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Elaeagnus Angustifolia Plant Extract Inhibits Proliferation and Invasion of Human Colorectal Cancer Cells Via EGFR1, Akt and β-Catenin Pathways

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Abstract

The Elaeagnus angustifolia (EA) plant used in traditional and alternative medicine possesses a broad spectrum of effectiveness including antioxidant, anti-inflammatory, antimicrobial, and anticancer potencies. Previous studies from our group reported EA's anti-cancer effect against human oral and breast cancer; yet its outcome against other cancer types including colorectal (CRC) remain unexplored. As such, we herein investigated the effect of aqueous EA flower extract on two CRC cell lines (HCT-116 and LoVo) with KRAS mutations to explore its plausible underlying anti-cancer mechanisms of action. Our results revealed that EA treatment inhibits cell proliferation and alters cell-cycle progression in both CRC cell lines. Moreover, treatment with EA extract significantly reduces the colony formation and invasive ability of HCT-116 and LoVo cells, a phenotype accompanied with a significant upregulation of E-cadherin and downregulation of vimentin and β--catenin, two important epithelial-mesenchymal transition (EMT) markers. Along this, our molecular pathway analysis further revealed a significant suppression in EGFR1, Akt, and β-catenin expression in EA treated CRC cell lines, presenting EGFR/RAS, PI3K/AKT, and Wnt/β-catenin as plausible EA targeted signaling pathways to control ongoing oncogenic events.

Keywords: Colorectal Cancer; Elaeagnus Angustifolia; EGFR/RAS; PI3K/AKT; Wnt/B-Catenin

Introduction

Colorectal cancer (CRC), which accounts for 10% of all cancer-related deaths worldwide, is categorized as the second deadliest cancer in the world, with approximately 900,000 deaths per year [1]. Furthermore, CRC represents the third most common type of cancer in males and the second most common in females [2]. Currently, CRC comprises 13% of all malignant tumors in the gastrointestinal tract [3]. Both genetic and environmental risk factors are associated with the onset of CRC. It is estimated that 35% of CRC incidences are sporadic [4]. Family history has a strong association with CRC occurrence, an example of which is familial adenomatous polyposis, a hereditary colon cancer without polyposis [5]. In addition, aging, lifestyle as well as pathogenic infections are among the risk factors allied with CRC. Although most CRC patients are older than 50 years, CRC tends to be more aggressive when diagnosed in young patients [6]. According to the underlying mechanism causing the disease, CRC could be either colitis-associated (CAC) or sporadic (SCC). CAC mostly occurs in the young population with inflammatory bowel disease or family history of CRC, accounting for more than 10% of deaths [7,8]. CAC occurs due to the accumulation of mutations in intestinal epithelial cells by inflammation sequence from dysplasia to carcinoma [9]. SCC, on the other hand, is the most common type of non-allied family history CRC as it develops with the individual's lifestyle and practices such as being obese, diabetic, and/or consuming alcohol [10,11].

Mechanistically, the occurrence of CRC is generally associated with multiple genetic and epigenetic variations including mutations in oncogenes and tumor suppressor genes [12]. KRAS is the most frequently mutated gene in CRC, and the occurrence of KRAS mutation usually indicates an early event of transitioning from normal epithelium to adenoma [13]. Although, KRAS mutations are detected at early stages of CRC, these mutations have been also reported to be significantly associated with metastasis. Patients with KRAS mutations, for instance, exhibit a higher tendency of lung metastasis compared to those with other type of mutations [14]. Recent studies have also shown that genetic mutations in KRAS affect the recurrence risk of CRC, and upon activation, leads to poor prognosis and low overall survival rate [15-17].

Similar to other types of cancer, traditional CRC control and treatment practices include surgery, chemotherapy, and radiotherapy [18]. Recently, however, several studies have flagged the adverse cytotoxicity and other side effects of some of those habitually adopted practices as well as the development of tumors resistance over the course of treatment [19,21], calling for novel, alternative, and effectual targeted therapies to prevent CRC progression and control its metastasis, with minimal to no side effects.

Natural resources including herbal products have been therefore, contemporarily presented as the called for alternative anti-cancerous treatment regimen [22]. Among various plant species, Elaeagnus angustifolia (EA), commonly known as Russian olive, is one example of a medicinal plant that could plausibly possess anti-CRC potential, given its richness in bioactive compounds including phenolic acids, steroids, and flavonoids, nutrients, and antioxidants [23]. In fact, a wide variety of flavonoids exist in EA, specifically flavonoid glycosides, which is found in different parts of EA [24]. Flavonoid glycosides such as Quercetin, Isorhamnetin were found to be abundant in EA and responsible for several biological activities such as antioxidant and anticancer [25-27]. Additionally, EA has been shown to be beneficial for human health and used for centuries in treating different ailments like tetanus [28,29]. Along the past years, several studies reported EA's therapeutic potency including its antifungal, antibacterial, antimutagenic, anti-inflammatory, antioxidant, and gastro-protective effectiveness [23,28,30]. Additional studies have also shown an anti--cancerous effect of EA flower extract against on oral and breast cancers [31,32]. As such, we explored herein the potential effect of EA aqueous flower extract on CRC cell lines and its plausible underlying mechanism of action.

Material and Methods

Plant Extract

EA flowers were collected and obtained from Montreal, Quebec, Canada in the month of June, and aqueous flower extract was prepared as previously described by our group [31]. In brief, flowers were dried at room temperature and stored in the dark. For fresh extract preparation, 3 g of dried flowers were boiled in 100 mL of water for 15 min-

utes at 100°C. The obtained solution was then cooled for 30 minutes, filtered using a sterile filter unit (0.45 µm pore size), and stored at 4°C. For experimental use, 25, 50, 100, 150, and 200 µL/mL dilutions were prepared in cell culture media.

Cell Lines and Cell Culture

HCT-116 and LoVo, CRC cell lines with KRAS mutations, used in this study were obtained from the American Type Culture Collection (ATCC). To test the selective effect of the EA aqueous flower extract against cancerous cell lines, we used normal colon cells immortalized by E6/E7 oncoproteins of HPV type 16 (NCE-1 E6/E7) as the control group [33]. All cell lines were cultured in the complete growth medium, Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (Life Technologies), and 1% penicillin and streptomycin (Life Technologies). Cells were maintained in an incubator at 37° C with 5% CO_2.

Cell Viability Assay (Alamarblue™ Assay)

The effect of EA aqueous flower extract on cell viability was tested against the two CRC cell lines (HCT-116 and LoVo) and the normal immortalized colon cell line (NCE-1 E6/E7) using AlamarBlue™ cell viability reagent (Invitrogen, Thermo Fisher Scientific). Briefly, in a 96-well plate, cells ($1x10^4$ cells/well) were seeded in 100 µL of DMEM media and incubated for 24 hours. For the control wells, 100 µL of DMEM media was added (negative control). For treated well, cells were incubated with increasing concentrations of EA extract (25, 50,100, 150, and 200 µL/mL) for 48 hours. Post treatment period,10 µL of AlamarBlue™ reagent was added to each well and incubated for 3 hours, after which the shift in fluorescence was measured at 570 nm (excitation) and 600 nm (emission), in a fluorescent plate reader (Tecan Infinite M200). Cell viability was then calculated based on the fluorescence of treated cells relative to the negative control.

Cell Morphology Analysis

HCT-116, LoVo and NCE-1 E6/E7 cell lines were seeded in a 6-well plate at a density of 100 x103 cells/well for 24 hours. The next day, old media was replaced with either fresh media (negative control) or media supplemented with 100 or 200 µL/mL of EA flower aqueous extract (treated groups) for 48 hours. Cell Morphological changes were examined using a Leica SP8 UV/Visible inverted microscope (Leica Microsystems, Wetzlar, Germany) and representative images were taken with a Leica DFC550 digital camera (Leica Microsystems, Wetzlar, Germany).

Cell Cycle Analysis using Flow Cytometer

Both CRC cell lines were seeded in 100mm Petri dishes at a density of 200 $x10^3$ cells/dish and incubated for 24 hours. To synchronize cells into the G0 phase of the cell cycle, all cells were starved for 24 hours with serum-free media. After starvation, cells were treated with 100 and 200 µL/mL of EA flower aqueous extract for 48 hours. Subsequently, all cells (floating and adherent) were harvested by centrifugation and the obtained pellet was washed twice with cold 1X Phosphate Buffer Saline (PBS). After washing, cells were fixed with 70% ice-cold ethanol and kept at -20°C overnight. For flow cytometric analysis, DNA obtained from cells was stained with 50 µg/mL FXCycle PI/RNase staining solution (Thermo Fisher Scientific) and then treated with RNase (50 µg/mL) (Thermo Fisher Scientific) at 37°C, for 30 minutes according to the manufacturer's protocol. Cell cycle analysis was performed using the BD Accuri™ C6 Plus flow cytometry (BD AccuriTM C6 Flow Cytometer,), and data was analyzed and quantified using the FlowJo™ V10 software.

Cell Invasion Assay

To study the effect of EA flower aqueous extract on HCT-116 and LoVo invasion ability, Boyden chamber plates (Corning) were used. In brief, serum free media was added to the lower chamber and cells were seeded at a density of 50 $x10^3$ cells/ well in the upper chamber with or without treatment for 48 hours. Post treatment period, cells were fixed with 3.7% formaldehyde for 10 minutes, washed 3 times with 1X PBS, stained with 5% crystal violet in ethanol, and visualized under the microscope. Images were taken with the Leica DFC550 digital camera (Leica Microsystems). For further validation of invaded cells, cells were stained with 4',6-Diamidino-2-Phenylindole (DAPI) and visualized under the EVOS fluorescence microscope. For quantification of invasive cells from different fields, Image J software was used.

Soft Agar Colony Formation Assay

To detect the ability of EA flower aqueous extract to prevent CRC cells colony formation, soft agar colony formation assay was performed as previously described [31]. In brief, DMEM medium with 0.4% agar was added in each well and HCT-116 and LoVo cell lines were seeded at a density of 100 $x10^3$ cells/well containing 0.3% agar plated on the top of the first layer for both control and treated groups. The formation of colonies was examined every 2 days for 4 weeks. Representative pictures were taken with the Leica DFC550 digital camera (Leica Microsystems) and formed colonies in each group were quantified from different fields.

Western Blot Analysis

For protein extraction, HCT-116 and LoVo cell lines were seeded in 100mm Petri dishes at a density of 2 $x10^6$ cells/dish and incubated for 24 hours. Old media was then discarded and replaced with either fresh media (for control group) or 100 and 200 µL/mL of EA flower extract (for treated groups), for 48 hours. Proteins were subsequently extracted from cells using lysis buffer as previously described [31]. Samples were then reduced and denatured in SDS-PAGE sample buffer (Thermo Fisher Scientific) for 20 minutes at 95°C. For proteins detection, membranes were incubated at 4°C, overnight with 1:1000 anti-E-cadherin, anti-vimentin, anti- β-catenin, anti-phospho-β-catenin, anti-Akt, anti-phospho-Akt, (Cell Signaling Technology), anti-EGFR (Abcam), anti-phospho-EGFR (Santa Cruz Biotechnology), and anti-GAPDH (Cell Signaling Technology). Then, membranes were incubated with: 1: 10,000 Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology) and 1:1000 Anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology). The ECL Western blotting substrate (Pierce Biotechnology) was used to detect protein bands. Blots were imaged using the iBrightCL1000 imaging system and quantified by the Image J software. Bands intensity representing relative protein expression was analyzed relative to the GAPDH loading control.

Statistical Analysis

Data was analyzed using the IBM SPSS software, and a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to calculate significant difference between compared groups. All experiments were conducted at least three independent times and measurement represent the mean ± SD. IC_{50} metrics was calculated from the non-linear regression equation.

Results

EA Treatment Reduces CRC Cell Lines Viability and Proliferation

To assess the effect of aqueous EA flower extract on CRC cell lines proliferation and viability, HCT-116, LoVo, and normal immortalized NCE-1 E6/E7 cell lines were treated with different concentrations of aqueous EA flower extract. As expected, increasing concentrations of EA treatment significantly inhibited cell proliferation and reduced viability of both CRC cell lines. As depicted in Figure 1A, the recorded viability of HCT-116 cell line was 35.65 ± 1.03 at 150µL/mL and 13.76 ± 2.00 at 200 µL/mL of EA concentrations. Likewise, LoVo cell lines displayed a 50.28 ± 3.9 viability at 150µL/mL and a 44.55 ± 0.8 viability at 200 µL/mL of EA concentration (Figure 1B). However, the viability and proliferation ability of NCE-1 E6/E7 normal immortalized cells were not affected by these EA concentrations (Figure 1C). From this experiment, the half maximal inhibitory concentration (IC_{50}) values of EA extract in HCT-116 and LoVo cell lines were also calculated to be 83.27 µL/mL ± 1.9 in HCT-116 cells and 155.67 µL/mL ± 3.6 in LoVo cells. Accordingly, 100 and 200 µL/mL concentrations of EA were adopted and used in all subsequent experiments.

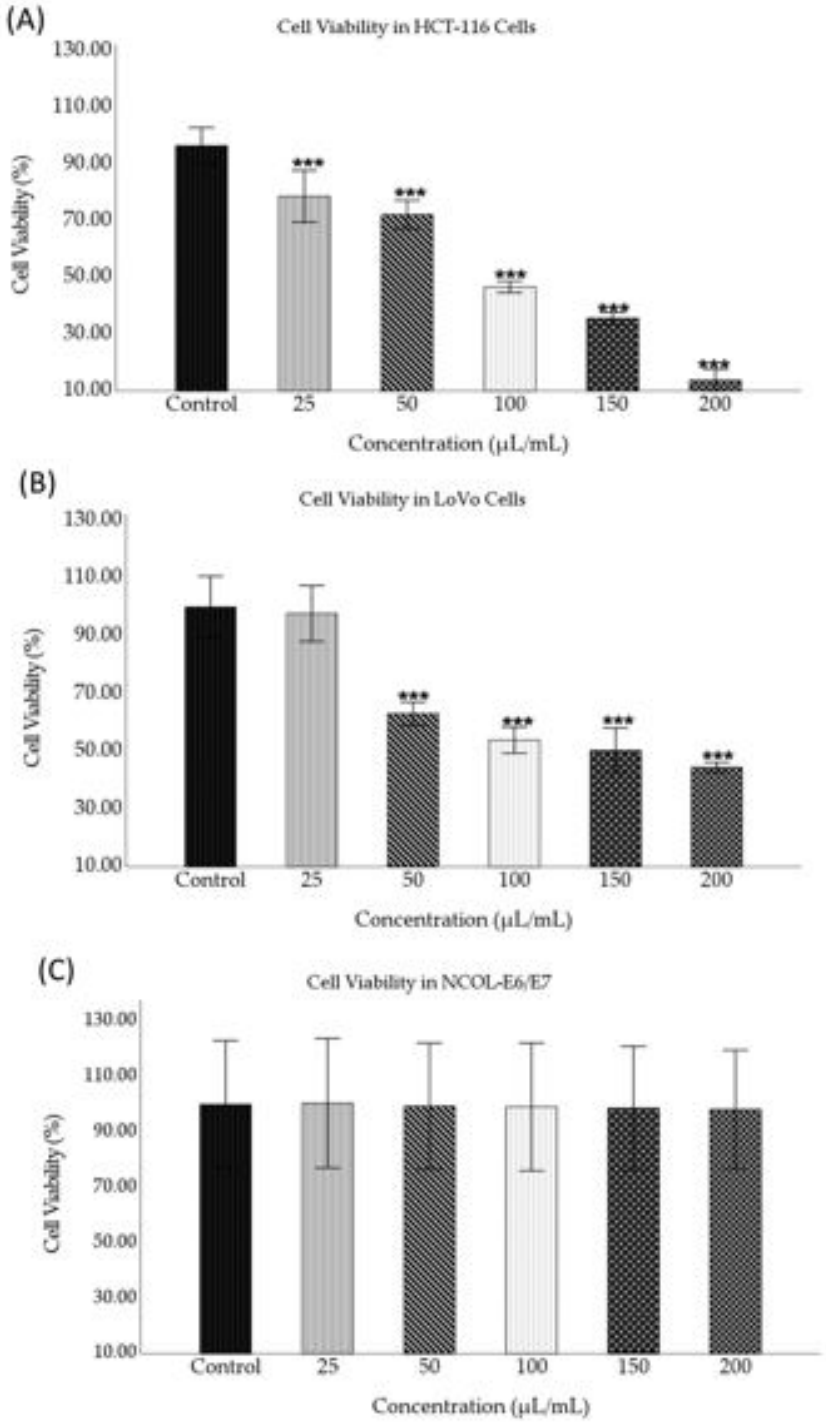

Figure 1: Effect of EA treatment on CRC cells viability and proliferation. Significant decrease in viability and proliferation of CRC cell lines (A) HCT-116 and (B) LoVo but not of the normal immortalized call line (C) NCE -1 E6/E7 48 hours post EA treatment as compared to the non-treated control group. A one-way ANOVA followed by Turkey's multiple comparisons test was used to calculate significant difference between compared groups. Error bars represent the mean ± SEM and asterisks indicate significance, with *** representing $p<0.001$

EA Treatment Exhibits Morphological Changes in CRC Cell Lines

To examine whether the detected effect of EA treatment on cell proliferation and viability (Figure 1) is associated with morphological changes in EA treated cells, we examined the cell morphology of HCT-116, LoVo and NCE-1 E6/E7cell lines treated with 100 and 200 µL/mL of EA aqueous flower extract. As anticipated, EA treatment notably affected the morphology of both CRC cell lines and enhanced cell-cell adhesion tendency. As shown in Figure 2A-B, CRC cell lines treated with EA for 48 hours underwent morpho-

logical alterations, with a switch from a multilayered system of "mesenchymal-like" cells into monolayered "epithelial--like" cells, suggesting a potential ability of EA treatment to inhibit epithelial-mesenchymal transition (EMT) in CRC cells. Furthermore, and within a period of 48 hours of 200 µL/mL EA treatment, CRC cells were observed to start detaching and floating from the surface, indicating a plausible ability of EA to induce cellular death as well. Interestingly, a similar effect of treatment with EA flower extract on NCE-1 E6/E7cells morphology was not detected (Figure 2C).

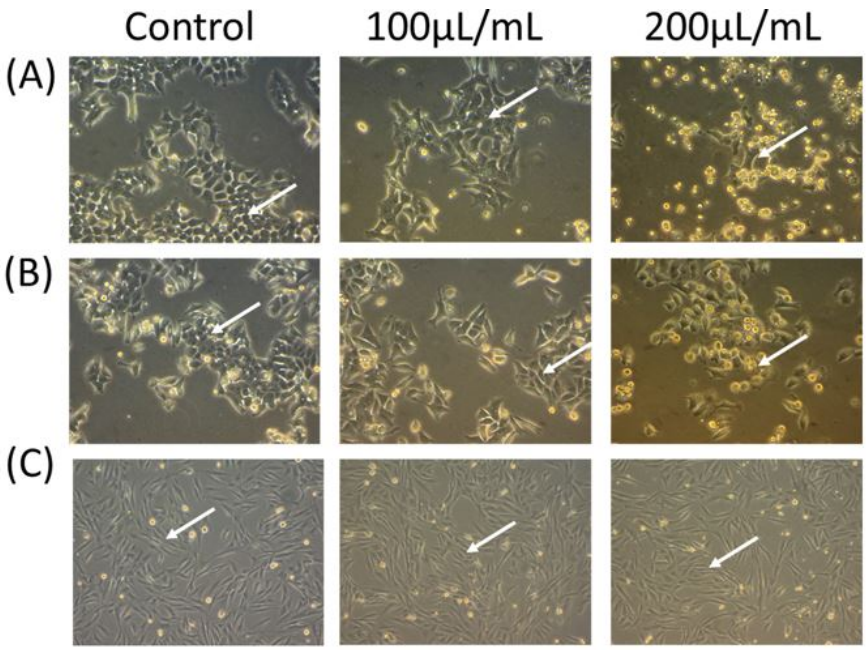

Figure 2: Effect of EA treatment on the morphology or CRC cells. Morphological changes in CRC cell lines (A) HCT-116 and (B) LoVo, but not in the normal immortalized cell line (C) NCE-1 E6/E7, 48 hours post EA treatment as compared to the non treated control group. As displayed using arrows, treatment with EA extract induces epithelial transition and formation of a monolayer of cells in both CRC cell lines but not in control cells. Also, and unlike the condition in NCE-1 E6/E7 cells, the two CRC cell lines start detaching from the surface of the tissue culture dish, indicating cell death 48 hours post treatment with 200 mL/mL EA. Images were taken at 10x magnification

EA Treatment Affects CRC Cells-Cycle Progression

To detect the effect of EA treatment on cell-cycle progression, HCT-116 and LoVo cells cycle analysis was performed by means of flow cytometry. As illustrated in Figure 3, treatment with 200 µL/mL of EA flower extract caused a significant accumulation of HCT-116 cells at G2/M phase (Figure 3A). On the other hand, treatment with 100 and 200 µL/mL of EA flower extract caused a significant accumulation of LoVo cells at the S phase (Figure 3B).

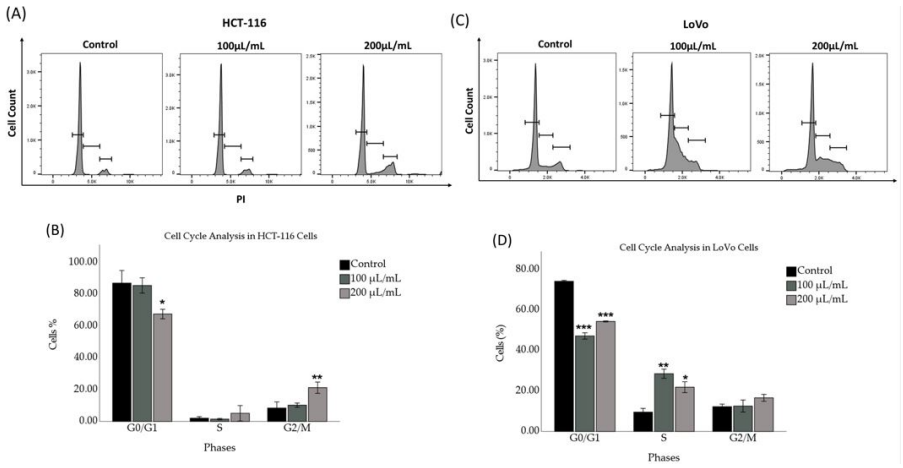

Figure 3: Effect of EA treatment on CRC cells cycles progression. Flow cytometry data analysis depicting deregulated cell cycle progression in (A-B) HCT-116 and (C-D) LoVo CRC cells 48 hours post EA treatment. A one-way ANOVA followed by Turkeys multiple comparisons test was used to calculate significant difference between compared groups. Error bars represent the means ± SD Asterisks indicate significant results between the control and the treated groups, with *representing p<0.05, ** representing p<0.01, and *** representing p<0.001

EA Treatment Affects CRC Cells Invasion Ability

To explore the effect of EA treatment on inhibiting CRC invasion and metastasis, we performed a cell invasion assay on HCT-116 and LoVo cells. As shown in Figure 4, treatment with 100 and 200 µL/mL of EA flower aqueous extract significantly decreased the cell invasion ability of HCT-116 cells by 38.7 and 97%, respectively (Figure 4A), and of LoVo cells by 85.2 and 94.5% (Figure 4B), correspondingly.

EA Treatment Affects Colony Forming Ability of CRC Cells

To further investigate the effect of EA treatment on colony forming ability of both CRC cell lines, we performed a soft agar colony formation assay. As shown in Figure 5, a significant decrease in the number of colonies formed by both HCT-116 and LoVo cell lines treated with the EA flower extract as compared to their untreated control group was detected. At 100 µL/mL concentration of EA treatment, approximately 15% and 13% colonies only were seen in HCT-116 (Figure 5A) and LoVo (Figure 5B) cells, respectively. Interestingly, and at 200 µL/mL concentration of EA treatment, no colonies in either cell lines were detected (Figure 5).

Effect of EA Treatment on the Expression Patterns of EMT Key Biomarkers and Proteins Involved in Signaling Pathways Culminating in Oncogenesis in CRC Cells

EMT biomarkers are known to be involved in the complex developmental program that permits cancer cells to curb their epithelial features transitioning to mesenchymal ones, subsequently allowing cells to acquire mobility and a migratory ability from the primary site [34]. As such and based on the above findings and the evident ability of EA treatment to control several oncogenic features in CRC cell lines, we further investigated the expression patterns of distinct EMT key biomarkers including E-cadherin, vimentin and β-catenin in EA treated HCT-116 and LoVo cell lines. As shown in Figure 6, and in comparison, with the untreated control group, HCT-116 (Figure 6A) and LoVo (Figure 6B) cell lines treated with 100 and 200 µL/mL of EA aqueous flower extract exhibited an enhanced E-cadherin and a significant decrease in vimentin expressions. Interestingly, EA treatment also inhibited the expression of other proteins involved in signaling pathways culminating in oncogenic phenotypes, including phospho-β-catenin, phospho-Akt and phospho-EGFR1 in both CRC cell lines (Figure 6).

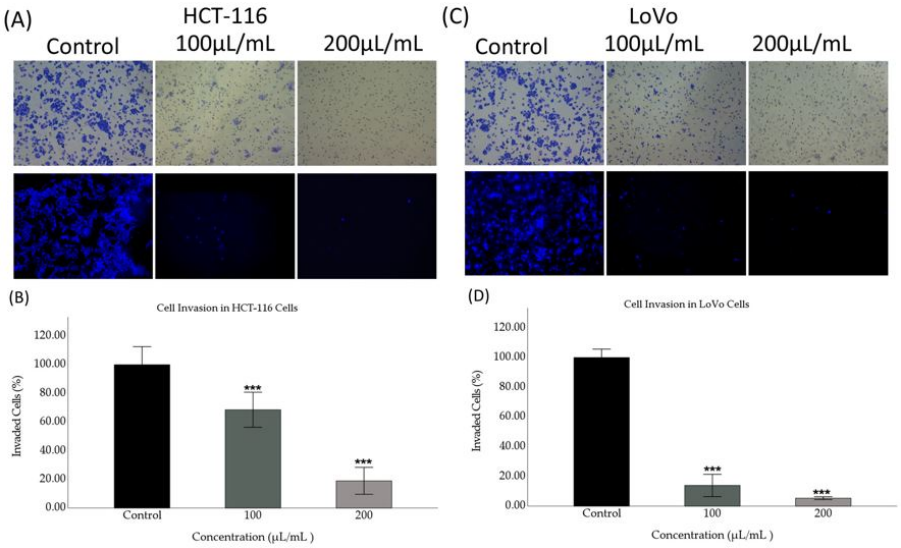

Figure 4: Effect of EA treatment on CRC cells invasion. Cell invasion analysis revealinginhibition of invasion ability of (A-B) HCT-116 and

(C-D) LoVo CRC cells 48 hours post EA treatment. A one-way ANOVA followed by Turkeys multiple comparisons test was used to calculate significant difference between compared groups. Error bars represent the means ± SD Asterisks indicate significant results between the control and the treated groups, with *** representing p<0.001. images were taken at 10x magnification

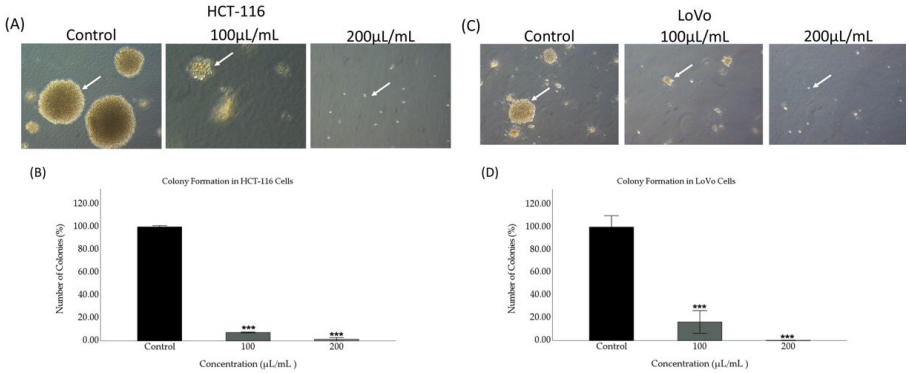

Figure 5: Effect of EA treatment on CRC cells colony forming ability. Colony formation assay analysis revealing suppression of (A_B) HCT-116 and (C-D) LoVo CRC cells colony forming ability 48 hours post EA treatment. A one-way ANOVA followed by turkey's multiple comparisons test was used to calculate significant difference between compared groups. Error bar represent the means ± SD Asterisks indicate significant results between the control and the treated groups, with *** representing p<0.001. images were taken at 10x magnification

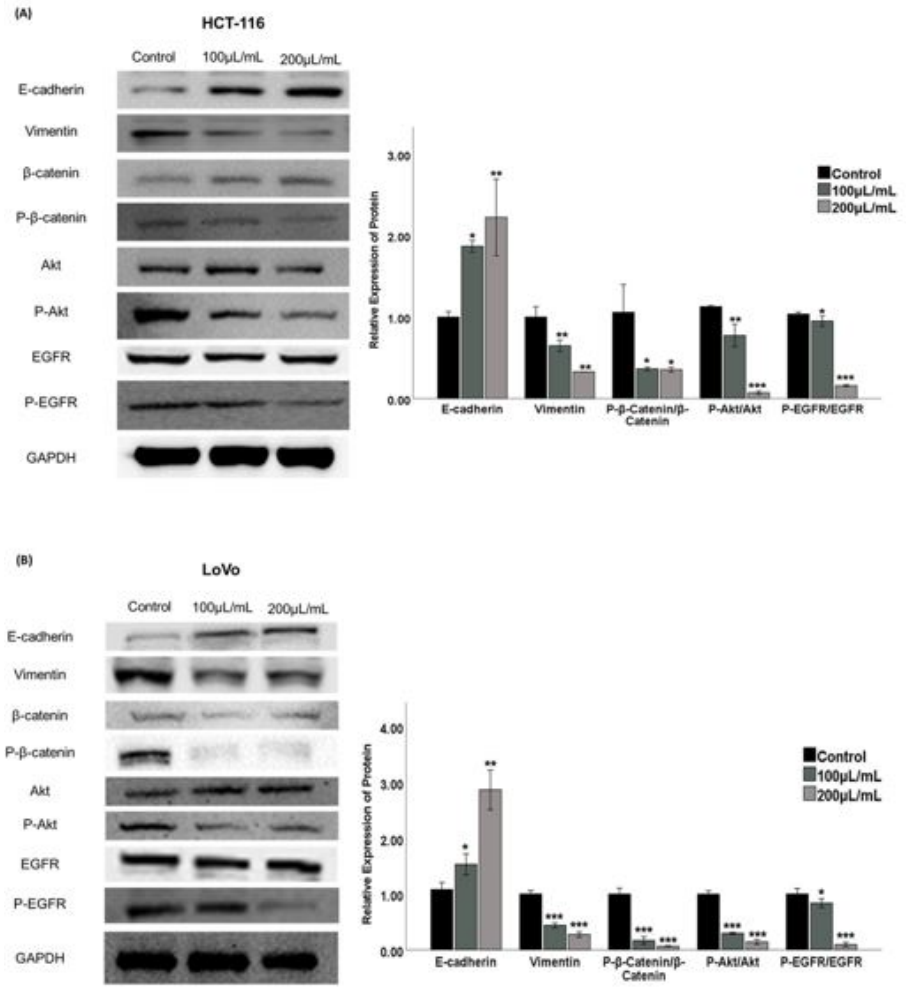

Figure 6: Effect of EA treatment on the expression patterns of EMT biomarkers and proteins involved in signaling pathways culminating in oncogenesis in CRC cells. EA treatment for 48 hours increases the expression of E-cadherin, and decreases the expression of Vimentin,

phospo-β-catenin, phospo-Akt, and phosphor-EGFR1, in both CRC cell lines as compared to the untreated control cells. GAPDH was used as a loading control. A one-way ANOVA followed by turkeys multiple comparisions test was used to calculate significant difference between compared groups. Error bars represent the mean ± SD and Asterisks indicate significant results between control and treated groups, with at * representing o<0.05, ** representing p<0.01 and *** representing p<0.001

Discussion

For years, the primary choice for CRC treatment has been chemotherapeutic drugs [35]. Though broadly adopted, this traditional treatment regimen impacts CRC patients differently, particularly at the level of toxicity and efficacy. In addition to chemotherapy's severe side effects, tumour resistance to some systemic chemotherapies including 5-FU and Irinotecan has been also reported over the treatment course [36], an actuality that called for alternative treatment plans. In this study, we explored for the first time the potential anti-cancer effect of EA medicinal plant extract on CRC using HCT-116 and LoVo CRC cell lines with KRAS mutation, by particularly revealing EA's ability to modulate several cancer hallmarks including cell viability and proliferation, cell cycle progression, EMT development, colony formation, and cell invasion ability. We also traversed the plausible effect of EA treatment on several signalling pathways underlying oncogenic events. As conjectured, our data shows that treatment with EA aqueous flower extract inhibits cell proliferation and deregulates cell-cycle progression in both HCT-116 and LoVo CRC cell lines, a finding consistent with previous work from our group reporting an effect of EA extract on human oral cancer cells [32] and on HER2-positive in addition to triple negative human breast cancer cells [31,37]. Interestingly, treatment with EA flower extract exhibited no effect on the proliferation ability of human normal immortalized colon cells, a finding that also aligns with our previously reported data showing no effect of EA treatment on human immortalized mammary epithelial cell lines (HNME-E6/E6 and MCF 10A) [31,37]. Collectively, these results indicate a selectivity of EA aqueous extract towards cancerous cells.

This EA anti-cancer effect could be plausibly tailored to its phytochemical composition, particularly its abundance in flavonoids that are known to exhibit antioxidant, anti-inflammatory, antiperoxidative, and anti-cancer sequels by inhibiting cell proliferation and/or causing cell apoptosis of human malignancies [30,38-40]. Consuming flavonoids has been, indeed, shown to decrease the risk of CRC incidence and recurrence [38,41]. For example, the Chinese herbal medicine Scutellaria baicalensis, which is highly rich in flavonoid Baicalin, exhibits an anticancer potency by particularly inhibiting CRC cells proliferation and deregulating their cell cycle, arresting them in S and G2/M phases [40]. Along this, grapes seeds extract, which are highly rich in flavonoids [42], induce LoVo cells cycle arrest in S phase after 48 hours of treatment [43]. This supports our findings in the present investigation revealing the impact of EA treatment on deregulating cell cycle progression by arresting HCT-116 and LoVo cells in the G2/M and S phases, respectively.

Besides its effect on cell cycle arrest, our data reveals the ability of EA treatment to provoke morphological cell alterations, such as enhancing cell-cell contact, suggesting the aptitude of EA treatment to inverse EMT into MET or inhibit EMT progression in CRC cells. Since EMT events play crucial roles in cancer invasion and metastasis, in addition to their involvement in the process of normal development and wound healing [44,45], then EMT represents a hallmark of human cancer invasiveness [46]. Indeed, a strong association between EMT, CRC metastasis, tumor invasiveness, resistance to apoptosis and chemotherapies has been previously reported [46,47]. Our data herein displays an ability of EA treatment to block the progression of EMT and consequently cell invasion in CRC, an event accompanied by an upregulation of E-cadherin and a downregulation of vimentin, two important biomarkers of EMT and key regulators of cell invasion and metastasis [48]. This reported ability of EA treatment to attune CRC by modulating E-cadherin and vimentin levels adjacently concords with previous studies demonstrating a high association between CRC development and progression and loss of E-cadherin expression [49]. On the other hand, clinicopathological investigations reveal vimentin overexpression in 17% of CRC cases, with it being absent in normal mucosa samples [50]. Vimentin expression has been also considerably correlated with short survival rate, distant metastasis, and high tumor grade [50]. Another indicator of tumor growth is cells colony forming ability. As such, we also examined this fea-

ture in our study and reported an inhibitory effect of EA treatment on CRC cells colony forming ability. Taken together, our present data presents EA flower extract as a potential therapeutic candidate for CRC management. This is backed by our preliminary data regarding the outcome of EA on CRC Drosophila Melanogaster model of KRAS mutation.

Vis-à-vis the molecular pathway, we show that EA treatment inhibits the phosphorylation of β-catenin, Akt, and EGFR1 in both CRC cell lines. This is consistent with our previous findings reporting the ability of EA treatment to restrain Erk1/2 and β-catenin signaling pathways in oral and breast cancers [31,32]. Also, other studies have reported that nucleic β-catenin is mutated in more than 85% of CRC tumors and that the accumulation of phosphorylated β-catenin is strongly associated with poor CRC prognosis [51]. Additionally, the tyrosine kinase receptor EGFR, which is overexpressed in 90% of CRC tumours, plays an essential role in CRC progression [52]. The activation of Akt, a downstream target of EGFR, has been also reported to be associated with CRC development and progression [53]. The ability of EA treatment to modulate the phosphorylated levels of β-catenin, EGFR, and Akt is presumed to be related to its rich flavonoids content. A recent in silico study identified flavonoids as effective EGFR inhibitors, re-counting this to their drug likeness properties with good docking score [54]. Moreover, flavonoids have been shown to inhibit cancer cell proliferation by suppressing EGFR and Akt given their pro-oxidant property [55]. Altogether, these results support the signalling pertinent findings of this study demonstrating the ability of EA treatment to inhibit CRC development and progression by plausibly targeting EGFR/RAS, PI3K/AKT, and Wnt/β-catenin signaling pathways known to be involved in oncogenic events.

Despite the significant findings and implications of our study on the inhibitory effects of EA plant extract on CRC cells, there are certain limitations that should be acknowledged. The lack of known anti-cancer agents or natural products for direct comparison makes it challenging to contextualize the efficacy of the EA extract. Although our findings demonstrate significant inhibitory effects on cell proliferation and invasion, it would be beneficial for future studies to include comparative analyses with established anti-cancer agents or other natural products with reported activities against CRC. Moreover, future investigations focusing on EA composition analysis would enhance our understanding of the extract's mechanism of action and facilitate the development of targeted interventions. Finally, these results need to be confirmed in vivo using different animal models of human cancers including CRC.

Conclusions

In this study, we illustrate for the first time an anti-CRC effect of EA aqueous flower extract on KRAS mutation CRC cell lines, HCT-116 and LoVo. We herein report EA's selectively in inhibiting CRC cells proliferation and its ability to deregulate CRC cells cycle progression. We also demonstrate the ability of EA treatment to block CRC cells invasion and colony formation, and to suppress EMT progression by altering the expression patterns of key EMT biomarkers. At the signalling level, we show EA's ability to modulate key players in EGFR/RAS, PI3K/AKT, and Wnt/β--catenin pathways involved in oncogenic events. Our findings open the door for future studies directed towards deciphering the exact mechanism of action of EA, identifying EA's specific active phytochemical components responsible for this anti-cancer effect in vivo, especially since our preliminary data regarding the outcome of EA on CRC Drosophila Melanogaster model show significant inhibition of tumour development in this KRAS mutation animal model.

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Author Contributions

Conceptualization: A-E.A.M.; Methodology: lab work: A.F.H., resources: A-E.A.M., A.K., and L.K. Data curation: A.F.H., A-E.A.M., A.K., and L.K.; Manuscript draft preparation: A.F.H.; Manuscript writing, reviewing, and editing, A-E.A.M., A.K., L.K., and A.F.H.; funding acquisition: A-E.A.M., and A.K.

All authors have read and agreed to the published version of the manuscript.

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Disclosure Statement

The authors report there are no competing interests to declare.

Data Availability Statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

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