



Anticancer activity of eugenol is not related to regulation of the oncogenic transcription factor *Forkhead Box M1*

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ABSTRACT. Genome-wide gene expression profiling of cancers has consistently identified the FOXM1 as one of the most commonly upregulated genes in cancer cells that plays an essential role in the regulation of a wide spectrum of biological processes, including inhibition of apoptosis. Since the anticancer activity of EUG reported in the literature is related to induction of apoptosis in cancer cells, we hypothesized that there is a correlation between the EUG-induced apoptosis effect and downregulation of FOXM1. A series of experiments were conducted to evaluate the effect of EUG on cellular viability of cancer cells (MTT) and its potential regulatory effect on FOXM1 protein levels (western blots). Our findings confirm the anticancer effect of EUG on different human cancer cell lines as previously reported in the literature (SKBR3 LC₅₀: 318.6; HT29 LC₅₀: 525.5; and HepG2 LC₅₀: 2090.0 μ M). However, we demonstrated that EUG does not regulate the FOXM1. The results evidenced the anticancer effect of EUG on three cancer cell lines and showed that the EUG- apoptosis induced effect is not related to regulation of FOXM1 at the protein level. Further studies must be done to provide information on the mechanism of action of this agent.

Keywords: cytotoxicity, apoptosis, essential oil, clove.

Atividade anticarcinogênica do eugenol não se deve à modulação do fator de transcrição oncogênico *Forkhead Box M1*

RESUMO. Estudos do genoma de células tumorais identificaram o FOXM1 como o fator de transcrição mais expresso, desempenhando papel essencial em uma gama de processos biológicos, incluindo a inibição da apoptose celular. A atividade anticarcinogênica do EUG, relatada na literatura, está relacionada à indução de apoptose em células cancerosas, por isso geramos a hipótese de que pode existir correlação entre este efeito indutor de apoptose e a supressão do FOXM1. Um conjunto de experimentos foi realizado com o objetivo de avaliar o efeito do EUG na viabilidade celular (MTT) e o potencial regulatório sobre o nível de proteínas do FOXM1, em células cancerosas (western blots). Nossos resultados corroboram o efeito anticarcinogênico do EUG relatado na literatura em diferentes linhagens celulares (SKBR3 LC₅₀: 318.6; HT29 LC₅₀: 525.5; e HepG2 LC₅₀: 2090.0 μ M). Entretanto ficou demonstrado que o EUG não interfere no nível proteico do FOXM1. Em nosso estudo demonstramos o efeito citotóxico do EUG em três linhagens celulares de câncer, sendo evidenciado que o efeito indutor de apoptose promovido pelo mesmo não é dependente da regulação do fator de transcrição FOXM1. Estudos mais detalhados serão conduzidos no intuito de esclarecer os mecanismos de ação desse agente anticarcinogênico.

Palavras-chave: citotoxicidade, apoptose, óleo essencial, cravo-da-índia.

Introduction

Genome-wide gene expression profiling of cancers has consistently identified the Forkhead Box M1 (FOXM1) as one of the most commonly upregulated genes in the early stages of carcinogenesis (Gartel, 2010). The FOXM1 plays an essential role in the regulation of a wide spectrum of biological processes, including cell proliferation, cell cycle progression, cell differentiation, angiogenesis

and apoptosis (Halasi & Gartel, 2013). This transcription factor has been regarded as the “Achilles’ heel of cancer”, and represents one of the most promising therapeutic targets for the development of novel anticancer agents (Radhakrishnan & Gartel, 2008). Consequently, several research groups have suggested the need to establish comprehensive and multidisciplinary programs dedicated to study the role and regulation of FOXM1 by chemical compounds.

Medicinal plants are a rich source for obtaining molecules to be explored therapeutically. Many compounds isolated from plants remain as sources of medicines, and over 60% of the current anticancer drugs were originated from natural sources (Cragg, Grothaus, & Newman, 2009). Moreover, there are many studies published recently regarding the antitumor, antimetastatic, and the cancer preventive activities of natural compounds (Kingston, 2011). The wide diversity of biologically active plant-derived compounds and their wide structural diversity make it promising to continue screening plants for compounds that could be useful in prevention and treatment of cancer (Kreuger, Grootjans, Biavatti, Vandenabeele, & D'Herde, 2012).

In this regard, the eugenol (4-allyl-2-methoxyphenol; EUG), a biologically active phenolic component of *Syzygium aromaticum* (the clove) has been traditionally used in Asian countries as a popular medicine, mainly as antiseptic, analgesic and antibacterial agent (Carrasco et al., 2009). This compound has a wide variety of applications in the food industry and has been used as a precursor in the synthesis of certain compounds by the pharmaceutical and cosmetic industry (Polzin, Stanfill, Brown, Ashley, & Watson, 2007). Many studies in the literature reported that EUG possesses various biological properties, such as antioxidant, antiseptic, analgesic, antibacterial, antimutagenic, and anti-inflammatory effects. Recently, the anticancer activity of EUG against various cancer cell lines was demonstrated. In these papers, the antiproliferative activity of EUG was attributed to its ability to induce apoptosis in cancer cells by modulating the Bcl-2 family proteins (Jaganathan & Supriyanto, 2012).

Since the overexpression of FOXM1 and its subsequent elevated transcriptional activity have been correlated with the direct upregulation of a wide variety of proteins associated with inhibition of apoptosis, such as Surviving and Bcl-2 (Xu et al., 2012), it might indicate correlation between the EUG-induced apoptosis effect and downregulation of FOXM1. However, to the best of our knowledge the effect of EUG on FOXM1 remains to be determined and constitutes the focus of this study.

In this context, we conducted a series of *in vitro* experiments aimed at evaluating the potential cytotoxic activity exerted by EUG on three different human cancer cell lines and its potential regulatory effect on FOXM1 protein levels.

Material and methods

Natural compound

The compound EUG (catalog number E51791; 99% purity) was purchased from Sigma (St Louis, MO, USA).

Cancer cell lines and culture conditions

Human breast cancer SKBR3 cells (ATCC HTB-30, Manassas, VA, USA), human colorectal adenocarcinoma HT29 cells (ATCC HTB-38, Manassas, VA, USA), and human hepatocellular carcinoma HepG2 cells (ATCC HB-8065, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin. Cells were grown in 75 cm² tissue culture flasks at 37°C in a 5% CO₂ humidified incubator.

Cell viability assay procedure

The effect of the test compound on the viability of SKBR3, HT29, and HepG2 cell lines, was estimated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Jaganathan, Mazumdar, Mondhe, & Mandal, 2011). Briefly, cells were trypsinized, neutralized and diluted with culture medium, and seeded into 96-well culture plates at a density of 3 x 10⁴ per well (200 µL), and were allowed to attach overnight at 37°C in a 5% CO₂ humidified incubator. Then, the compound EUG dissolved in DMSO was added to the corresponding 96-well plate, in serum free media, and incubated for 24 hours at 37°C in a 5% CO₂ humidified incubator. The final concentrations obtained in 1% of DMSO (0.01, 0.1, 1, 10, and 100 µM) are not toxic to the cells, as previously reported (Chattopadhyay et al., 2012; Nakhjavani, Zarghi, & H'Shirazi, 2014; Sudan & Rupasinghe, 2014). After that time, the media was aspirated out and the wells washed with 200 µL of phosphate buffered saline (PBS) before the addition of the MTT solution (100 µL well⁻¹) in PBS (0.5 mg mL⁻¹). The plate was then incubated for 2 hours at 37°C in a 5% CO₂ humidified incubator. Finally, the MTT solution was aspirated out and the insoluble formazan crystals were dissolved in a solution of 0.01 M HCl in isopropanol (100 µL well⁻¹) and the absorbance of each well was read at 570 nm in a plate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, Biotek Instruments, USA). Cell viability in the control wells was taken as 100% of viability and used to calculate the percent of viability and the concentration of test compound that

decreases 50% of cell viability (LC_{50}). The EUG was assayed at six concentrations in quadruplicate and the experiment was repeated three times.

Western blot procedure

To determine the effect of the test compounds on FOXM1 expression, the western blots were carried out, as proposed by (Wei et al., 2015) with minor modifications. Only the human breast cancer SKBR3 cells were used since these cells present high FOXM1 expression and high sensitivity to modulation of this transcription factor (Wonsey & Follettie, 2005). Briefly, SKBR3 cells were seeded into 6-well plates at a density of 3×10^4 per well, and were allowed to attach overnight at 37°C in a 5% CO_2 humidified incubator. Then, EUG dissolved in DMSO was added into the corresponding 6-well plate, in serum free media, and then incubated for 24 hours at 37°C in a 5% CO_2 humidified incubator. The final concentrations obtained were 18, 37, 75, 150, and 300 μM in 1% of DMSO. Afterwards, the media was aspirated out and the wells fasted for one hour with PBS before the addition of 100 μL per well of laemmli 2X buffer (Sigma, USA) to lyse the cells. The homogenate of proteins was then collected into sterile Eppendorf tubes, heated at 95°C for 5 min, sonicated, and loaded onto 8% acrylamide gel for 45 min at 150 volts followed by protein transfer to a nitrocellulose membrane using a semi-dry transfer cell (Trans-blot SD, Bio-Rad Laboratories, USA) for 30 min at 25 volts. The membranes were covered with 10 mL of blocking buffer (5 milk powder; 0.5 Tween 20; and 0.58% NaCl; in double distilled water). After 2 hours, the blocking buffer was removed and the membranes were probed with specific primary antibody (mouse monoclonal IgG_{2a}, Santa Cruz Biotechnology, USA) in blocking buffer (1:250) and incubated overnight at 4°C. Then, the blots were washed and incubated with the secondary antibody (goat anti-mouse IgG-HRP, Santa Cruz Biotechnology, USA) in blocking buffer (1:2000) for one hour at room temperature and developed using chemiluminescence based on horseradish peroxidase-conjugated secondary antibodies color development system (Amersham ECL Prime, GE Healthcare Bio-Sciences, Sweden). The β -actin was used as the internal control to ensure equal sample loading. Densitometric analyses were carried out using image digitizing software (Versadoc Imaging System, Bio-Rad Laboratories, USA). The percentage of FOXM1 was correlated with that of DMSO-treated wells, which were set to a value of 100% FOXM1.

Statistical analysis

Data were expressed as mean values $\pm$ standard error of the mean (SEM). Statistical significance was

tested using one-way analysis of variance (ANOVA), followed by the Tukey's test for comparison of means. The difference was considered significant when the p values were smaller than 0.05.

Results and discussion

We first investigated the cytotoxic effect of EUG on different human cancer cell lines (SKBR3, HT29, and HepG2) using the MTT assay. The SKBR3 and HT29 cells presented clear sensitivity to EUG (Figures 1A and B). However, the SKBR3 cells showed the highest sensitivity to EUG and the LC_{50} for HT29 was clearly lower than the LC_{50} obtained for SKBR3 cells. Lastly, the HepG2 cells exhibited high resistance to EUG, as shown in the Figure 1C. This indicates that EUG has differential cytotoxicity against different human cancer cell lines.

Values of LC_{50} (the concentration that leads to 50% survival) are presented in Table 1.

Table 1. Concentration of eugenol that leads to 50% survival (LC_{50}) in different human cancer cell lines calculated by MTT method.

Cell line	$LC_{50}(\mu M)$
Breast cancer cells (SKBR3)	318.6 ± 0.119
Colorectal adenocarcinoma cells (HT29)	525.5 ± 0.103
Hepatocellular carcinoma cells (HepG2)	$2,090.0 \pm 0.578$

Values represent mean values $\pm$ SEM for each cell line. Values were calculated by linear regression with $R^2 > 0.99$.

The effect of EUG on FOXM1 protein levels was determined by western blots after the MTT results, whereas the highest concentration used here was the LC_{50} obtained by MTTs (Table 1). As shown in Figure 2, the concentrations of EUG tested (300.00, 150.00, 75.00, 37.50, and 18.75 μM) were not able to regulate the expression of FOXM1, since there was no significant difference in cell viability between control cells and treated cells.

Some studies have suggested that the EUG-induced apoptosis plays the most important role in the chemopreventive action of EUG on human cancer cells. Researchers suggested that the EUG-induced apoptosis effect on cancer cell lines is related to the modulation of the Bcl-2 family proteins (Manikandan, Vinothini, Priyadarsini, Prathiba, & Nagini, 2011) and levels of p53 (Jaganathan & Supriyanto, 2012). In this regard, the literature support that increased expression of FOXM1 can downregulate the Bcl-2 family proteins and suppress the accumulation of senescence markers, such as p53 (Koo, Muir, & Lam, 2012). Therefore, we hypothesized that there is correlation between the EUG-induced apoptosis effect and downregulation of FOXM1 transcription factor.

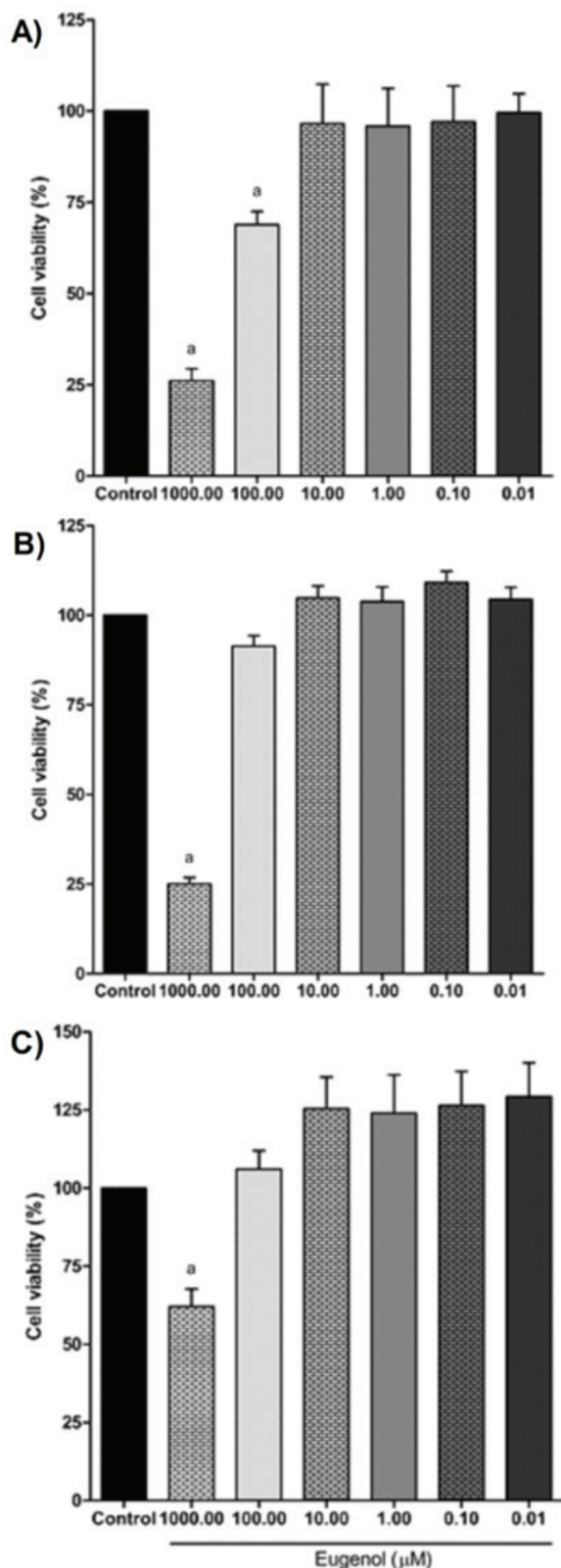


Figure 1. Effect of eugenol on cell viability of SKBR3 (A), HT29 (B), and HepG2 cells (C), determined by MTT method. Values represent mean values $\pm$ SEM for each treatment. ^aSignificant difference at $p < 0.05$ compared with control cells.

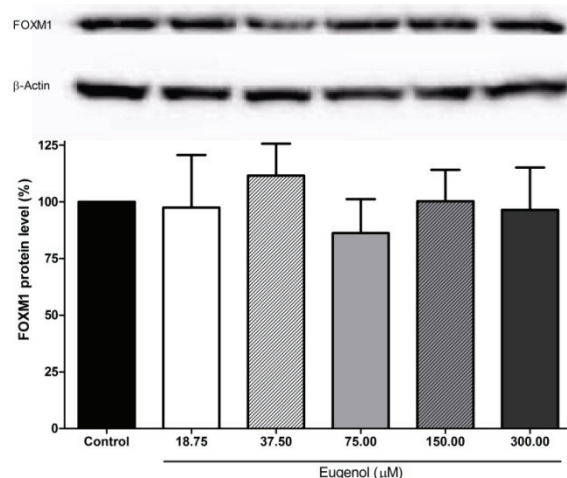


Figure 2. Effect of eugenol on FOXM1 protein levels in SKBR3 cells determined by western blots. Values represent mean values $\pm$ SEM for each treatment. ^aSignificant difference at $p < 0.05$ compared with control cells.

We demonstrated by means of western blots that EUG does not interfere with FOXM1 protein levels and then its cytotoxic effect is not related to the regulation of FOXM1. It does not mean that EUG may not interfere with the FOXM1 pathway, but indicates that EUG does not downregulate the FOXM1 production. Manikandan, Vinothini, Priyadarsini, Prathiba, and Nagini (2011) investigated the EUG-induced apoptosis in gastric carcinogenesis and related the mechanism via the mitochondrial pathway by modulating the Bcl-2 family proteins (Manikandan et al., 2011). Moreover, Jaganathan and Supriyanto (2012) demonstrated that osteosarcoma cell proliferation is inhibited by EUG and that increased levels of p53, PARP, and caspase 3 cleavage accompany the EUG-induced apoptosis (Jaganathan & Supriyanto, 2012). Lastly, Pisano et al. studied the EUG-induced apoptosis effect on melanoma and neuroblastoma cells and related the possible association of caspases 3 and 6 on this mechanism (Pisano et al., 2007). We suppose that these mechanisms might be involved in the EUG-induced apoptosis in the SKBR3, HT29, and HepG2 cell lines, as well and it could explain the results obtained in our experiments. Future studies will be carried out to confirm these suppositions.

Our results corroborate the cytotoxic effect of EUG on different human cancer cell lines, as previously reported in the literature (Vidhya & Devaraj, 2011). Cancer cells possess different expression profiles for FOXM1 and other transcription factors and proteins (Wonsey & Follettie, 2005). It may explain the variation on the LC₅₀ obtained for SKBR3, HT29, and HepG2 cell lines. We may still infer that only SKBR3 and HT29

cells could be considered to be the therapeutic targets for *in vivo* experiments with EUG, since the LC₅₀ for HepG2 cells is a very high concentration to be reached in the plasma (2,090.0 µM), either in animals or humans.

Conclusion

EUG remains as a promising tool for both *in vivo* and *in vitro* studies. Nevertheless, before being considered as a candidate for clinical trials, further studies must be done to provide information on the mechanism of action of this agent.

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