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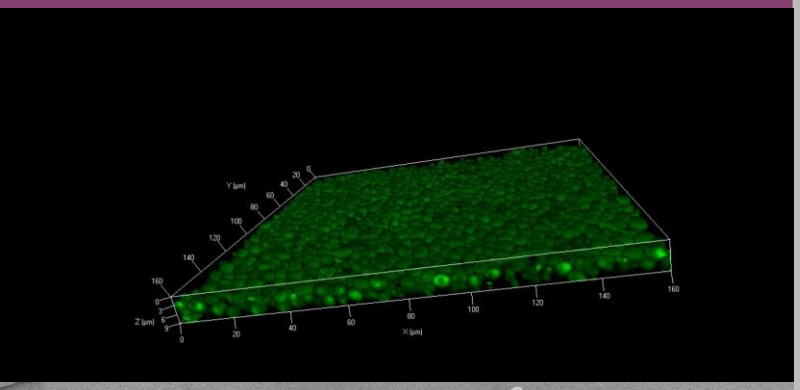

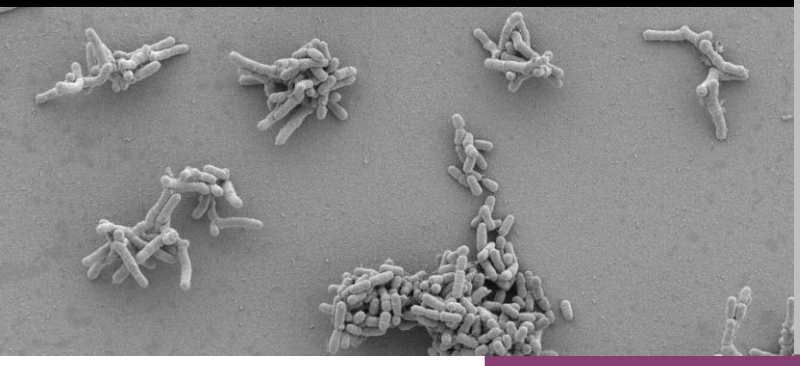

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Aims and Scope:

International Journal of Orofacial and Health Sciences (IJOHS) is a peer reviewed biannual international journal dedicated to publish high quality of scientific research in the field of orofacial sciences, health sciences and interdisciplinary fields, including basic, applied and clinical research. The journal welcomes review articles, original research, case reports and letters to the editor. Areas that are covered include but are not limited to dental sciences, oral microbiology and immunology, oral maxillofacial and craniofacial surgery and imaging, dental stem cells and regenerative medicine, dental biomaterial, oral maxillofacial genetic and craniofacial deformities.

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CONTENTS

Analysis of the anti-cancer effect of ethyl-p-methoxycinnamate extracted cekur (*Kaempferia galanga*) on cancer cell lines with wild-type and null p53

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Abstract

This study aimed to examine the in-vitro anti-cancer potential of ethyl-p-methoxycinnamate (EPMC), the major constituent of *Kaempferia galanga (K. galanga)* in selected human lung adenocarcinoma cells line A549 (p53 wild-type) and H1299 (p53 null). The involvement of p53 pathway in the anti-cancer effect of EPMC on selected cells was determined using MTT assay and Real-time PCR. The MTT results show that EPMC induces cytotoxicity in a dose-dependent manner in A549 cancer cell lines containing the p53 wild-type gene. Meanwhile, our RT-PCR results indicate that the apoptotic activity of EPMC does not involve the p53 pathway. Overall, these results indicate that EPMC compounds of *K. galanga* stimulates in vitro cytotoxic and apoptotic activity unrelated to the p53 pathway.

Keywords: Ethyl-P-Methoxycinnamate, Kaempferia Galanga, p53

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Introduction

Cancer is a major public health problem worldwide. There are many factors that may contribute to the development of cancer. In many cases, cancer involves mutations in protein-encoding genes that regulate cell division. Eventually, more genes become mutated because the genes that normally repair DNA damage become themselves mutated and cease to function. The amplification of mutations in the cell causes further abnormalities in the cell and its daughter cells.

TP53 (tumor suppressor gene p53) plays a significant role in protecting cells from malignancies. It is well-known that p53 suppresses tumor formation and protects against DNA damage by inducing cell cycle arrest, DNA repair or apoptosis (Wang and Sun, 2010). p53 induces cell cycle arrest by trans-activating genes such as p21 (CDK-inhibitor 1, cyclin dependent kinase) (Chiang *et al*., 2013). Furthermore, p53 initiates apoptosis via trans-activating pro-apoptotic proteins such as PUMA (p53 upregulated modulator of apoptosis) (Bai and Wang, 2014), BAX (Bcl-2-associated X protein) or FAS (cell surface death receptor) (Wang and Sun, 2010).

However, the p53 is often mutated in cancer (Klein and Vassilev, 2004).

Evidence suggests that, mutations or deletions in the TP53 gene are present in nearly 50% of human cancers and primarily results in impaired tumor suppression function (Wang *et al*., 2012). Loss of p53 functionality leads to the proliferation of damaged cells that may subsequently transfer the mutations to the next generation. It is believed that it is through this mechanism that deregulation of p53 often leads to the formation of tumors (Khoury and Domling, 2012).

Due to the high occurrence of p53 mutations in human tumors, this tumor suppressor is a key target for novel anticancer therapies. Several research teams have dealt with the possibility of restoring p53 function to treat cancer. Many novel molecules have been identified so far to restore p53 wild-type conformation and thereby recover its tumor suppressive function (Hientz *et al*., 2017).

Thus far, a number of studies has reported that EPMC, a major constituent of volatile oil of *K. galanga* possesses in vitro anti-cancer activities on various cancer cell lines such as human colon cancer SW620, cervical cancer C33A, breast cancer MCF-7 cell lines and oral cancer HSC-3 and Ca922 cell lines (Amuamuta *et al., 2017;* Omar *et al., 2017;* Omar *et al., 2016;* Ichwan *et al., 2019).*

Despite these promising results, the question of the role of *K. galanga* in the p53 pathway remains unknown. Therefore, the objective of this study was to determine the in vitro anti-cancer potential of EPMC extracted from *K.*galanga against human cancer cell lines A549 (lung cancer, p53 wild-type) and H1299 (lung cancer, p53 null). The study also aims to explore the involvement of p53 pathway in anticancer mechanism of EPMC extract on human cancer cell lines.

Materials and Methods

Cell subculturing and maintenance

The human lung adenocarcinoma cells A549 (wild-type p53) and H1299 (null p53) were kindly provided by Prof. Dr. Masa Aki Ikeda, Tokyo Medical and Dental University, Japan. The cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin mixture in a humidified incubator, 5% CO_2 at 37°C.

MTT assay

Cell viability was measured by the ability of the cells to convert soluble MTT into an insoluble formazan crystal. The assay was performed according to the protocol previously described (Ichwan *et al*., 2014) with slight modifications. Exponentially growing cells were subcultured in 96-well plates at an initial density of $2x10^4$/ well. The cells were exposed to pre-defined concentrations of EPMC and doxorubicin for 24 hours.

After 24 hours of incubation, the medium was removed and the cells were washed with PBS. Cell viability was determined by adding 20 µL MTT 3-(4, 5-dimetyhlthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide at a concentration of 5mg/ml (in PBS). Cells were incubated at 37°C in humidified atmosphere for 4 hours. The formazan crystals were dissolved in 100 µL of dimethylsulfoxide (DMSO) for 1 hour. The absorbance was measured in a spectrophotometer at a wavelength of 570nm (reference: 630 nm).

Total RNA extraction

The total RNA Mini Kit was acquired from Geneaid (Taiwan). RNA extraction was performed following the manufacturer's protocol.

Complementary DNA (cDNA) preparation

cDNA synthesis was conducted using ReverTra Ace ® qPCR RT Master Mix Kit (Toyobo, Japan) based on manufacturer's instructions. A total of 8 µl of solution consisting of 4X DN Master Mix, RNA template, and nuclease-free water was prepared. After incubation for 5 min, 5× Master Mix was added to the solution to a total of 10 µl of reverse transcription solution. Then, the solution was incubated at 37 °C for 15min and heated at 98 °C for 5 min to obtain cDNA.

Quantitative PCR (qPCR)

The mRNA expressions of p21 and PUMA were determined by qRT-PCR. The RT-qPCR was performed using the Quantitect SYBR® Green PCR (Qiagen) kit according to manufacturers's instructions. The qRT-PCR reaction mixture consists of 2x QuantiTect SYBR Green PCR master mix, pre-developed gene expression assays, primers mix and H_2O to a final volume of 14 µL were prepared. The relative expression level of the mRNA sample was normalized by the amount of β-actin, a housekeeping gene that was used as endogenous control.

Statistical analysis

Non-parametric Kruskal-wallis was used to test the significant difference of PUMA upregulation between doxorubicin and EPMC.

Results and Discussion

In brief, we used MTT assay to test whether EPMC induced cytotoxicity in human lung adenocarcinoma cell lines A549 (p53 wild-type) and H1299 (p53 null). The cells were exposed to graded concentrations of EPMC (0-20 µg/mL). EPMC was found to decrease cells viability in a dose-dependent manner in the presence of p53 (Figure 1). However, in the absence of p53, EPMC did not induce cytotoxicity and cell death (Figure 2).

The findings of this study would seem to suggest that presence of the p53 gene is a key factor for sensitivity to anticancer agents (El Deiry *et al*., 2003). The H1299 cell line has a recognized p53 gene deletion in both alleles. Thus, p53-null cells succumb to tumorigenesis. Unlike p53-null, p53 wild-type have the ability to suppress malignant growth of transformed cells as well as tumors. Therefore, compounds that activate wild-type p53 would have an application for the treatment of wt-p53 containing human cancer. Nevertheless, mutated p53 confers to the resistance of tumor cells to anticancer drugs by inhibiting p53-dependent pathway (Volgestein *et al*., 2000).

The cytotoxicity of EPMC on human lung cancer cell line was compared against doxorubicin, a well-known chemotherapeutic agent as a positive control. EPMC and doxorubicin both induced the cytotoxic activity of p53 wild-type.

We next sought to determine whether EPMC was involved in the p53 pathway in A549 and H1299 cell lines through the expression of p53 mediated target genes (Beckerman *et al*., 2010). The p53 mediated target genes assessed in the study were p21 and PUMA.

As shown in Figure 3, p21 expression levels were not induced in both cancer cell lines after incubation with EPMC. However, PUMA expression levels in both cancer cell lines increased after treatment with EPMC (Figure 4). Non-parametric Kruskal-Wallis statistical analysis was used to test for significant differences in PUMA upregulation upon treatment with doxorubicin and EPMC. No significant difference in PUMA upregulation between doxorubicin and EPMC was found. This indicates that EPMC is a potent treatment candidate for cancer. Furthermore, from the graph obtained it showed that the increment was more pronounced in A549 (p53 wild-type).

In a previous study, PUMA was reported to play an important role in benzyl isothiocyanate (BITC)-induced apoptosis. In the study, treatment with BITC clearly increased the level of PUMA in cells with wild-type p53 (MCF-7) (Anthony *et al*., 2012).

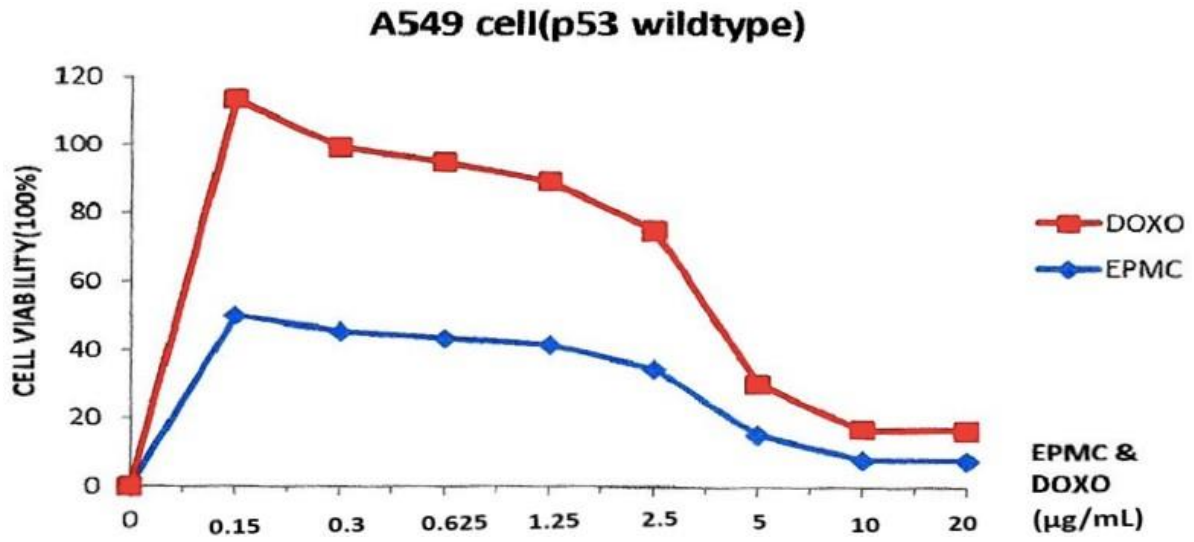

Figure 1. Ethyl-p-methoxycinnamate (EPMC) treatment schedules, EPMC dependent cytotoxicity and induced cell death in the presence of p53.

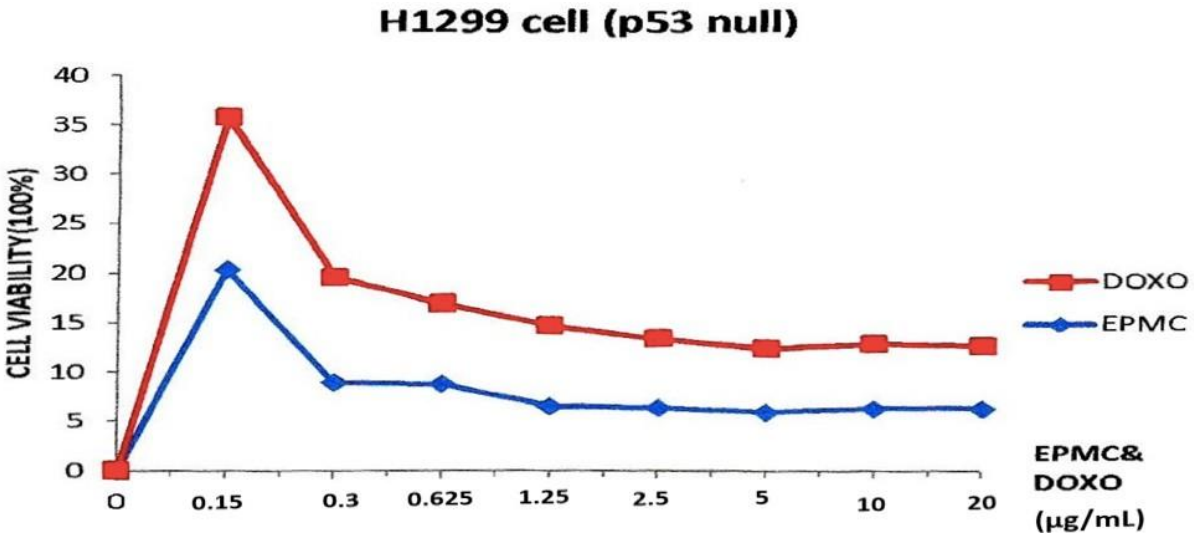

Figure 2. Ethyl-p-methoxycinnamate (EPMC) does not induce cytotoxicity and cell death in dose dependent manner in the absence of p53.

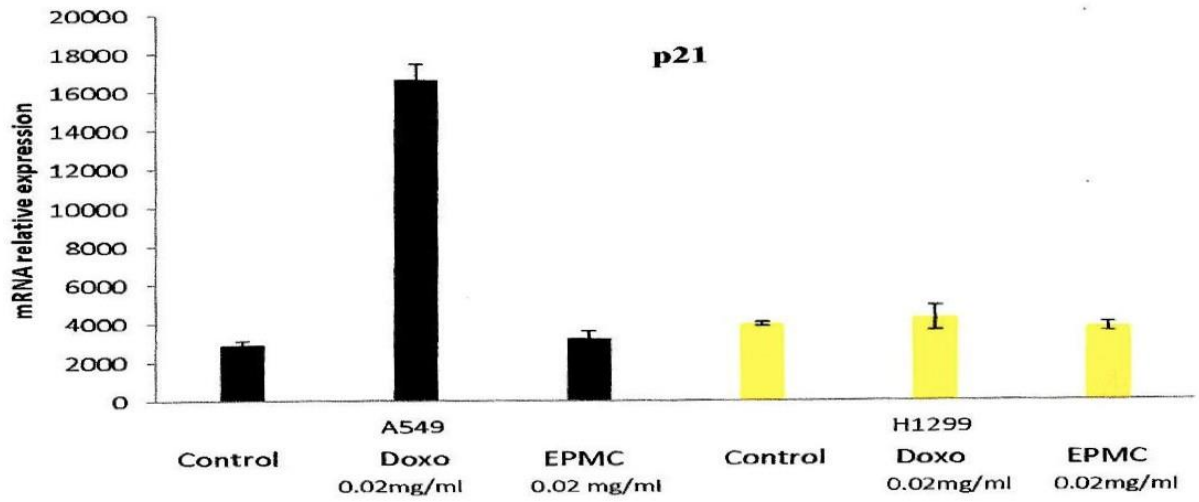

Figure 3. The graph shows that mRNA expression was not induced by EPMC incubation in both; A549 (p53 wild-type) and H1299 (p53 null) cancer cell line.

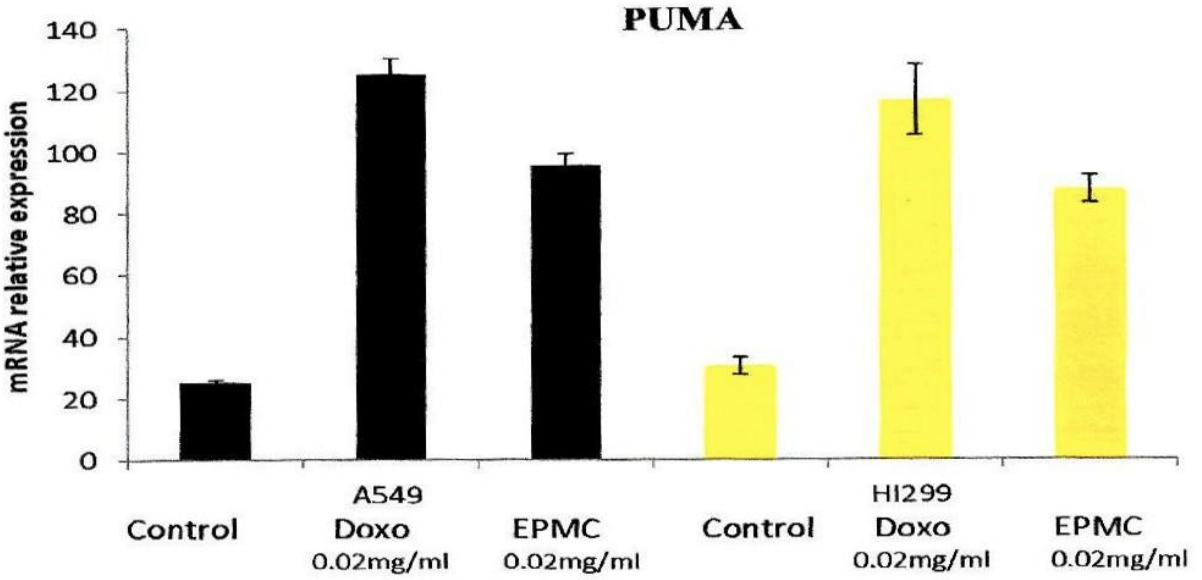

Figure 4. RT-PCR analysis revealed that mRNA expression levels were upregulated in both; A549 (p53 wild-type) and H1299 (p53 null) cancer cell line.

Nevertheless, the upregulation of PUMA mRNA expression does not indicate the involvement of the p53 pathway. This is because PUMA can also be activated independently and thus plays a role in p53-independent apoptosis as in the case of the p53 homolog p73, which is

able to engage the PUMA promoter at the p53 response elements (Li *et al*., 2006).

Thus, in this study EPMC is shown to stimulate cytotoxic and apoptotic effects on human lung cancer cell lines. However, the apoptotic effect of EPMC does not involve the p53 pathway.

Acknowledgement

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