African Journal of Food Science Vol. 4(8) pp. 495 - 502, August 2010
Available online http://www.academicjournals.org/ajfs
ISSN 1996-0794 ©2010 Academic Journals

Full Length Research Paper

Effects of oil palm phenolics on tumor cells *in vitro* and *in vivo*

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Accepted 18 June, 2010

The effects of oil palm phenolics (OPP) on cell lines were demonstrated in this study. OPP dose-dependent studies in selected cell cultures showed that OPP inhibited the proliferation of mouse IgA-secreting myeloma (J558), estrogen-receptor-positive human breast adenocarcinoma (MCF7) and human lung carcinoma (A549) cells at all doses. However, OPP enhanced the proliferation of Madin Darby Canine Kidney (MDCK) and *Aedes albopictus* larvae (C6/36) cells at all concentrations, whereas, growth of Syrian Baby Hamster Kidney (BHK) cells was only enhanced with low doses of OPP. 5% OPP in combination with 5% fetal calf serum (FCS) was shown to be as effective as 10% FCS on the growth of BHK and A549 cells. *In vivo* studies using J558 myeloma cells showed that OPP caused tumor regression. These findings imply that OPP has anti-proliferative effects on tumor cells *in vitro* and *in vivo*, but conversely enhances the growth of normal cells.

Key words: *Elaies guineensis*, oil palm, phenolics, cancer, cell lines, apoptosis.

INTRODUCTION

The oil palm (*Elaies guineensis*) is an important oil crop that contains large quantities of phytochemicals, which can either be lipid-soluble or water-soluble. Numerous studies have been carried out in recent years to explore the anti-cancer properties of oil palm which is rich in lipid-soluble vitamin E (tocopherols and tocotrienols) and carotenes, as well as water-soluble phenolics (Sundram et al., 2003). Red palm olein is a major source of carotenoids which effectively inhibit some types of cancer. A diet containing palm oil, when compared to diets containing other oils exerted an inhibitory effect on the development and incidence of experimentally-induced breast cancer in rats (Sylvester et al., 1986). In animal models, palm oil has been shown to offer a significant protection against 12-*O*-tetradecanoyl-phorbol-13-acetate-mediated skin tumorigenesis in Swiss albino mice (Kausar et al., 2003). The tocotrienol-rich fraction of palm oil has been shown to inhibit the growth and proliferation of estrogen receptor-negative and estrogen receptor-positive human breast cancer cells (Guthrie et al., 1997). Other minor palm oil constituents including β-carotene, have been shown to individually enhance the anti-cancer activity of natural killer cells and tumor necrosis factor-α *in vitro* and *in vivo* (Prabhala et al., 1993). Another minor constituent of the oil palm which may have potential anti-cancer properties is the recently discovered water-soluble oil palm phenolics (OPP), which consist mainly of phenolic acids isolated from the palm oil mill vegetation liquor through a solvent-free patented process (Sundram et al., 2001; Sambanthamurthi et al., 2008; Sambandan et al., 2009).

An important mechanism of cytotoxicity in the prevention and treatment of cancer is the apoptosis or

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Abbreviations: OPP, Oil palm phenolics; **IgA,** immunoglobulin A; **DMEM,** Dulbecco's modified eagle media; **RPMI,** roswell park memorial institute media; **MEM,** minimum essential media; **FCS,** fetal calf serum; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; **PBS,** phosphate-buffered saline; **TUNEL,** TdT-mediated dUTP nick end-labeling.

programmed cell death pathway (Weinberg, 2007). Apoptosis is characterized by several morphological and biochemical changes including blebbing of plasma membrane, condensation of cytoplasm and nucleus, as well as degradation of DNA (Stellar, 1995). The inability of cells to undergo apoptosis is a key factor in the pathogenesis of malignancies. Recently, there has been increasing interest on the use of palm oil and other oil palm components in tumor inhibition and regression (Nesaretnam, 2008; Yu et al., 2008). In relation to this, several studies have been carried out on tocopherol- and tocotrienol-induced apoptosis (Yu et al., 1999; McIntyre et al., 2000). In this study, the authors determined the effects of water-soluble OPP (also known as palm juice) on tumor and normal cell lines, and further checked for the mechanism of cell death caused by OPP in cell lines which were inhibited by the extract. They also determined the *in vivo* effects of OPP on J558 myeloma tumor growth in syngeneic BALB/c mice.

MATERIALS AND METHODS

Cell lines

Six selected cell lines were cultured in different growth media in 25 cm^3 cell culture flasks. The cell lines used were mosquito larvae of *Aedes albopictus* (C6/36), Syrian Baby Hamster Kidney (BHK), Madin Darby Canine Kidney (MDCK), estrogen receptor-positive human breast adenocarcinoma (MCF7), mouse IgA-secreting myeloma (J558) and human lung carcinoma (A549). The cells were grown in Dulbecco's Modified Eagle Media (DMEM) (for BHK, MCF7, A549 and J588), Roswell Park Memorial Institute Media (RPMI) (for C6/36) and Minimum Essential Media (MEM) (for MDCK) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 100 U/ml penicillin-streptomycin. All cell cultures were incubated at 37˚C in 5.0% CO_2 except for MDCK (37˚C in O_2) and C6/36 (28˚C in O_2). The stock OPP was kindly provided by the Malaysian Palm Oil Board (MPOB).

Dose experiments

For all the selected cell lines except for the J558 cell line, 1 X 10^5 viable cells were distributed into each well in a 96-well flat-bottom microtiter plate and sterile OPP was added in concentrations of 200, 500, 1000, 1500 and 2000 µg/ml before topping up with the appropriate maintenance media (DMEM, RPMI 1640 or MEM). Control wells were prepared and each dose of the extract was studied in triplicates. The plates were incubated in optimal conditions for each cell line and changes in cellular morphology, proliferation and viability were observed on days 1, 3, 5 and 7 using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. For the J558 cell line, sterile OPP was added in concentrations of 200, 400, 600, 800 and 1000 µg/ml, and observations were carried out 24, 48 and 72 h after incubation. The reasons for these discrepancies were (i) the J558 cell line was more sensitive to the effects of OPP and (ii) the J558 cell line grew faster than the other cell lines, achieving maximal growth on the third day for the control, after which the cells would start dying due to nutrient constraint.

MTT assay

The MTT assay was performed according to the method of Hansen et al. (1989). Briefly, 20 µl of MTT labeling reagent was added to each well after incubation. After four hours, 100 µl of solubilization solution was added to each well prior to overnight incubation. The absorbance of the samples was measured using an ELISA reader (DYNATECH MR5000, Guernsey Channel Islands) at 570 nm wavelength, with a reference wavelength of 650 nm.

Detection of apoptotic DNA ladder

Cellular DNA from incubated cells was extracted at the end of the incubation period using the apoptotic DNA ladder kit (Roche, Germany). Briefly, cells treated with different concentrations of OPP were harvested from microtiter plates (approximately 2 x 10^6 cells per well), centrifuged at 1000 rpm for ten minutes and then washed with phosphate-buffered saline (PBS). The resulting pellet was resuspended with PBS and mixed with 200 µl of binding buffer, prior to incubation at room temperature for ten minutes. Isopropanol (100 µl) was added and the cell suspension was vortexed. All suspensions were then pipetted into the upper reservoir of a filter tube and centrifuged at 8000 rpm for one minute.

The filter was washed twice with the washing buffer and centrifuged at 8000 rpm for one minute. The filter was centrifuged at 13000 rpm for ten seconds to remove residual liquid prior to being transferred to a fresh microcentrifuge tube. DNA was eluted from the filter with the addition of 200 µl of prewarmed (at 70˚C) elution buffer, followed by centrifugation at 8000 rpm for one minute. The eluted DNA was treated with RNase and quantified. 2 µg of sample DNA and control DNA (from apoptotic U937 cells) were electrophoresed in a 1% agarose gel for 1.5 h at 75 V.

Detection of apoptotic cells using the TdT-mediated dUTP Nick End-labeling (TUNEL) enzymatic labeling assay

The Dead End Colorimetric Apoptosis Detection System (Promega, USA) was used for the detection of apoptosis in OPP-treated cells. The assay was performed according to manufacturer's instructions. Briefly, cells treated with different concentrations of OPP were harvested from microtiter plates (approximately 2 x 10^6 cells per well), centrifuged at 1000 rpm for ten minutes, then washed with PBS, resuspended with PBS and laid onto slides. The slides were air dried and fixed in 10% formalin at room temperature for 25 min, then washed twice with PBS at room temperature for five minutes. Cell permeabilization was carried out by immersing the slides in 0.2% Triton x-100 at room temperature for five minutes.

The slides were then washed twice with PBS and 100 µl of equilibrium buffer was added prior to incubation at room temperature for five to ten minutes. The slides were blotted dry prior to addition of 100 µl of TdT reaction mix and incubated at 37˚C for 60 min in a humidifying chamber. The reaction was terminated using a 2 x saline-sodium citrate solution at room temperature for five minutes. The slides were washed twice and blocked with 0.3% hydrogen peroxide. The slides were then washed twice before adding 100 µl of diluted streptavidin in horseradish peroxidase solution, followed by an incubation at room temperature for 30 min. Following this, the slides were washed twice before the addition of 3,3'-diaminobenzidine mixture, and allowed to develop prior to stopping the reaction by rinsing in deionized water. The slides were then mounted and viewed under a light microscope.

OPP as a growth additive in cell cultures

BHK and A549 cell cultures were selected to be grown in DMEM containing (i) 10% FCS, (ii) 5% OPP with 5% FCS, and (iii) 10% OPP. The cells were incubated in optimal conditions and grown in triplicates. The stock solution of OPP used was 1000 µg/ml. When the cells were 100% confluent, they were passaged to a total of five

passages, into new flasks where similar preparations of DMEM medium were used. DNA was extracted from the cells at the end of the fifth passage to detect for any changes at the molecular level.

***In vivo* effects of OPP on tumor development in BALB/c mice**

Six to eight weeks old BALB/c mice were divided into four groups (groups A-D), of 12 mice each. Groups A, B and C were given OPP as the drinking source at three different dilutions: 25, 50 and 100% OPP, respectively. The OPP dilutions were freshly prepared and given daily. Group D, the control group, was given normal plain water. Tumor growth was observed daily. The mice were sacrificed 21 days post-inoculation. The resulting tumors were collected, weighed and measured (length, width, and height). In a separate experiment, mice were treated with 100% OPP with and without the inclusion of cyclophosphamide (200 - 300 mg/kg body weight).

RESULTS

Effects of OPP on the growth and viability of cells

OPP exhibited different effects on the growth and viability of normal cells and tumor cells. Using the MTT assay, the number of viable tumor cells from the J588 and A549 cell lines showed an overall decrease over the 72 h and seven days of incubation respectively (Figures 1A-1B). However, there was a general increase in the number of viable MCF7 cells from days 1 - 5 of incubation, which declined on day 7 (Figure 1c). The controls were maintained constantly throughout the incubation periods, and had higher numbers of viable cells when compared to the treatment groups. The cells were generally less viable and less proliferative at higher doses of OPP, with the highest inhibition occurring at the maximal OPP concentration used in each case. In contrast, all doses of OPP on normal cells (BHK, C6/36, MDCK) showed a general increase in the number of viable cells until day 5 (Figures 1d - 1f). Overall, there were major increases in the number of viable and proliferative cells, with 1500 µg/ml OPP being the most effective dose for MDCK cells, 200 µg/ml for BHK cells and 500 µg/ml for C6/36 cells.

Detection of DNA degradation using the apoptotic DNA ladder

Electrophoresis of DNA extracted from J558 tumor cells treated with OPP showed nucleosomal fragmentation pattern in the agarose gel, compared to intact chromosomal DNA from untreated cells (Figure 2). Similar results were obtained for the A549 and MCF7 tumor cells. However, in the normal cells (MDCK, BHK, C6/36), no fragmentation or DNA smearing was observed (Figure 3).

Detection of apoptotic cells using the Tdt-mediated dUTP nick end-labeling (TUNEL) assay

Apoptotic cells were detected using colorimetric detection system in all tumor cells treated with OPP (Figure 4). There was an increase in the number of apoptotic cells in groups incubated with higher concentrations of OPP. There were no apoptotic cells detected in normal and untreated cells. This was consistent with the results of the DNA fragmentation assay.

Effect of OPP as a growth additive

Results obtained were similar in both tumor (A549) and normal (BHK) cell lines. Preliminary findings showed that a combination of 5% OPP with 5% heat-inactivated FCS in DMEM was as effective as conventional growth medium containing 10% FCS (Figure 5). However DMEM containing 10% OPP did not enhance the growth of cells which did not reach a state of confluence. Morphologically, the BHK and the A549 cells differed. BHK cells grown in DMEM containing 10% OPP lost their fibroblastic appearance and did not adhere to the flask. Although the floating cells had the tendency to clump, cytopathic effect was not observed. A significant morphological change was observed in A549 cells grown in 10% OPP whereby cells increased three to five times in size. Cell fusion also occurred, resulting in giant cells prior to lysis.

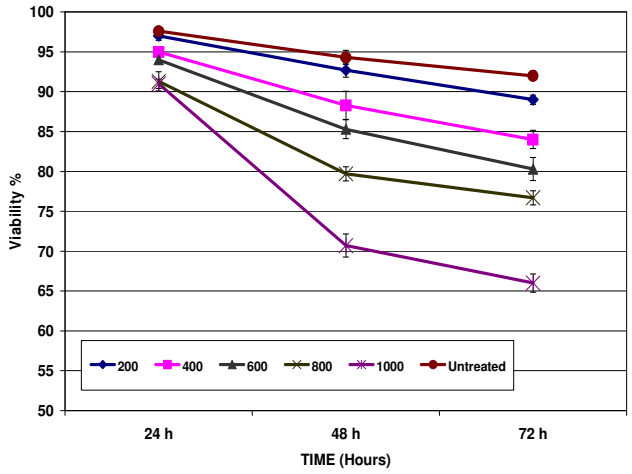
OPP reduces tumor growth *in vivo*

Anti-tumor properties of OPP were studied using six to eight weeks old BALB/c mice. The *in vivo* experiments revealed a decrease in tumor weight, volume and number in all groups treated with OPP (Table 1). The decrease was dependent on the dose of OPP with groups of mice treated with 100% OPP having a lower number, weight and volume of tumors (22.7%, 0.55 g and 643 mm^3, respectively) when compared to the control group. In mice given 50% OPP, tumor appearance percentage, weight and volume increased (37.5%, 0.52 g and 736 mm^3 respectively) and there was a further increase in tumor size and volume in the group of mice given only 25% OPP (Table 1).

In a separate experiment, mice were treated with 100% OPP with and without the inclusion of cyclophosphamide. The results showed that the control group that received cyclophosphamide had the highest number of tumors (100%), while in the control group without cyclophosphamide, the number of tumors was slightly reduced (77.3%). However, the average tumor volume and weight were almost equal in both groups. The group of mice given 100% OPP with cyclophosphamide had a lower number of tumors (40%) when compared to the control groups. Average tumor incidence, volume and weight were significantly reduced in the group of mice given only 100% OPP (22.7%, 0.55 g and 643 mm^3, respectively), when compared to the control groups.

DISCUSSION

The palm oil milling process generates vegetation liquor



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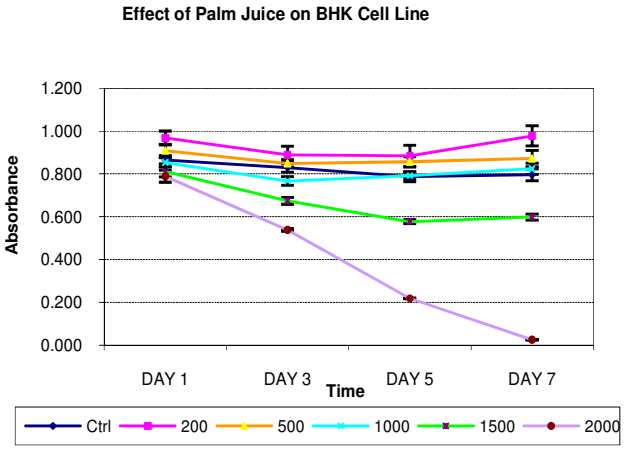

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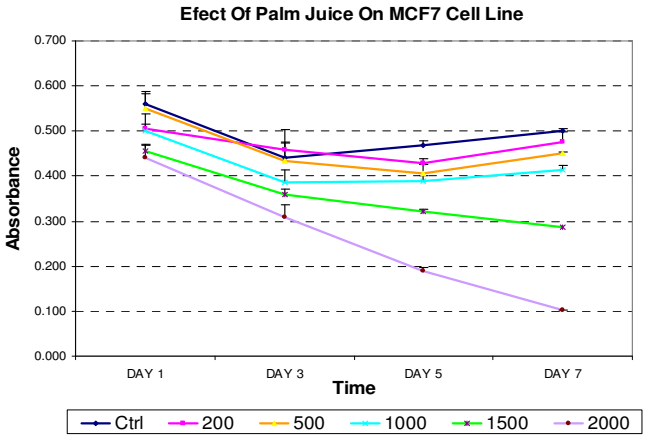

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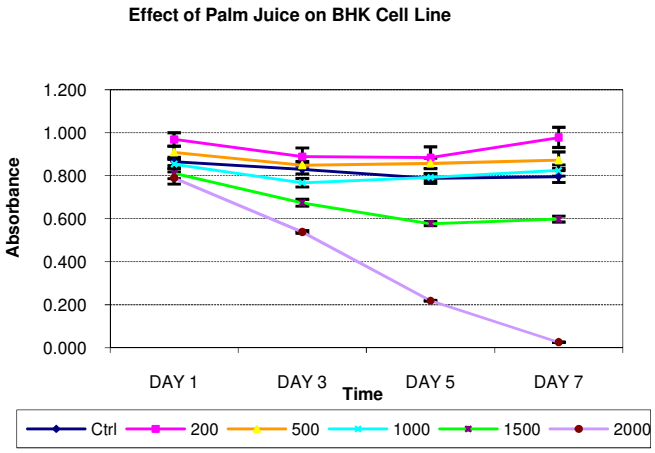

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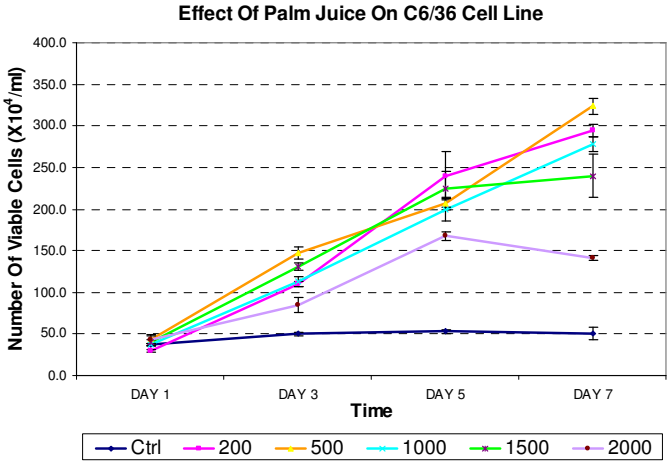

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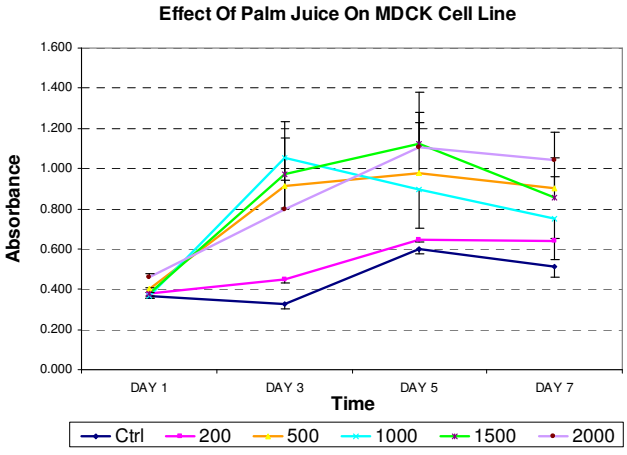

F

Figure 1. Effect of various concentrations of OPP on the viability of different cell lines. A = J558; B = A549; C = MCF7; D = BHK; E = C6/36; F = MDCK.

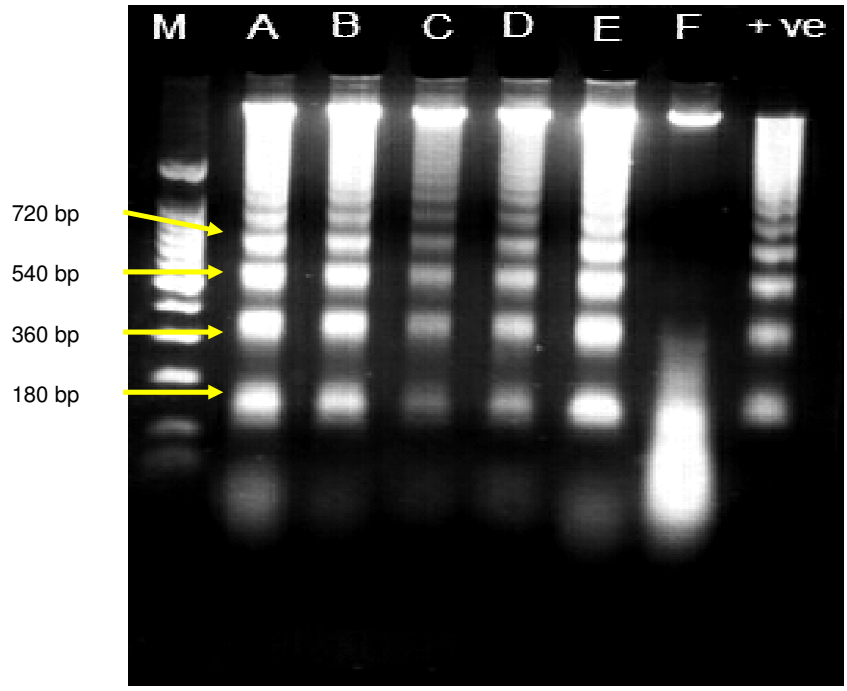

Figure 2. Apoptotic DNA ladder electrophoresis (J558 cells). Lane M = 100 bp molecular weight marker; Lanes A to E = DNA extracted from cells treated with different concentrations of OPP (A = 200 µg/ml; B = 400 µg/ml; C = 600 µg/ml; D = 800 µg/ml and E = 1000 µg/ml); Lane F = DNA extracted from untreated cells (negative control); Lane +ve = DNA extracted from apoptotic U973 cells (positive control).

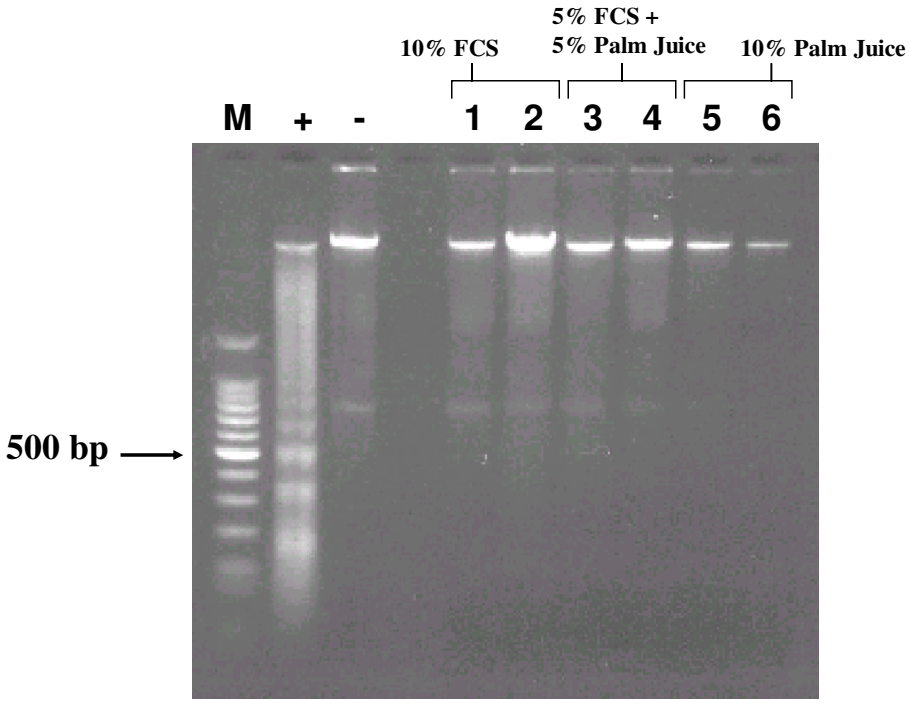

Figure 3. Apoptotic DNA ladder electrophoresis (BHK cells). Lane M = 100 bp molecular weight marker; Lane + = DNA extracted from apoptotic U973 cells (positive control); Lane - = DNA extracted from untreated cells (negative control); Lanes 1 and 2 = DNA extracted from cells grown in 10% FCS, Lanes 3 and 4 = DNA extracted from cells grown in 5% FCS with 5% OPP; Lanes 5 and 6 = DNA extracted from cells grown in 10% OPP

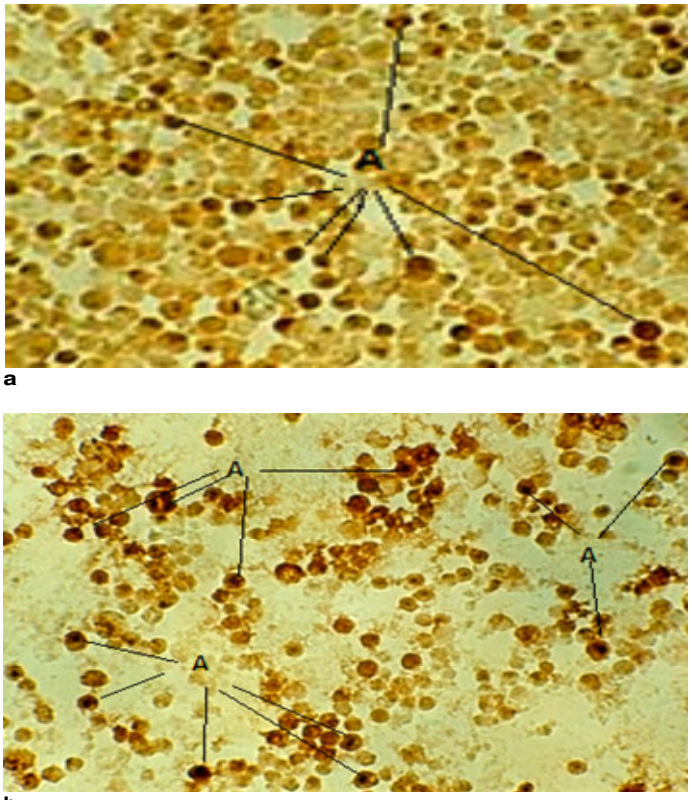

Figure 4. Apoptotic cells detected using the TUNEL assay in J588 cell cultures treated with OPP, photographed at a magnification of X40. A points to apoptotic cells; I = J588 cells treated with 200 µg/ml OPP; II = J588 cells treated with 1000 µg/ml OPP.

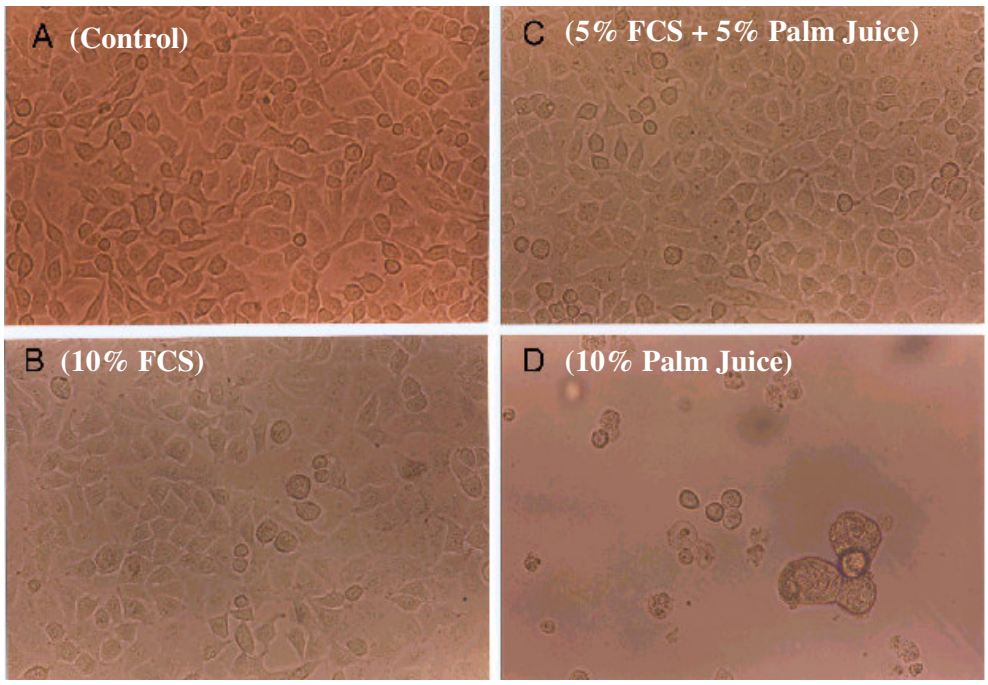

Figure 5. Morphology of BHK cells grown in various preparations of DMEM. A = Negative control; B = BHK cells grown in 10% FCS; C = BHK cells grown in 5% FCS with 5% OPP; D = BHK cells grown in 10% OPP.

Table 1. Effects of OPP on tumor formation and growth in BALB/C mice. 25% = Fed 25% OPP in drinking water; 50% = Fed 50% OPP in drinking water; 100% = Fed 100% OPP in drinking water; control = control group fed plain water.

Groups (%)	Total number	% tumor formation	Average tumor volume/ mm^3 (*p* value)	Average tumor weight/ g (*p* value)
25	24	62.5	1443 (0.168)	0.84 (0.432)
50	24	37.5	736 (0.007)	0.52 (0.007)
100	44	22.7	643 (0.006)	0.55 (0.002)
Control	44	77.3	1617	1.16

which is rich in water-soluble phytochemicals. Isolation and concentration of the phytochemicals from this aqueous stream result in OPP. *In vitro* exposure of tumor cells to various concentrations of OPP revealed dose-dependent growth arrest and cell death effects of the extract. In the dose experiments, when estrogen receptor-positive human breast adenocarcinoma (MCF7), human lung carcinoma (A549) and mouse IgA-secreting myeloma (J558) cell lines were incubated with difference doses of OPP, the extract showed a distinct inhibitory effect on the viability and proliferation of the cells at all doses. These findings are concurrent with the studies on palm oil vitamin E (tocotrienol-rich fraction) tested on human breast cancer cells, where similar inhibitory actions were reported (Guthrie et al., 1997; Nesaretnam et al., 2000; Ng et al., 2000). The accelerated decrease in the viability of the carcinoma cells started after 24 h of treatment with the extract, and was highest at the end of the incubation periods. This effect was more pronounced with higher concentrations of OPP. In contrast, OPP was shown to enhance the growth and proliferation of normal vertebrate and invertebrate cell lines in a dose-dependent manner. Enumerated by the MTT assay, OPP doses below 2000 µg/ml were found to enhance the growth of the C6/36, BHK and MDCK cell lines. However, cell viability decreased dramatically after day 7. This could be due to lack of nutrients or space for cell growth.

In general, cell death can occur by either necrosis or apoptosis (programmed cell death). To determine the cell death mechanism of the tumor cells, two methods of detecting DNA fragmentation were used. Nuclear fragmentation was detected in all samples of tumor cells treated with various concentrations of OPP, indicating that OPP had induced apoptosis in these cells *in vitro*. These results were further confirmed using the TUNEL assay, which showed that the intensity of cells undergoing apoptosis was greater with higher concentrations of OPP used. The selective induction of cell death in tumor cells could be due to the presence of gallic acid in OPP, as that reported by Inoue et al. (1995) and Yoshioka et al. (2000). Agrawal et al. (2004) also showed that the tocotrienol-rich fraction of palm oil activated p53, resulting in DNA fragmentation and ultimately apoptosis. However, further studies would have to be carried out in order to determine the actual mechanisms of apoptosis induction by OPP. These would include the measurements of caspase-3 protein activity and early changes in membrane permeability (Martin and Green, 1995; Levkau et al., 1998).

In addition, OPP has the potential to be used as a growth additive in cell culture media. In the present study, DMEM with 5% FCS and 5% OPP was as effective as DMEM with 10% FCS in growing both tumor (A549) and normal (BHK) cells. This contrast to the anti-proliferative effect shown by the growth enhancement of A549 cells in medium containing FCS supplemented with OPP could be rationalized by the higher concentration of FCS, which could be responsible for masking the inhibitory effect of OPP. The presence of OPP (10%) alone in DMEM however, caused growth inhibition and resulted in abnormal morphological changes in the cells but not within their DNA. A combination of 5% FCS and 5% OPP was thus regarded as an optimal alternative to 10% FCS, whereby OPP can be used as an additive in cell culture media.

In vivo studies in BALB/c mice showed the inhibitory effects of OPP on tumor formation and growth in mice inoculated with J588 cells. The inhibitory effect reached 70% inhibition in animals treated with 100% OPP compared to untreated controls. Mice in OPP-treated groups developed tumors which were significantly smaller up to 50% in size and volume from untreated groups. Furthermore, the extent of growth inhibition varied with the concentration of OPP given, whereby maximum inhibition was achieved in mice given 100% OPP. This shows that the tumor regression properties of OPP are dose-dependent.

However, in a controlled immune system suppressed mice, the tumor incidence increased from 22.7 - 40% in mice which received 100% OPP and cyclophosphamide. These results indicate that the immune system has a vital role to play in tumor suppression, and this property may be enhanced in OPP-treated mice. Similar findings have been shown by He et al. (1997) where a diet containing tocotrienol suppressed the growth of melanoma in mice. In conclusion, this study demonstrates the potential of OPP as a natural product that possesses anti-proliferative effects on tumor cells (both *in vitro* and *in vivo*) while enhancing the growth of normal cells. Further studies should be carried out to fully understand the mechanisms behind the anti-cancer activity of this extract for its possible application in the prevention and treatment of

cancer.

ACKNOWLEDGEMENT

The authors thanked the Malaysian Palm Oil Board (MPOB) for providing the OPP samples and for partially funding the studies.

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