

# Different Mechanisms of Seed Kernel Extract from *Mangifera indica* on the Growth of Two Colon Cancer Cell Lines

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## Abstract

Flesh extract of *Mangifera indica* possesses anti-proliferation effect on different types of cancer cells. However, the effect of its seed kernel extract (MSP) on the growth of human colorectal carcinoma cells (CRC) has not yet been evaluated. Phenolic species of MSP were extracted and measured by colorimetry. Two CRC cell lines (Colo 320DM and SW480) were treated with MSP and assessed for viability by trypan blue exclusion, for cell cycle distribution by flow cytometry, for apoptosis by annexin V labeling, for mitochondria potential by rhodamine 123 staining and for changes in the levels of proteins involved in cell cycle control or apoptosis by immunoblotting. MSP inhibited the proliferation (12.5 µg/mL - 50 µg/mL) of Colo 320DM and SW480. MSP inhibited proliferation by blocking cell cycle progression at G1 (SW480) or S (Colo 320DM) phase and inducing apoptotic death. Western blotting indicated that MSP-blocking cell cycle was associated with cyclin levels. MSP-treated Colo 320DM and SW480 also showed activation of caspase 8, 9 and 3. MSP induces cell cycle arrest and apoptotic death in two CRC cell lines. The results indicate that MSP is a potential novel chemoprevention and treatment agent for colorectal cancer.

## Keywords

Mangoseed Kernel Extract, Colorectal Carcinoma, Cell Cycle, Apoptosis

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## 1. Introduction

Colorectal carcinoma (CRC) is the most common cancer in Taiwan since there were 12,488 newly-diagnosed CRC patients in 2009. CRC is a progressive disease which often begins from benign tumors or inflammatory disorders. After gradual accumulation of gene mutations, chromosomal instability and epigenetic changes, the malignant carcinoma would form and threaten the patients' life [1]. Epidemiological studies show a correlation between increased consumption of phenolic compounds and reduced risk of many diseases, including cancer [2] [3].

Fruit seeds contain many protective phenolic compounds, making them of interest to the nutraceutical industry [4]. *Mango* (*Mangifera indica*) is a fruit tree grown throughout southern Asia, with India, Malaysia, Thailand and Taiwan as the main centers of commercial production [5]. Recent studies showed that the peel, flesh and seed of Mango contain rich amount of bioflavonoids [6]. Mango juice could inhibit the transformation of mouse fibroblast Balb/3T3 cells and the cell cycle progression of human promyelocytic leukemia HL-60 cells [7]. Feeding the Mango flesh to Swiss albino mice could protect its liver cell transformation induced by carcinogens [8]. Mango extract also showed the inducing ability of apoptosis to prostate cancer LNCaP cells [9]. A component of Mango bark named mangiferin could protect benzo(a)pyrene-induced carcinogenesis of lung cells [10]. Mangiferin regulated CDC2/cyclinB1 expression to suppress the growth of HL-60 cells [11]. The anti-cancer ability of mangiferin and its derivatives was mediated by inflammation-associated pathway on breast cancer MCF-7 and MDA-MB-231 cells [12]. Mango flesh extract exhibited inhibited effect on the cell growth of leukemia Molt-4 cells, lung adenocarcinoma A549 cells, breast cancer MDA-MB-231 cells, prostate cancer LNCaP cells and colorectal cancer SW480 cells [13]. Abu Bakar and colleagues revealed that the ethanol extract from the seed kernel of *Mangifera pajang* could inhibit the growth and induce G2/M arrest of cell cycle and apoptosis on MCF-7 and MDA-MB-231 cells [14], indicating the potential role of Mango seed kernel extract on cancer treatment or prevention. Here, we investigated the effect of the ethanol extract from the seed kernel of *Mangifera indica* (MSP) on the growth, cell cycle and apoptosis of two CRC cells and revealed the potential role of MSP on CRC chemoprevention and treatment.

## 2. Materials and Methods

### 2.1. Materials

Roswell Park Memorial Institute (RPMI) media 1640, Leibovitz L-15 and Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin and antibiotics were purchased from Gibco Ltd. (Paisley, UK). Proteinase inhibitor cocktail, sodium orthovanadate, NaF, sodium pyrophosphate, Triton X-100, ammonia persulfate, *N,N,N',N'*-tetramethylethylenediamine (TEMED), Tween 20, rhodamine 123, propidium iodide, gallic acid and catechin were purchased from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL). Acrylamide was purchased from Bio-Rad (Hercules, CA). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was purchased from Millipore (Bedford, MA). Mouse monoclonal anti-caspase 3, cyclin A, cyclin D1 and Cyclin E antibodies were purchased from Zymed (San Francisco, CA). Goat polyclonal anti-poly [ADP-ribose] polymerase (PARP), Tp53 and CIP 1/p21 antibodies and goat anti-rabbit, anti-mouse and rabbitanti-goat secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from R&D Systems (Minneapolis, MN). Annexin V conjugated with FITC was from Gene Research (Taipei, Taiwan).

### 2.2. Cell Line

Human CRC cell lines SW480 and Colo 320DM and rat small intestine epithelial cell IEC-6 were obtained from the Bioresource Collection and Research Center, Taiwan. SW480, established from a primary adenocarcinoma of a colon cancer patient with Duke's stage B, were cultured in 90% Leibovitz L-15 supplemented with 10% heat-inactivated FBS. Colo 320DM, derived from a moderately differentiated adenocarcinoma of a colon cancer patient, were cultured in 90% RPMI medium 1640 supplemented with 10% heat-inactivated FBS. IEC-6 were cultured in 90% DMEM supplemented with 0.1 Unit/ml bovine insulin and 10% FBS. All media used here were supplemented with 25 U/mL penicillin and 25 µg/mL streptomycin as antibiotics. The cells were incubated at 37°C in a 95% air/5% CO<sub>2</sub> and water-saturated atmosphere, except that SW480 were in room air. All experiments were carried out on cell lines passaged 5 - 20 times. *Mango* fruit was purchased from a certified *Mango*

farm (Tainan, Taiwan).

### 2.3. MSP Preparation

*Mango* fruit was washed and peeled, and then the seeds were dried in a 70°C oven for at least 48 h. The dried seeds were then ground using a stainless-steel grinder (RT-02, Rong Tsong Iron Factory Incorporation, Taiwan). The powder was sealed and stored at -20°C, or applied to extraction of the polyphenol compounds. For extraction, *Mango* seed powder was refluxed with a 10 times (v/w; mL/g) ratio of 70% ethanol solution overnight. The crude extract was filtered through No 1 filter paper, and then centrifuged at 3000 rpm for 30 min. The supernatant was concentrated using a rotary evaporator under reduced pressure in a water bath at <35°C and, then, freeze-dried. The final crude extract was defined as MSP, in which polyphenol species named total phenols, total flavonoids and condensed tannins were measured by colorimetry methods as previously described [15]. Briefly, the amount of total phenolics in MSP, estimated by the Folin-Ciocalteu method was performed by mixing the samples (0.25 mL) with 3.5 mL of distilled water in screw-capped test tubes followed by addition of 0.5 mL of Folin-Ciocalteu solution. After 3 min, 1 mL of sodium carbonate (20%) was added, and the test tubes were properly shaken before they were incubated in a boiling water bath for 1 min. The tubes were then allowed to cool in darkness. A blue coloration was developed, and the absorbance was read at 685 nm. Results were expressed in mg of gallic acid equivalent/g dry mass MSP. The total flavonoid content of LCSP, measured by the AlCl<sub>3</sub> method, was assessed by mixing aliquots of 1.5 mL of MSP with equal volumes of a solution of 2% AlCl<sub>3</sub>·6H<sub>2</sub>O (2 g in 100 mL methanol). The mixture was vigorously shaken, and absorbance was read at 367.5 nm after 10 min of incubation. Flavonoid contents were expressed in mg catechin equivalent/g dry weight of MSP. Condensed tannins of MSP, determined by the vanillin method were performed by mixing an aliquot of LCSP with a final volume of 5 mL of vanillin reagent (0.5% vanillin in glacial acetic acid containing 4% HCl). The formed pink chromogen was read at 510 nm. The concentration of condensed tannins was expressed as catechin equivalent of LCSP. The total phenols, total flavonoids and condensed tannins in MSP were 481.97 mg, 29.83 mg and 22.48 mg per gram dry weight of MSP, respectively.

### 2.4. Cell Proliferation Assay

Two CRC cell lines and IEC-6 cells were plated at 100,000 cells in 60-mm tissue culture dishes. After 18 h of culture, cells were treated with different concentrations of DMSO-dissolved MSP (0, 12.5, 25 and 50 µg/mL). At 24 h, cells were collected by trypsinization, stained with trypan blue, and the cell number in suspension was counted in duplicate using a hemocytometer. Data were the average of three independent experiments.

### 2.5. Cell Cycle Analysis

MSP treated cells were harvested by trypsinization and washed with phosphate-buffered saline, then ethanol-fixed in 70% ethanol at -20°C for at least 30 min. Fixed cells were collected by centrifugation and reconstituted in phosphate-buffered saline. Cells were then stained with propidium iodide solution (20 µg/mL propidium iodide and 10 µg/mL RNase A) at 37°C in the dark for 30 min. The stained cells were examined by flow cytometry using FL-2A to score DNA content of cells. Percentages of G1, S and G2/M cell cycle phases were determined with Modfit software (Verity Software House, Inc., Topsham, ME).

### 2.6. Apoptosis Analysis

Apoptosis measurement was carried out using annexin V to label cell surface phosphatidylserine of apoptotic cells [16]. Briefly, treated cells were trypsinized and washed twice with phosphate-buffered saline, then suspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl<sub>2</sub>). Cells were stained with a final concentration of 2 µg mL<sup>-1</sup> of annexin V conjugated with fluorescein isothiocyanate (FITC) at room temperature in the dark for 30 min. Flow cytometry measured fluorescence intensity with the FL-1H channel detecting FITC. Untreated cells served as the negative control.

### 2.7. Mitochondrial Membrane Potential ( $\Delta\Psi_m$ )

$\Delta\Psi_m$  measurement was carried out essentially as described by Hsu *et al.* [17]. Briefly, the MSP-treated cells

were harvested and suspended at a density of  $1 \times 10^6$  cells/ml in fresh medium. The cells were then stained with rhodamine 123 at a final concentration of 10  $\mu\text{g/ml}$  for 30 min at 37°C. Cells were then washed twice with fresh medium, and the fluorescence intensity of cells was immediately examined by flow cytometry. Ten thousand cells without cell debris were analyzed, and the rhodamine 123 negative cells were defined as those with lower fluorescence intensity than untreated cells.

## 2.8. Immunoblotting

MSP-treated cells were washed with ice-cold phosphate-buffered saline and lysed in homogenization buffer (10 mM Tris-HCl at pH 7.4, 2 mM EDTA, 1 mM EGTA, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1:100 proteinase inhibitor cocktail) on ice for 30 min. After centrifugation for 30 min at 13,000 rpm at 4°C to remove insoluble materials, the lysate was determined the protein concentration by BCA protein assay kit and then separated on SDS-PAGE. The resolved bands were electrotransferred to PVDF membranes using a semi-dry blot apparatus (Bio-Rad) at 3 mA per  $\text{cm}^2$  of the gel in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol) at room temperature for 30 min. Immunoblotting was performed by incubating PVDF membranes with 5% non-fat milk in Tris-buffered saline supplemented with Tween 20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween 20) for 1 h at room temperature for blocking the residue free protein binding sites on PVDF. The membrane was incubated with different primary antibodies in 3% non-fat milk in TBST at 4°C for 18 h. After repeating washing with TBST, the membrane was incubated with secondary antibodies conjugated with HRP. Immunoblots were developed using enhanced chemiluminescence and the luminescence visualized on a chemoluminescence detection system (Bio-Rad).

## 2.9. Statistical Analysis

All data are expressed as means + standard deviation (SD) unless stated otherwise. Differences between groups were calculated using the Student's unpaired *t*-test. Dose-dependent effect was calculated using simple linear regression.  $P < 0.05$  was considered statistically significant. All statistical analyses were performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL).

## 3. Results

### 3.1. MSP Inhibits CRC Cell Growth

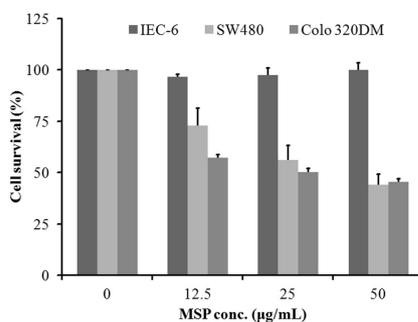
As shown in **Figure 1**, compared with untreated cells, survival decreased with increasing MSP dose in two CRC cells but not in normal cell line IEC-6. The survival ratio of Colo 320DM and SW480 were decreased by MSP treatment, with more than 25% inhibition at 12.5  $\mu\text{g/mL}$  and 50% inhibition at 50  $\mu\text{g/mL}$ . The survival ratio of MSP-treated normal intestinal epithelium IEC-6 cells was unremarkable change compared with untreated control.

### 3.2. MSP Blocked Cell Cycle of CRC Cells

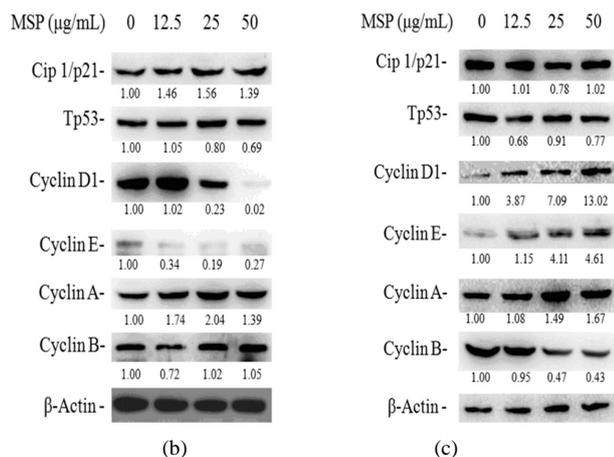
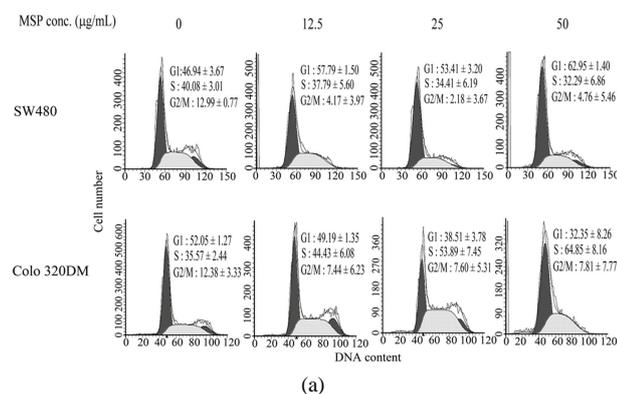
To determine the cellular and molecular mechanism of growth inhibition of MSP on CRC cells, we investigated cell cycle progression and the change of the expression levels of cell cycle controlling proteins after MSP treatment. As shown in **Figure 2(a)**, G1 phase increased and G2/M phase decreased in SW480 treated with more than 12.5  $\mu\text{g/mL}$  MSP, The S phase increased and G1 phase decreased in Colo 320DM treated with MSP. Immunoblotting analysis showed that cyclin D1, cyclin E and Tp53 levels were gradually decreased and p21 and cyclin A were increased in SW480 cells after MSP treatment (**Figure 2(b)**). Cyclin B level was unremarkable change in MSP-treated SW480 cells. The levels of cyclin D1, cyclin E and cyclin A were increased and cyclin B and Tp53 were decreased in MSP-treated Colo 320DM cells (**Figure 2(c)**). P21 level was unremarkable change in MSP-treated Colo 320DM cells.

### 3.3. MSP-Induced Apoptotic Death of CRC Cells

Phosphatidylserine translocation was assessed to determine apoptosis of MSP-treated CRC cells by staining with FITC-conjugated annexin V. Annexin V positive cells gradually increased in Colo 320DM cells and SW480 cells after which were treated with more than 12.5  $\mu\text{g/mL}$  MSP (**Figure 3(a)**, **Figure 3(b)**). The cells with mitochondria



**Figure 1.** The dose dependent response of four colorectal carcinoma (CRC) cell lines to *longan* seed extract (LSP).

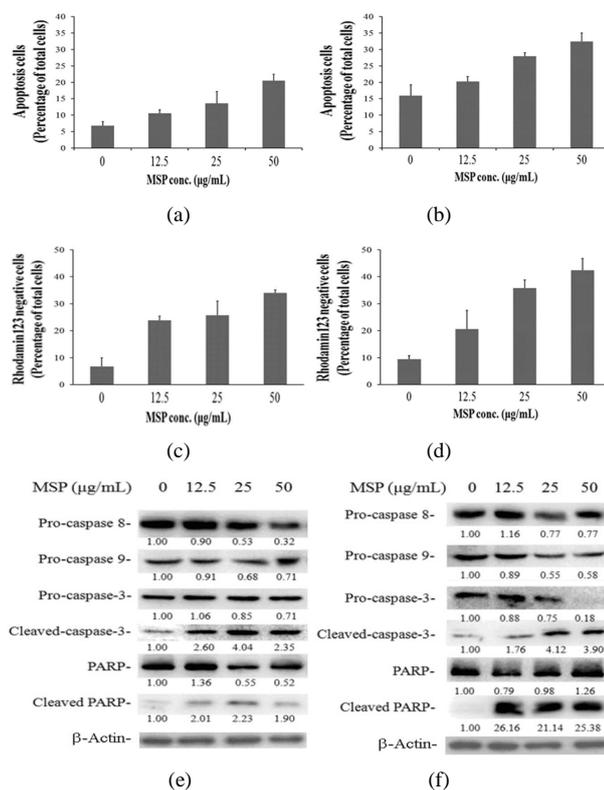


**Figure 2.** Cell-cycle blockage in CRC cells by MSP.

membrane potential loss, assessed by measurement of rhodamine 123 staining also decreased in MSP-treated Colo 320DM and SW480 cells (**Figure 3(c)**, **Figure 3(d)**). Pro-caspase 8 and pro-caspase 3 levels were decreased whereas cleaved-caspase 3 and cleaved PARP were increased in MSP-treated SW480 cells (**Figure 3(e)**). In MSP-treated Colo 320DM, pro-caspase 8, 9, and 3 levels were decreased whereas cleaved caspase 3 and cleaved PARP were increased (**Figure 3(f)**).

#### 4. Discussion

We here investigated MSP growth inhibition in two CRC cell lines. MSP inhibited proliferation of Colo 320DM and SW480 cells. To our best knowledge, this is the first evidence that MSP inhibits proliferation of CRC cells. Recent studies show that Mango extract or its important component mangiferin could suppress the growth of lung, colon, prostate, breast cancer cells and leukemia cells [18]. The seed kernel extract from a relative species



**Figure 3.** MSP-induced apoptosis in CRC cells.

of Mango named *Mangifera pajang* possessed the inhibition ability to breast cancer cell proliferation [14], indicating the potential role of Mango seed kernel extract on cancer treatment or prevention. Here, we directly demonstrated that MSP inhibited proliferation in at least two CRC cell lines.

Cell cycle arrest and apoptosis are two main cellular mechanisms that polyphenols inhibit the growth of CRC cells [15] [19]. MSP induces cell cycle arrest and apoptosis in Colo 320DM and SW480 cells, indicating that MSP could regulate both cell cycle machinery and apoptosis signaling and suppresses CRC cell growth. However, the detail effects seem to be different in these two CRC cells. MSP arrests cell cycle of SW480 cells in G1 phase, which associates with downregulation of cyclin D1 and E and upregulation of p21. Not the same as SW480 cells, Colo 320DM cells are arrested by MSP treatment in S phase of cell cycle, which may be from the upregulation of cyclin E and downregulation of cyclin B [20]. Cyclin D1 and cyclin E are both key regulators to CDK4 and 6, which phosphorylate retinoblastoma protein and results in retinoblastoma protein to degeneration. The retinoblastoma protein degeneration releases transcriptional factor E2F, triggers the protein expression for DNA synthesis and leads cells entering S phase of cell cycle [21] [22]. CIP1/p21 is the negative regulator to cyclin E/CDK 6 complex. MSP-inducing CIP 1/p21 expression and downregulates cyclin D1 and E may lead SW480 cells to be arrested in G1 phase. However, the effect of MSP in cell cycle of Colo 320DM cells is quite different to SW480. MSP arrests Colo 320DM cells in S phase of cell cycle. Cyclin D1, E and A are all increased and only cyclin B is downregulated by MSP treatment in Colo 320DM. Downregulated cyclin B is often associated with G2/M phase arrest of cell cycle [23], the result provided here does not seem to comply this rule. This may be associated with the expression of cyclin E and suppression of cyclin B level. A recent report reveals that upregulation of cyclin E and concurrent downregulation of cyclin B prevent S phase exit [20]. This may be the main event that MSP-treated Colo 320DM cells are arrested at S phase rather than G2/M phase.

Apoptosis induction is another possible mechanism of MSP in CRC cells. In the present study, we showed that MSP induced apoptosis significantly in Colo 320DM and SW480 cells at concentrations more than 12.5 µg/mL. MSP-induced apoptosis was mediated by caspase 3 activation. Many reports suggest that polyphenolic-induced apoptosis in CRC cells was mediated by activation of caspase 3 and the elevating cleavage fragments of its substrate PARP was a functional marker to imply caspase 3 activity [24] [25]. MSP treatment increases

protein levels of cleaved caspase 3, the active form of caspase 3, and the cleaved PARP in Colo 320DM and SW480 and closely correlated with apoptotic induction. These results indicate that MSP-induced apoptosis in Colo 320DM and SW480 is mediated by caspase 3 activation.

MSP-induced caspase 3 activation and apoptosis may operate through caspase 8-mediated apoptosis. Caspase 8 is one of the cysteine proteases involved in apoptosis. The resting stage of caspase 8 is an inactive single polypeptide chain zymogen procaspase, named pro-caspase 8. When ligand binding-induced trimerization of death receptors occurs, the receptor-specific adapter protein Fas-associated death domain (FADD) is recruited. This protein complex then recruits pro-caspase 8 and is activated by proteolytic cleavage. Activated caspase 8 then communicates the apoptosis signal either by directly cleaving and activating downstream caspases such as caspase 3 or by cleaving the BH3 Bcl2-interacting protein, which results in increasing mitochondria permeability and the release of cytochrome c from mitochondria to cytosol, promoting activation of caspase 9 in a complex with dATP and Apaf-1 [26]. In the present result, pro-caspase 8 level is decreased after MSP treatment in both SW480 and Colo 320DM and concomitantly mitochondria membrane potential of both CRC cells is disruption and pro-caspase 9 level is decreased. Taken together, we conclude that MSP induces caspase 8 activation and triggers caspase 9 cleavage and then activates caspase 3 to provoke CRC cells apoptosis.

## 5. Conclusion

In conclusion, MSP inhibited CRC cells proliferation mainly through cell cycle arrest at either G1 or S phase, through regulating levels of cyclins. MSP may operate through apoptosis by activating caspase 8 and directly triggers caspase 3 activation or passes through mitochondria membrane potential disruption and the cleavage of pro-caspase 9. We found that MSP treatment possesses anti-proliferative effect in CRC cell lines, suggesting its potential as a novel chemopreventive agent for CRC.

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