

# Ethanollic Extract of Red Okra Pods Induces Aberrant Spindle Segregation and Apoptotic Cell Death by Disrupting the Wnt Signaling Pathway in Colon Cancer Cells

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## What's Known

- $\beta$ -catenin localizes to centrosomes during mitosis and plays a pivotal role in orchestrating the precise execution of spindle-mediated chromosome segregation.

## What's New

- The present study highlights the capacity of ethanollic extract of red okra pods to induce apoptosis in SW480 cells by modulating the Wnt/ $\beta$ -catenin signaling pathway.
- Treatment with the mentioned extract reduces  $\beta$ -catenin levels, promotes abnormal spindle apparatus segregation, and ultimately leads to colorectal cancer cell apoptosis.

## Abstract

**Background:** In approximately 80% of colorectal cancer cases, mutations in the adenomatous polyposis coli (*APC*) gene disrupt the Wnt/ $\beta$ -catenin signaling pathway, a crucial factor in carcinogenesis. This disruption may result in consequences such as aberrant spindle segregation and mitotic catastrophe. This study aimed to analyze the effectiveness of the ethanollic extract of red okra (*Abelmoschus esculentus*) pods (EEROP) in inducing apoptosis in colorectal cancer cells (SW480) by inhibiting the Wnt/ $\beta$ -catenin signaling pathway.

**Methods:** The  $IC_{50}$  of EEROP in SW480 cells was determined by treating the cells with varying doses of EEROP, ranging from 0 to 1000  $\mu$ g/mL. Apoptosis assay and signaling pathway analysis were performed through immunofluorescence staining and Western Blotting on SW480 cells treated with 250  $\mu$ g/mL of EEROP for 72 hours.

**Results:** EEROP treatment induced apoptosis in SW480 cells, marked by elevated levels of active caspase-3 ( $P < 0.001$ ) and cleaved poly-(ADP-ribose) polymerase (PARP)-1. Moreover, it notably decreased  $\beta$ -catenin protein levels, resulting in an augmented occurrence of cells displaying abnormal spindle segregation during mitosis ( $P = 0.04$ ).

**Conclusion:** EEROP treatment reduces  $\beta$ -catenin protein levels, promotes abnormal spindle apparatus segregation, and finally leads to apoptotic cell death in CRC cells.

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**Keywords** • *Abelmoschus esculentus* • Apoptosis • Colorectal cancer • Wnt signaling pathway

## Introduction

The molecular signaling pathway of the Wntless-related integration site (Wnt) is responsible for regulating not only embryonic development but also the normal physiological processes of adults, such as cell proliferation, differentiation, and homeostasis.<sup>1,2</sup> There are two types of Wnt signal transduction pathways: canonical and non-canonical. The canonical pathway, also known as the Wnt/ $\beta$ -catenin signaling pathway, is responsible for modulating cell proliferation and survival,

while the non-canonical pathway enhances cell migration.<sup>2</sup> Wnt signaling contributes to the metabolism of various organs including the intestine. Upon activation, Wnt maintains tissue homeostasis by regulating the turnover of stem cells and adult cells located at the bottom of the intestinal crypts. However, abnormal activation of Wnt signaling is associated with colorectal cancer (CRC) progression.<sup>1</sup>  $\beta$ -catenin gene mutations cause CRC, in addition to mutations in the adenomatous polyposis coli (*APC*) gene, accounting for 80% of CRC incidence.<sup>3</sup>

The signaling pathway of Wnt/ $\beta$ -catenin begins when the Wnt protein binds to a complex receptor comprising low-density lipoprotein receptor-related proteins (LRP)-5 or LRP-6 and 10 members of the Frizzled (FZD) family of proteins. This binding activates the Dishevelled (Dvl) cytoplasmic protein, which further inhibits glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ). Consequently,  $\beta$ -catenin translocates into the nucleus and initiates target gene transcription by binding to the transcription factor, namely T-cell factor/lymph enhancer factor 1 (TCF/LEF).<sup>4</sup> Conversely, GSK3 $\beta$ , together with Axin and APC (known as the destruction complex), phosphorylates three residues (Thr41, Ser37, and Ser33) of  $\beta$ -catenin in the absence of Wnt ligands.<sup>3, 4</sup>  $\beta$ -catenin phosphorylation is also carried out by casein kinase 1 (CK1) at the Ser45 residue.<sup>3</sup> This series of  $\beta$ -catenin phosphorylation ultimately leads to  $\beta$ -catenin ubiquitination and degradation via the proteasome pathway.<sup>5</sup> In human cancers including CRC, the inhibition of  $\beta$ -catenin phosphorylation allows cells to proliferate continuously.<sup>6</sup> Therefore, targeting the Wnt/ $\beta$ -catenin signaling pathway, either at the level of the  $\beta$ -catenin destruction complex or the nuclear/transcription factor complexes, holds versatile therapeutic potentials for CRC treatment.<sup>3, 4</sup>

Beyond its function as a transcription factor in Wnt signaling,  $\beta$ -catenin also localizes at the centrosomes, actively promoting mitotic progression and cell proliferation.<sup>7</sup> The centrosome plays a pivotal role in organizing the microtubule cytoskeleton.  $\beta$ -catenin, during both interphase and mitosis, localizes at centrosomes to ensure the accurate disjunction of centrosomes and the formation of bipolar spindles.<sup>8</sup> NIMA-related kinase 2 (Nek2) is the main regulator of  $\beta$ -catenin at centrosomes and the  $\beta$ -catenin phosphorylation is independent of GSK3 $\beta$ . The phosphorylation of  $\beta$ -catenin by Nek2 results in its stabilization at the centrosome, constituting a mechanism for centrosome segregation by retaining  $\beta$ -catenin during mitosis instead of removing it. Despite

this insight, the specific role of  $\beta$ -catenin in promoting centrosome segregation and the function of the phosphorylated, stabilized pool of  $\beta$ -catenin at centrosomes remain unclear.<sup>9</sup> Moreover, it is yet to be determined whether Nek2 phosphorylation primarily shields  $\beta$ -catenin from destabilizing phosphorylation by GSK3 $\beta$  or if the Nek2 phosphorylation sites directly contribute to  $\beta$ -catenin's functional role at centrosomes.<sup>10</sup>

Colorectal cancer is ranked fourth and second among the most common cancers and the causes of ailment-associated death worldwide, respectively. The total mortality of CRC has exceeded 600,000 deaths annually.<sup>11, 12</sup> Treatments for patients with CRC have rapidly advanced. However, many patients are susceptible to recurrence following surgery. Thus, an appropriate adjuvant needs to be identified to reduce the mortality rate. In recent years, much research has been conducted to investigate the use of herbal products for cancer prevention and therapy.<sup>13</sup>

*Abelmoschus esculentus* (red okra) is a plant from the Malvaceae family. It contains antioxidant components, including quercetin and flavonoids.<sup>14</sup> Several studies have investigated the anti-inflammatory and anti-cancer potentials of the crude extract of red okra in breast and cervical cancers.<sup>15, 16</sup> However, no study has reported the anti-cancer activity of EEROP in CRC cells. The objective of the recent study is to investigate the potential activity of EEROP, as an anti-cancer agent, in the SW480 cell line and explore the signaling pathways affected by treatment with EEROP.

## Materials and Methods

### Ethics Committee Approval

The study was approved by Universitas Airlangga Faculty of Dental Medicine Health Research Ethical Clearance Commission, with approval number: 270-2/HRECC.FODM/V/2022.

### Preparation of EEROP

The preparation of EEROP followed a previously established protocol.<sup>15</sup> Briefly, red okra pods (5 Kg) with specimen vouchers (1205/IPH.06/HM/XI/2019), from traditional markets in Surabaya were washed and then air-dried in the absence of direct sunlight. Subsequently, the dried okra pods were ground into a powder, which was further cold-macerated by 3 L of absolute ethanol (Merck, 64-17-5, Germany) for 24 hours while stirring periodically. The maceration process was repeated three times. The extract was then filtered and concentrated using a rotary evaporator.

### Cell line and Cell Culture

Human CRC cancer cells (SW480) were obtained from the American Type Culture Collection (ATCC, USA). The SW480 cell line is known for its genetic stability, which is essential for maintaining consistent and reproducible experimental results over time. Additionally, SW480 cells carry mutations in the APC gene, a mutation found in approximately 80% of sporadic colorectal tumors. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, 10082147, USA) and 50 U/mL penicillin-streptomycin (Nacalai Tesque, 26253-84, Japan). To determine the inhibitory concentration ( $IC_{50}$ ) value, SW480 cells (5,000/well) were plated in a 96-well format. Following a 24-hour incubation, cell groups were subjected to treatment with either DMSO (control) or EEROP diluted in DMSO at varying concentrations ranging from 0 to 1000  $\mu$ g/mL for an additional 72 hours. Post-incubation, 10  $\mu$ L of 12 mM MTT solution was introduced to each well, followed by a 3-hour incubation period, and the reaction was halted with the addition of 100  $\mu$ L of STOP solution. Thorough mixing of the samples was ensured before measuring absorbance at 570 nm.<sup>17</sup> Meanwhile, the cells were treated with 250  $\mu$ g/mL of EEROP alone as part of another experiment. An equal volume of dimethyl sulfoxide (DMSO) was used in the control group because EEROP was dissolved in DMSO. All cultures were maintained in a 37 °C incubator containing 5% CO<sub>2</sub>.

### Immunofluorescence Staining

SW480 cells were seeded onto polylysine-coated glass coverslips in six-well plates. After treatment with 250  $\mu$ g/mL of EEROP, the cells on the coverslips were washed with phosphate-buffered saline (PBS) and fixed for 10 min in ice-cold absolute methanol (Merck, 67-56-1, Germany) and permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, 9002-93-1, USA) for 10 min. The cells were then washed after successful fixation. To prevent unspecific binding, blocking with 4% bovine serum albumin (BSA; Sigma Aldrich, 9048-46-8, USA) in PBS was done for 30 min at room temperature. The cells on the coverslips were then incubated with one of the primary antibodies. This study used antibodies against  $\alpha$ -tubulin (Sigma-Aldrich, T9026, USA), active/cleaved caspase-3 (Abcam, E83-77, UK), TPR (Santa Cruz Biotechnology, sc-101294, California), and pGSK3 $\beta$ S9 (Cell Signaling, 9336, USA). The primary antibodies were prepared by dilution of 4% BSA in PBS with a 1:100 ratio, followed by 4 °C overnight incubation.

Subsequently, the cells were washed with PBS three times. The cells were then incubated with secondary antibodies at a ratio of 1:100 with BSA in PBS for 2 hours at room temperature. After washing with PBS, the coverslips were mounted on the slide using ProLong Gold Antifade with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, P36941, California) and observed using a confocal laser scanning microscope (Olympus, Japan).<sup>18, 19</sup>

### Protein Isolation and Western Blotting

The cells were homogenized in 100  $\mu$ L of lysis buffer mixed with protease inhibitor cocktails (Merck, 11836170001, Germany) and incubated on ice for 20 min. Insolubilized material was centrifuged at 10,000 g for 10 min at 4 °C. The protein concentration was measured using UV spectroscopy at 280 nm, and 30  $\mu$ g of the total protein extract was loaded onto each well. The protein extract was separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck, 03010040001, Germany) using the wet transfer method.<sup>20</sup> The PVDF membranes were blocked with 5% skim milk in phosphate-buffered saline/tween (PBST) and incubated with primary antibodies in a ratio of 1:1000. The primary antibodies used for Western Blotting analysis included antibodies against poly (ADP-ribose) polymerase-1 (PARP1) (sc-7150, Santa Cruz), GSK3 $\beta$  p-Y216 (612312, BD Biosciences), GSK3 $\beta$  (05-412, Millipore),  $\beta$ -catenin (Sigma-Aldrich, PLA0230, USA), p-ERK (Sigma-Aldrich, SAB5700045), p-AKT (Sigma-Aldrich, SAB5600064, USA), AKT (Sigma-Aldrich, SAB3500216, USA), and  $\beta$ -actin (sc-47778, Santa Cruz) as an internal control. Actin expression is relatively stable and not significantly affected by most experimental treatments. This stability makes actin a suitable internal control for normalizing protein expression levels in Western Blotting. Subsequently, the membranes were exposed to secondary horseradish peroxidase-conjugated antibodies (ThermoFisher, USA). The membranes were exposed to an enhanced chemiluminescence (ECL) reagent (Sigma-Aldrich, USA), Immobilon Western Chemiluminescence Horseradish peroxidase (HRP) substrate (Millipore, Germany), and images were detected using a LAS-4000 image analyzer (GE Healthcare, USA).<sup>21, 22</sup>

### Bioinformatics Analysis

The data for GSK3B and CTNNB1 correlation analysis was retrieved from cBioportal (<https://>



www.cbioportal.org/). A correlation study was performed using Pearson analysis to determine the correlation coefficient. All bioinformatics data were retrieved and analyzed accurately.<sup>18, 21, 23</sup>

### Statistical Analysis

Statistical analysis was performed using GraphPad PRISM 7 software (GraphPad Software Inc., California, USA). Data were expressed as mean±SD. The normality of the data was assessed using the Shapiro-Wilk test. Based on the normality of the data, either the Student's *t* test or Mann-Whitney test was employed to assess statistically significant differences between the respective groups.  $P < 0.05$  was considered to indicate statistical significance.

## Results

### 50% Inhibitory Concentration ( $IC_{50}$ ) and the Effect of EEROP on the Growth of SW480 Cells

To determine the potent EEROP concentration for inhibiting 50% of SW480 cell growth, an MTT assay was conducted and the  $IC_{50}$  of EEROP was determined. In SW480 cells, the  $IC_{50}$  of EEROP within 72 hours of treatment was 306.2  $\mu\text{g/mL}$  (figure 1).

### EEROP-Induced Apoptosis of CRC Cells

To determine the effect of EEROP on the induction of apoptosis in CRC cells, immunostaining was performed for active caspase-3 (red) and counterstained with  $\alpha$ -tubulin (green). The DNA was stained with DAPI (blue). Counting the number of cells expressing active caspase-3 showed that treatment with EEROP significantly increased this number (figure 2A,  $P < 0.001$ ). Additionally, in the Western Blotting analysis of cleaved PARP-1, a representative marker of apoptosis, higher expression in the EEROP-treated group was

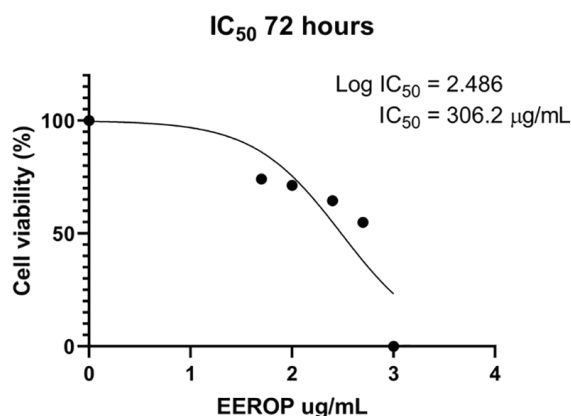


Figure 1: The  $IC_{50}$  of EEROP in SW480 cells after 72 hours of treatment.

observed (figure 2B).

### EEROP Reduced $\beta$ -catenin Protein Expression in CRC Cells

To identify the molecular mechanism corresponding to the induction of apoptosis after treatment with EEROP, Western Blotting we performed on several key proteins involved in the Wnt signaling, extracellular signal-regulated kinase (ERK), and Akt signaling. Treatment with 250  $\mu\text{g/mL}$  EEROP for 72 hours reduced the protein level of  $\beta$ -catenin in SW480 cells. The total protein level of GSK3 $\beta$ , a regulator of  $\beta$ -catenin expression, was not significantly changed. However, the expression of GSK3 $\beta$ -pY216 (the active form of GSK3 $\beta$ ) was increased (figure 3A). Immunofluorescence staining also showed that treatment with EEROP changed the expression and localization of GSK3 $\beta$ -pS9 (the inactive form of GSK3 $\beta$ ) (figure 3B). To validate the correlation between  $\beta$ -catenin (CTNNB1) and GSK3 $\beta$  (GSK3B), the microarray data available in the cBioPortal for Cancer Genomics was analyzed. A negative correlation was found between CTNNB1 and GSK3 $\beta$ , but it was not statistically significant ( $P = 0.269$ ) (figure 3C).

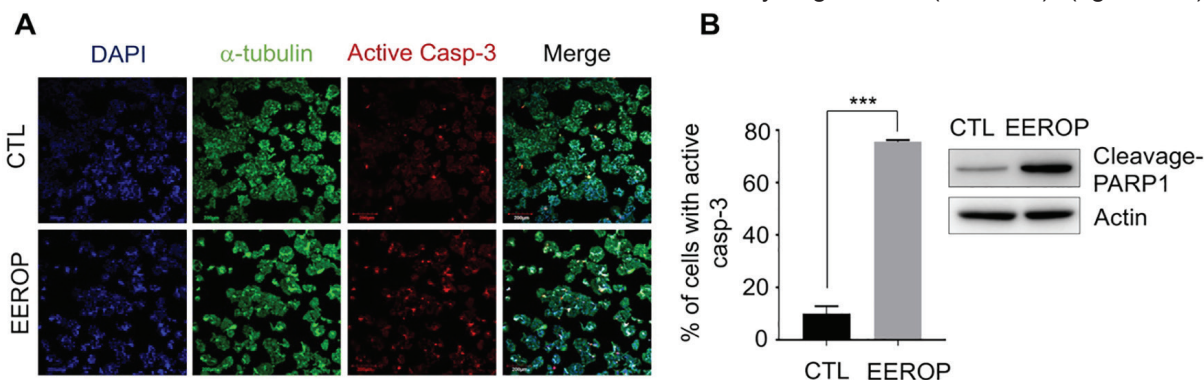
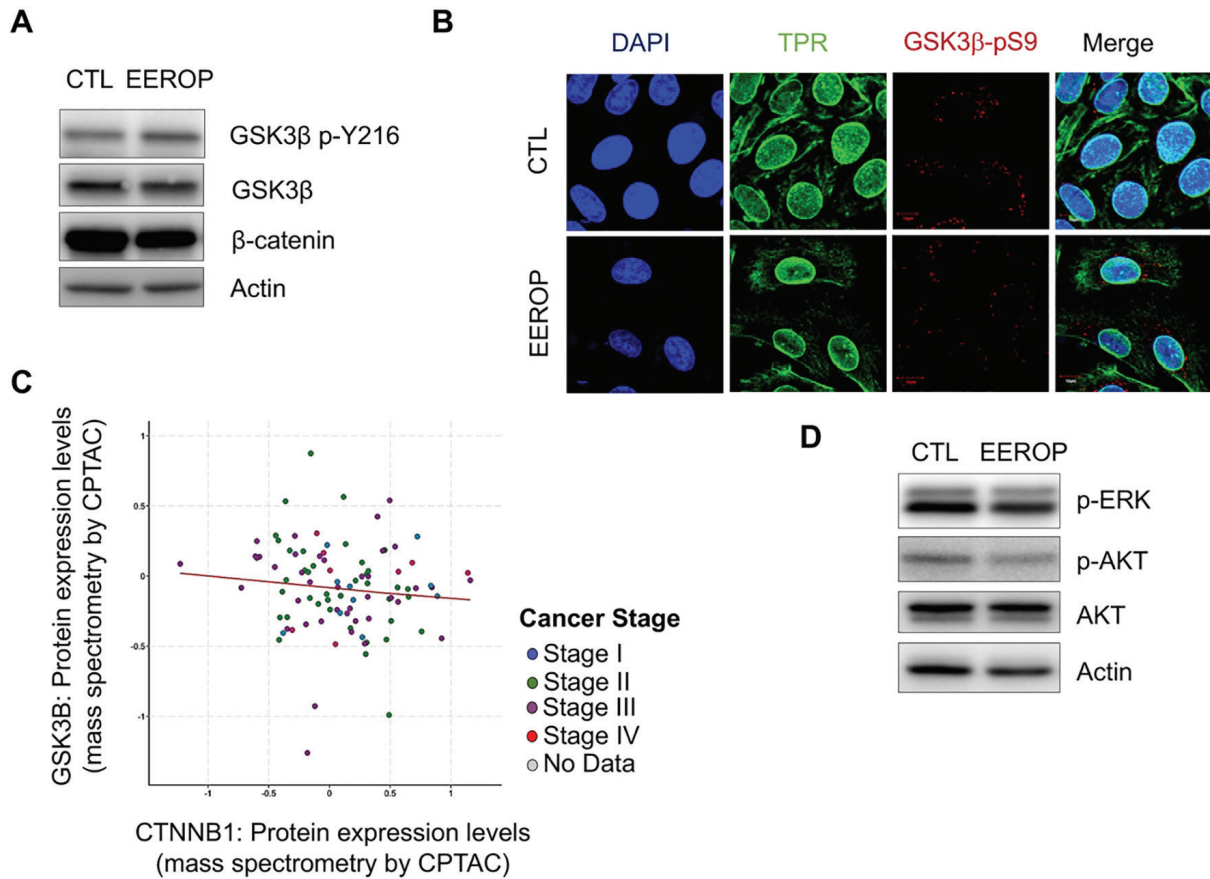


Figure 2: EEROP treatment for 72 hours induced apoptosis in SW480 cells, as shown by (A) immunofluorescence staining revealing 4',6-diamidino-2-phenylindole (DAPI),  $\alpha$ -tubulin and active caspase-3 in SW480 cells treated with 250  $\mu\text{g/mL}$  of EEROP. The *P* values are presented in the EEROP group,  $P < 0.001$ . (B) Western Blotting analysis demonstrated the expression of cleaved PARP-1. CTL: Control group; EEROP: Ethanolic extract of red okra pods-treated group.



**Figure 3:** (A) Protein levels and localization of key proteins in the Wnt/ $\beta$ -catenin signaling pathway in the control group and the EEROP-treated group by Western Blotting analysis. (B) Immunofluorescence staining of 4',6-diamidino-2-phenylindole (DAPI, blue), translocated promoter region (TPR, green), and glycogen synthase kinase 3 $\beta$ -phospho serine 9 (pGSK3 $\beta$ S9, red). (C) DMSO was administered to the control group, and 250  $\mu$ g/mL of EEROP was administered to the EEROP-treated group for 72 hours. Correlation analysis between catenin  $\beta$ 1 (CTNNB1) and glycogen synthase kinase 3 $\beta$  (GSK3B) protein levels was performed by an *in-silico* study using cBioportal. Pearson correlation coefficient=0.14, P=0.013. (D) Protein expression analysis of proteins involved in the extracellular signal-regulated kinase (ERK) and serine/threonine kinase (AKT) signaling pathways in SW480 cells treated with 250  $\mu$ g/mL of EEROP for 72 hours, comparing the control and EEROP-treated groups. CTL: Control group; EEROP: Ethanolic extract of red okra pods-treated group.

Additionally, to determine whether EEROP is involved in another signaling pathway, Western Blotting analysis was conducted on p-ERK, p-Akt, and Akt. It was confirmed that EEROP did not alter AKT and ERK signaling because there was no reduction in the p-Akt and p-ERK expressions in the EEROP group compared to the control group (figure 3C).

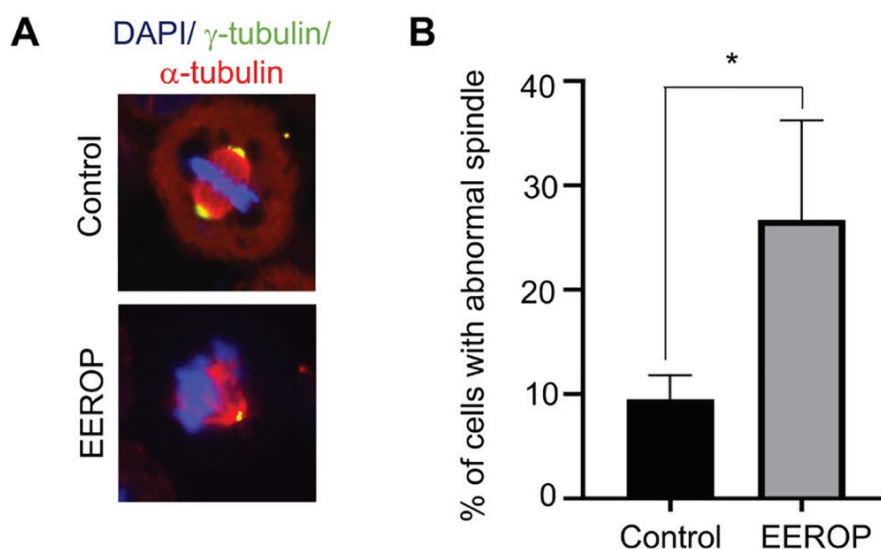
*Reduction of  $\beta$ -catenin Expression is Accompanied by an Increased Number of Cells with the Abnormal Spindle*

To examine the impact of reducing  $\beta$ -catenin protein levels, SW480 cells were treated with either DMSO or 250  $\mu$ g/mL EEROP for 72 hours. The cells were subjected to staining with  $\gamma$ -tubulin highlighting the centrosome in green,  $\alpha$ -tubulin indicating the spindle in red, and DAPI staining the DNA in blue. Our investigation revealed a significant increase in the number of cells exhibiting abnormal spindle segregation following treatment with EEROP (P=0.04) (figure 4A–B).

**Discussion**

The present study revealed an IC50 of 306.2  $\mu$ g/mL for EEROP in SW480 cells, significantly lower than the IC50 of EEROP in HeLa cells (776.8  $\mu$ g/mL) reported in a previous study.<sup>15</sup> EEROP has been documented to lower the levels of three oncogenes linked to the cell cycle in cervical cancer cells, namely MYC, TYMS, and MDM2.<sup>15</sup> Notably, our study represents the first report of the IC50 of EEROP in the SW480 cell line.

The impact of EEROP on the induction of apoptosis in SW480 cells was evident by the significant increase in cells expressing active/cleaved caspase-3 and cleaved-PARP1 expression. In addition to the increased number of cells exhibiting active caspase-3, the treatment with EEROP also led to elevated levels of cleaved-PARP1 expression. PARP-1 is recognized as one of the cellular substrates susceptible to caspase cleavage, and the cleavage of PARP-1 by caspases serves as a hallmark of apoptosis.<sup>24</sup>



**Figure 4:** Analysis of abnormal spindle segregation in SW480 cells of the control group and the EEROP-treated group. (A) The control group received DMSO, while the EEROP-treated group was treated with 250 µg/mL of EEROP for 72 hours. (B) The corresponding P value was indicated for the EEROP-treated group as P=0.04. CTL: Control group; EEROP: Ethanol extract of red okra pods-treated group.

In a prior study, the use of a distinct component of *A. esculentus*, specifically the flower flavonoid extract, demonstrated inhibition of CRC cell proliferation. This inhibition was achieved by inducing mitochondrial dysfunction, triggered by the activation of p53 and the induction of apoptosis. Moreover, *in-vivo* experiments revealed that okra flower extract exerted significant antitumor effects in tumor xenograft model mice, displaying efficacy without apparent toxicity or side effects.<sup>25</sup>

The anticancer potential of *A. esculentus* extends beyond its crude extract, as multiple studies have highlighted the anticancer properties of its bioactive compounds, such as lectin and quercetin.<sup>26-29</sup> Lectin, derived from *A. esculentus*, was shown to induce apoptosis and inhibit the growth of breast cancer (MCF7) and glioma (U87) cell lines, underscoring its effectiveness in promoting apoptosis.<sup>28, 29</sup> Additionally, the anticancer efficacy of quercetin has been extensively documented in both *in vitro* and *in vivo* studies involving diverse cell lines and animal models. Remarkably, the potent anticancer capacity of *A. esculentus* is highlighted by the significant toxic effect of quercetin on cancer cells, accompanied by minimal or no discernible side effects on normal cells.<sup>26</sup> This underscores the promising anticancer potential inherent in *A. esculentus*.

The development of CRC is a complex and multi-stage process characterized by the mutation of specific genes (*APC*, *KRAS*, and *P53*) and the modification of various signaling pathways (PI3K/AKT, Wnt/β-catenin, and so on). These pathways play crucial roles in

regulating CRC cell processes such as growth, differentiation, angiogenesis, apoptosis, and survival. Notably, the Wnt/β-catenin signaling pathway has been identified in numerous studies as a central mechanism driving colorectal carcinogenesis.<sup>30-32</sup> In our study, EEROP administration resulted in the downregulation of β-catenin levels, a key player in the Wnt/β-catenin signaling pathway, in a colon cancer cell line. This effect was accompanied by alterations in the phosphorylation state of GSK3β.

Given the essential role of GSK3β in the Wnt/β-catenin signaling pathway, its expression was measured in this study. GSK3β activity is regulated by phosphorylation at both the serine 9 (S9) residue in the inactive state and the tyrosine 216 (Y216) residue in the active state.<sup>33, 34</sup> In this study, Western Blotting and immunofluorescence of GSK3β expression and localization indicated that EEROP treatment regulated β-catenin expression through GSK3β activity modulation, specifically by increasing the expression of its active form. This suggests that EEROP may regulate the phosphorylation state of GSK3β, influencing β-catenin expression. As β-catenin is known to be involved in centrosome separation during mitosis, the significant reduction in β-catenin expression after EEROP treatment was accompanied by abnormal spindle segregation and apoptotic cell death. Several previous studies revealed that disrupting GSK3β activity not only impairs centrosome regulation in cancer cells but also triggers mitotic catastrophe, leading to apoptotic cell death.<sup>18, 35</sup>

Several natural compounds have been studied



for their effects on Wnt/ $\beta$ -catenin signaling, such as curcumin from *Curcuma longa* and genistein from soy, which have demonstrated anti-proliferative effects via Wnt-signaling pathway suppression.<sup>36, 37</sup> The role of red okra in CRC cells has not been previously reported. Red okra pods contain various bioactive compounds, including polyphenolic compounds, carotene, folic acid, thiamine, riboflavin, niacin, vitamin C, oxalic acid, and amino acids.<sup>14</sup> The polyphenolic compounds within *A. esculentus* may contribute to its therapeutic effect. This study, to the best of our knowledge, provides the first evidence that treatment with *A. esculentus* induces apoptosis in CRC cells by regulating Wnt/ $\beta$ -catenin signaling activity.

It is important to note that this study has limitations, including the use of cell culture instead of an animal model. While *in vitro* studies provide valuable insights into cellular and molecular mechanisms, further *in vivo* studies and clinical trials are necessary to validate the findings for clinical application.

### Conclusion

EEROP demonstrated the ability to induce apoptosis in SW480 cells through the regulation of the Wnt/ $\beta$ -catenin signaling pathway. The 72-hour treatment with EEROP notably increased the population of cells expressing caspase-3 protein and elevated the cleavage of PARP1 protein. Furthermore, there was a significant reduction in  $\beta$ -catenin expression, accompanied by changes in GSK3 $\beta$  phosphorylation. To strengthen the validity of these *in vitro* findings, further investigation using an animal model is essential.

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### Authors' Contribution

F.R.P.D. contributed to the conception of the work, performed MTT assay, protein isolation,

Western Blotting, and drafted the manuscript; S.P.A.W. contributed to the extraction of red okra pods and reviewed the manuscript; V.L. contributed to the bioinformatics analysis and drafted the manuscript; L.L.A.I. contributed to the immunofluorescence staining and drafted the manuscript; A.H. contributed to the statistical analysis and reviewed the manuscript. All authors made substantial contributions to the final manuscript revisions, have read and approved the final manuscript, and agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Conflict of Interest:** None declared.

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