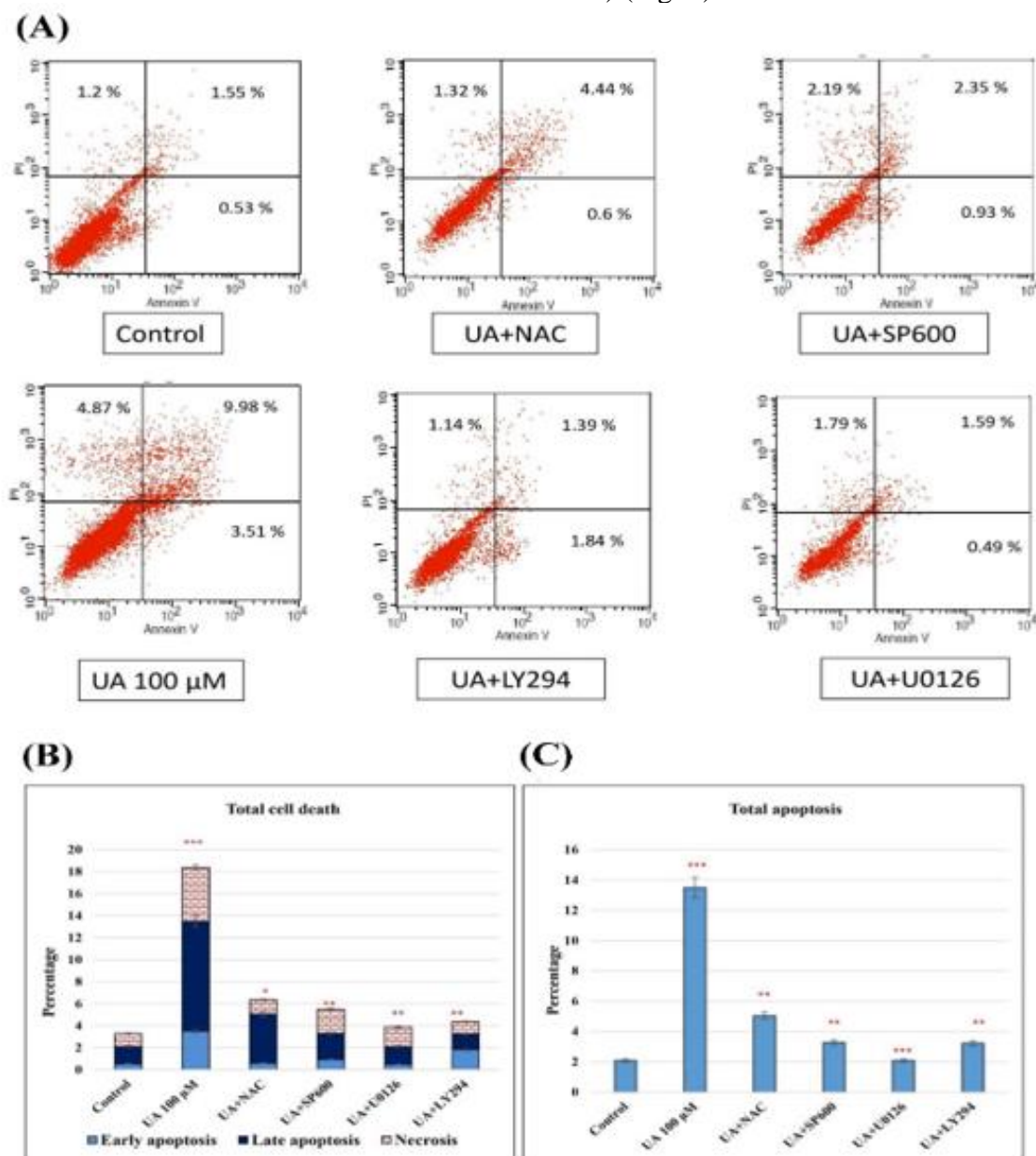


Fig. 1: Illustration of the Signaling Pathway Eliciting the Proapoptotic Effect of UA on HT29 Cells. The cells were subjected to a 1 μ M treatment of death inhibitors (NAC, SP600, U0126, and LY294) with a 1-hour pre-treatment of 100 μ M UA, followed by a 24-hour incubation period. The experiments were repeated three times for statistical analysis. Statistical analysis was conducted using the Chi-square test, and significance levels were denoted as * < 0.05 , ** < 0.01 , and *** < 0.001 . (A) Dot blots depicting the apoptotic effect assessed by Annexin V/PI assay, (B) Bar chart illustrating total cell death, (C) Bar chart presenting the overall apoptotic effect.

However, these inhibitors had an insignificant effect on metastatic SW620 cells. U0126 and LY294002 showed the greatest inhibitory impact, with 26.2% and 34.4%, respectively (Fig. 2). These data may suggest p-AKT/PI3K/mTOR as a UA signaling pathway in colon cancer.

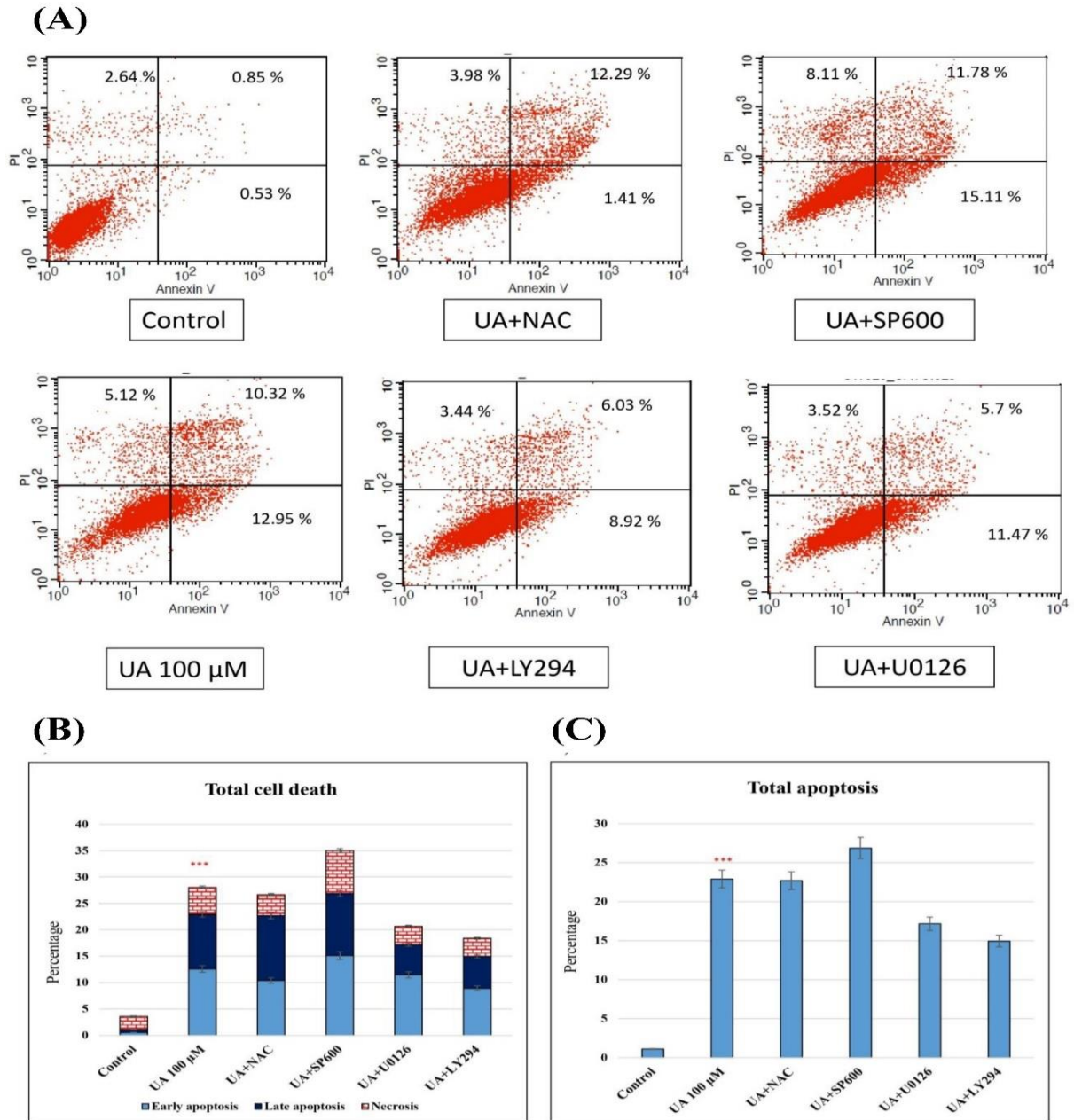


Fig. 2: Exploration of the Signaling Pathway Responsible for the Proapoptotic Impact of UA on SW620 Cells. The cells underwent treatment with 1 μM of death inhibitors (NAC, SP600, U0126, and LY294) with a 1-hour pre-treatment of 100 μM UA, followed by a 24-hour incubation period. The experiments were repeated three times for statistical analysis. Statistical analysis was conducted using the Chi-square test, indicating significance levels as * <0.05, ** <0.01, and *** <0.001. (A) Dot blots displaying the apoptotic effect assessed by Annexin V/PI assay, (B) Bar chart demonstrating total cell death, (C) Bar chart portraying the overall apoptotic effect.

The outcomes from western blot analysis once again underscored the robust impact of UA on the p-AKT/PI3K/mTOR pathway proteins. Specifically, in HT29 cells exposed to UA, the phosphorylated extracellular-signal-regulated kinase (p-ERK), which is necessary for the main cellular processes (Tangchirakhaphan *et al.*, 2018), manifested as p-ERK 1 (44 KDa) and p-ERK 2 (42 KDa). Their concentrations exhibited significant increases of 13.6%,

49.4% ($P < 0.001$), and 67.1% ($P < 0.001$) at 25, 50, and 100 μM of UA, respectively. Similarly, the expression levels of phosphorylated 5'adenosine monophosphate-activated protein kinase (p-AMPK), a regulator pivotal in maintaining cellular energy homeostasis (López-Cotarelo *et al.*, 2015), witnessed substantial increments of 30.8%, 62.2%, and 84.9% at 25, 50, and 100 μM of UA, respectively ($P < 0.001$) (Fig. 3).

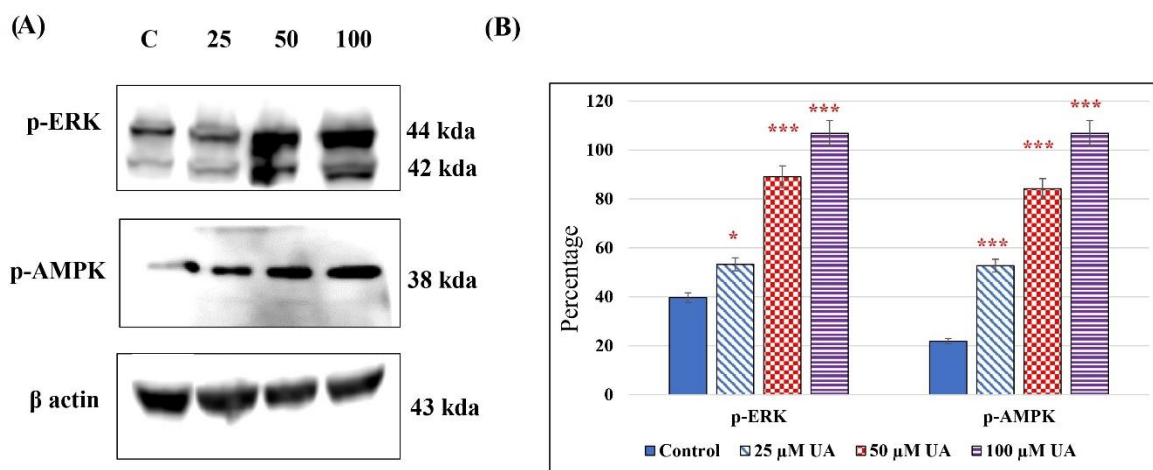


Fig. 3: Impact of UA on ERK/AKT Pathway Proteins in HT29 Cells. The investigation involved varying UA concentrations (0, 25, 50, and 100 μM) through immunoblot analysis. ImageJ software facilitated the quantification of densitometry. The experiments were repeated three times for statistical analysis. Statistical analysis was conducted using the Chi-square test, with a significance threshold of P -value < 0.05 . (A) Chemiluminescent images; (B) Bar chart presenting band intensities of each protein at different UA concentrations.

In SW620 cells, there was a noteworthy reduction in the levels of the apoptosis suppressor (p-AKT) and the cell growth activator Mechanistic Target of Rapamycin protein (mTOR) ($P < 0.001$). Conversely, the levels of the apoptosis suppressor Proto-Oncogene Protein (p-c-RAF) and the tumor suppressor (PTEN) exhibited substantial increases ($P < 0.001$), indicating a cellular defense mechanism against UA-induced apoptosis (Fig. 4A). The densitometric analysis demonstrated a decline

in the expression levels of p-AKT by 56.9%, 77.9%, and 100%, and mTOR by 60.1%, 63.6%, and 64.3% in cells treated with 25, 50, and 100 μM of UA, respectively ($P < 0.001$). In contrast, the expression levels of p-c-RAF increased by 24% ($P < 0.01$), 26.9% ($P < 0.001$), and 51.8% ($P < 0.001$) in cells treated with 25, 50, and 100 μM of UA, respectively. Similarly, the levels of p-PTEN significantly increased by 27.2%, 60.7%, and 95.1% in a dose-dependent manner (Fig. 4B).

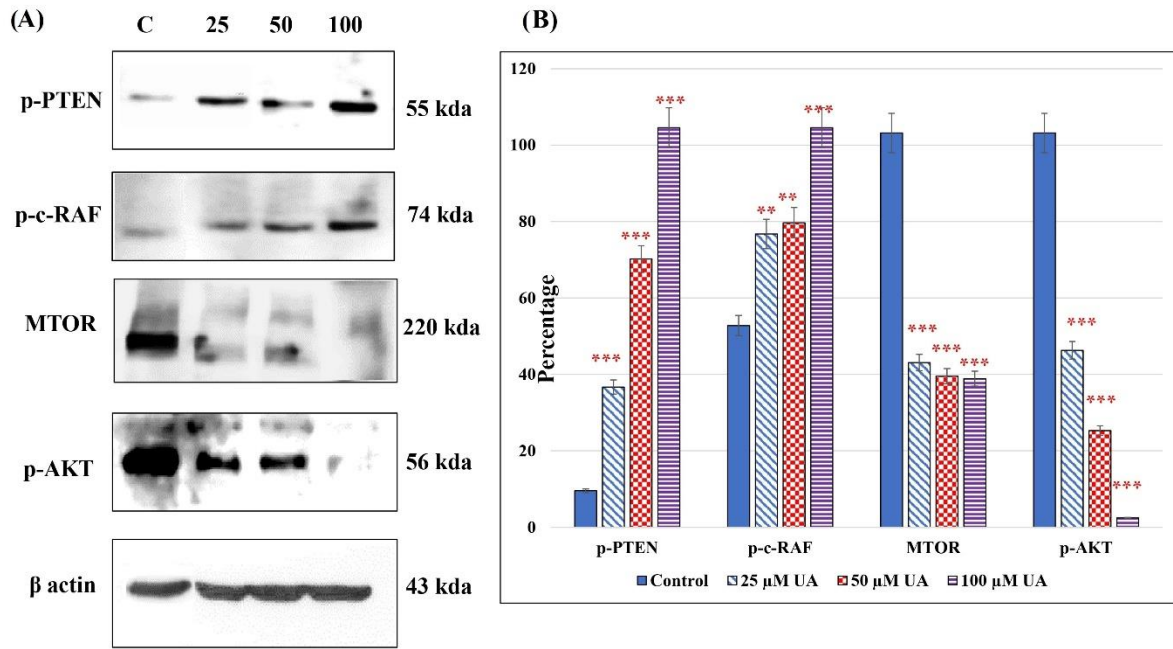


Fig. 4: Influence of UA on ERK/AKT Pathway Proteins in SW620 Cells. The study explored varied UA concentrations (0, 25, 50, and 100 μM) through immunoblot analysis. ImageJ software facilitated the quantification of densitometry. The experiments were repeated three times for statistical analysis. Statistical analysis was conducted using the Chi-square test, with a significance threshold of P -value < 0.05. (A) Chemiluminescent images; (B) Bar chart depicting band intensities of each protein at different UA concentrations.

DISCUSSION

Polyphenols, abundantly present in fruits, nuts, grains, and vegetables, have gained recognition for their role in cancer chemoprevention, exerting a combination of antiproliferative and pro-apoptotic effects (Moga *et al.*, 2016). Over the past two decades, polyphenols have been acknowledged for their diverse biological actions, impacting various signaling pathways implicated in the initiation, progression, and advancement of different malignancies (Alam *et al.*, 2018).

Recent studies have identified particular polyphenols that may protect against colon cancer. Resveratrol, a compound found in red grapes and wine, has been shown to decrease CRC growth by altering cell cycle progression and causing apoptosis (Jang *et al.*, 2022). Quercetin, found in onions and apples, has anti-inflammatory properties and may slow colon cancer cell growth (Zhang *et al.*, 2015). Curcumin, produced from turmeric, also has chemopreventive benefits by targeting various

signaling pathways implicated in colon cancer growth (Layos *et al.*, 2022). Furthermore, the interaction between phytochemicals and gut microbiota is gaining attention. UA, a metabolite derived from ellagitannins found in berries and nuts, has been associated with favorable effects on gut microbiota and potential protective effects against CRC (Marchesi *et al.*, 2016).

In the current study, the cancer cells were exposed to four apoptotic suppressors, U0126, SP600125, LY294002, and NAC pre-treatment with UA at the maximum dose (100 μM) in a study to examine the precise signaling route that would define UA proapoptotic actions in CRC. The flow Cytometry results revealed that U0126, which is an ATP- and ERK1/2 pathway inhibitor (You *et al.*, 2022) and protector against oxidative stress and ROS generation (Ong *et al.*, 2015), and LY294002, which is an AKT (Harikrishnan *et al.*, 2018) and phosphatidylinositol 3-kinase (PI3K) pathways inhibitor (Wang *et al.*, 2017). The immunoblotting study revealed that UA-

exposed HT29 cells had some protein modulation in the AKT/PI3K/mTOR pathway. After UA administration, the expression levels of p-ERK isomers p-ERK 1/2 were increased in HT29. p-ERK belongs to the "generic" MAPK signaling pathway, which controls and regulates a variety of cellular activities including proliferation, differentiation, and death.

U0126 serves as a chemical compound designed as a specific inhibitor targeting the MEK signaling pathway. The MEK kinase is a pivotal component of the MAPK/ERK signaling cascade, governing diverse cellular processes including cell proliferation, differentiation, and survival. The mode of action of U0126 involves the inhibition of MEK activity, leading to the blockade of downstream ERK activation (You *et al.*, 2022). This interference disrupts signal transmission within the MAPK/ERK pathway, a critical regulator of cell growth and division. Consequently, U0126 is frequently employed as a research tool in laboratories, facilitating the exploration of the specific roles played by the MAPK/ERK pathway in various cellular processes, notably, those implicated in cancer development (Ong *et al.*, 2018). Researchers utilize U0126 to examine the consequences of inhibiting MEK across diverse experimental contexts.

LY294002 is a chemical compound recognized as a specific inhibitor that targets the phosphoinositide 3-kinase (PI3K) signaling pathway (Wang *et al.*, 2017). PI3K is a crucial enzyme involved in diverse cellular processes, including cell growth, survival, and proliferation. By impeding the activity of PI3K, LY294002 disrupts downstream signaling events, particularly affecting the Akt/mTOR (protein kinase B/mammalian target of rapamycin) pathway (Hassan *et al.*, 2013). Commonly employed as a tool in laboratory studies, LY294002 aids in investigating the specific functions of the PI3K pathway and its involvement in cellular processes, with a particular emphasis on diseases characterized by pathway dysregulation, such as various cancers.

In the current research, the impact of UA on cellular signaling pathways was explored. At a concentration of 100 μ M of UA, the levels of phosphorylated adenosine monophosphate-activated protein kinase (p-AMPK), crucial for cellular energy balance, increased by 80%. This finding aligns with previous studies suggesting that ERK and ribosomal protein S6 kinase A (RSK) regulate AMPK phosphorylation, affecting its catalytic activity (Papa *et al.*, 2019; Yuan *et al.*, 2020). Activation of AMPK suppressed the development of kidney proximal tubular epithelial cells (PTECs) by depleting intracellular energy, consistent with our results, indicating that increased p-ERK1/2 levels triggered AMPK phosphorylation, leading to cell proliferation.

In SW620 cells, UA induced a strong apoptotic impact by decreasing the levels of apoptosis suppressors p-AKT and mTOR (cell growth activator). Conversely, levels of the apoptosis suppressor (p-c-RAF) and tumor suppressor (PTEN) increased, indicating significant apoptotic effects. AKT, a downstream effector of PI3K, is a well-known apoptotic suppressor, and AKT dysfunction or inhibition often accompanies higher levels of apoptotic indicators, such as cytochrome c and caspase-3 (Zhang *et al.*, 2013). mTOR, a regulator of cell survival and growth, was inhibited by UA, activating the PI3K-AKT pathway, which is a therapeutic strategy in various solid tumors. PTEN, a negative regulator of the PI3K pathway, slowed AKT cellular binding. Furthermore, c-RAF, a MAP3K in the ERK1/2 pathway, downregulated apoptosis suppressors and, when deactivated, impaired cellular division, migration, and differentiation while activating apoptotic generators.

Inhibiting the PI3K/AKT/mTOR and RAF/MEK/ERK pathways has emerged as a promising strategy for anticancer interventions. Preclinical studies have shown the synergistic anticancer effects of drugs targeting these pathways. UA, as evidenced in our study, induces apoptosis in CRC cells by modulating proteins in these signaling pathways. Its impact on reactive oxygen

species (ROS) generation and cell cycle arrest implies a role in modulating cellular DNA damage, particularly in the PI3K/AKT/mTOR pathway. Prior investigations have highlighted UA's antitumor activity in various cancers through different signaling pathways, emphasizing its potential as an adjuvant therapy in CRC treatment. Clinical trials across different CRC stages are essential to further validate these observations.

In line with our research outcomes, earlier investigations have indicated that Urolithin A (UA) exerts minimal influence on the Wnt, PI3K, and autophagic signaling pathways (Totiger *et al.*, 2019). Another study delineated UA's proapoptotic effects in SW620 cells, primarily through autophagy (Zhao *et al.*, 2018). Additionally, UA was demonstrated to induce cell cycle arrest and modulate MAPK signaling in Caco-2 cells (González-Sarrías *et al.*, 2009). Notably, pomegranate polyphenolics exhibited inhibitory effects on cancer growth in HT29 colon cancer cells by influencing both the miR-126/VCAM-1 and miR-126/PI3K/AKT/mTOR signaling pathways (Banerjee *et al.*, 2013).

Despite the useful insights gathered from this study, a few limitations should be recognized. The study was conducted exclusively *in vitro*, which may not have properly captured the dynamic interactions between tumor cells and the tumor microenvironment that occur *in vivo*. As a result, the effects of UA found in this study may vary when applied to animal models or human individuals. Also, the UA concentrations utilized in this investigation may not be physiologically relevant *in vivo*. Future research could look into the potential synergistic or antagonistic interactions of UA with other anticancer drugs or treatment modalities routinely utilized in CRC care. Recognizing these limitations is critical for guiding future research efforts targeted at better understanding the therapeutic value of UA in CRC treatment and overcoming translational barriers to practical implementation.

Conclusion:

In this study, we investigated the apoptotic pathway through which Urolithin A (UA) induces its inhibitory effects on colorectal cancer (CRC) cells. Exposure of HT29 cells to UA, followed by treatment with death suppressors, revealed significant protection against the pro-apoptotic action of UA, with U0126 and LY294002 substrates demonstrating notable inhibition. Conversely, these inhibitors showed insignificant effects on metastatic SW620 cells, suggesting a potential signaling pathway involving p-AKT/PI3K/mTOR in colon cancer. Further analysis via western blotting confirmed the impact of UA on the p-AKT/PI3K/mTOR pathway proteins. In HT29 cells, UA treatment led to significant increases in the concentrations of phosphorylated extracellular-signal-regulated kinase (p-ERK) and phosphorylated 5'adenosine monophosphate-activated protein kinase (p-AMPK) in a dose-dependent manner. Conversely, in SW620 cells, UA treatment resulted in decreased levels of apoptosis suppressor p-AKT and cell growth activator mTOR, while apoptosis suppressor p-c-RAF and tumor suppressor PTEN exhibited substantial increases, indicative of a cellular defense mechanism against UA-induced apoptosis. These findings underscore the intricate interplay between UA and various signaling pathways implicated in CRC progression and metastasis. UA's ability to modulate these pathways presents a promising avenue for further exploration of its therapeutic potential in CRC treatment.

Declarations:

Ethical Approval: Not applicable.

Conflict of Interest: The authors declare that they have no competing interests.

Authors Contributions: MIR and HH contributed to the study conception. MSE contributed to the design of the study, performing of experiments, data collection, analysis, and wrote the first draft of the manuscript. MIR and HH supervised the work, reviewed and edited the final manuscript. All authors have commented on the previous versions of the manuscript. All

authors have read and approved the final manuscript.

Availability of Data And Materials: The data that support the findings of this study are available from the corresponding author (Mohamad I. Rady) upon reasonable request.

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