Cytotoxic and genotoxic effects of the *ent*-kaurenoic acid and *ent*-kaurenoic acid enriched *Mikania glomerata* extract in V79

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Abstract. Ferreira NH, Ribeiro AB, Moraes MD, Peixoto AM, Bernardino MA, Moreira MR, Soares ACF, Heleno VCG, Veneziani RCS, Tavares DC. 2020. Cytotoxic and genotoxic effects of the *ent*-kaurenoic acid and *ent*-kaurenoic acid-enriched *Mikania glomerata* extract in V79. *Biofarmasi J Nat Prod Biochem* 18: 1-4. The *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel effectively inhibited the formation of *Streptococcus mutans* biofilm. Given the biological potential of this extract and its major component, the present study was carried out to evaluate the safety of the *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel and *ent*-kaurenoic acid alone in an in vitro test system. The results showed that the *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel was cytotoxic at concentrations up to 40.0 µg/mL. Genotoxic effects were observed in cell cultures treated with the highest concentrations tested of *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel (10.0 and 15.0 µg/mL) and *ent*-kaurenoic acid alone (2.5, 5.0 and 7.5 µg/mL) when compared to the control group. Therefore, the *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel demonstrated cytotoxicity and genotoxicity effects at the highest concentrations tested, while *ent*-kaurenoic acid showed to be genotoxic at the same concentrations present in the *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel in V79 cells. These results demonstrate that the *ent*-kaurenoic acid should be partly responsible for the genotoxicity of *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel.

Keywords: Clonogenic assay, cytotoxicity, *ent*-kaurenoic acid, genotoxicity, *Mikania glomerata*

INTRODUCTION

In a recent study, our research group described the *ent*-kaurenoic acid-rich extract from *M. glomerata* Sprengel (KAMg) as a new proposal for antimicrobial products for oral care. The results presented by the authors showed that KAMg was effective in inhibiting the formation of *Streptococcus mutans* biofilm (Moreira et al., 2016). Given the biological potential of this extract and its major component (*ent*-kaurenoic acid - KA – Figure 1), the present study was carried out to evaluate the cytotoxic potential of KAMg through the clonogenic efficiency assay and the genotoxic potential of KAMg and KA in the V79 (Chinese hamster lung fibroblasts) cell line through the cytokinesis-block micronucleus assay.

Figure 1. Chemical structure of *ent*-kaurenoic acid

MATERIALS AND METHODS

Procedures

Obtention of the *ent*-kaurenoic acid-rich *M. glomerata* extract and *ent*-kaurenoic acid

KAMg was prepared and characterized as described by Moreira et al. (2016). The KA content in the extract was determined after developing and validating a reverse-phase HPLC analytical method, which corresponded to about 48% of KA content in KAMg (Moreira et al., 2016). The KA samples used in this work were obtained from the leaves of *M. glomerata*, just as described by Soares et al. (2019).

Cell line and culture conditions

The Chinese hamster lung fibroblasts (V79 cell line) were employed for experimental assays. The cell line was maintained as monolayers in plastic culture flasks (25 cm²) in HAM-F10 plus DMEM (1:1; Sigma-Aldrich) culture medium supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma-Aldrich), and 2.38 mg/mL HEPES (Sigma-Aldrich), at 37°C in a humidified 5% CO₂ atmosphere. The V79 cells were used from the 4th until the 12th passage. This cell line has an average cycle of 12 h in the experimental conditions described.

Cytotoxicity assessment

The clonogenic efficiency assay was used to determine the cytotoxicity of KAMg. For this, cells were treated for 3 h with KAMg concentrations ranging from 0.3 to 160 µg/mL. Negative (no treatment), solvent (Methanol, MeOH, 0.4%), and positive (methyl methanesulfonate, MMS, Sigma-Aldrich, 110 µg/mL) controls were also included. Following, the cultures were trypsinized, and 300 cells were seeded per culture flask (three flasks per concentration). The experiments were carried out for 10 days. The culture medium was then removed, and the colonies were washed with PBS (phosphate-buffered saline), fixed (1:1:9 of methanol/acetic acid/distilled water), and stained with Giemsa (1:20 in phosphate buffer, pH 7.0) for 20 minutes. Individually stained colonies were counted, and the colony-surviving fraction was calculated as follows (adapted from Franken et al. 2006):

$$SF (\%) = (A/B) \times 100$$

Where; A corresponds to the number of colonies found in the corresponding treatments and B to the number of colonies found in the negative control.

Genotoxicity assessment

The cytokinesis-block micronucleus assay was used to determine the genotoxicity of KAMg and KA. The concentrations of KAMg were chosen based on the results of the clonogenic efficiency assay, using cytotoxicity as selection criteria, according to OECD 487 (2016). The concentrations of KA were chosen according to the content of KA in KAMg, which corresponded to 48%. To assess the genotoxicity, 1×10^6 cells were seeded in culture flasks, incubated for two cycles (24 h) in 5 mL of complete HAM-F10/DMEM medium, washed with PBS, pH 7.4, and then submitted to the treatment in serum-free medium for 3 h with KAMg (2.5, 5.0, 10.0 and 15.0 µg/mL) or KA (1.25, 2.5, 5.0 and 7.5 µg/mL). Negative control (no treatment), solvents (MeOH, 0.4% for KAMg and dimethylsulfoxide, DMSO, Sigma-Aldrich, 1% for KA), and positive (MMS, 44 µg/mL) controls were also included. At the end of this period, cells were washed twice with PBS. A fresh serum-supplemented medium containing 3 µg/mL cytochalasin-B (Sigma-Aldrich) was added, and the cells were incubated for an additional 17 h. At harvest, the cells were trypsinized (0.025%), and a hypotonic solution of 1% sodium citrate at 37°C was added. The cells were then fixed in methanol: acetic acid (3:1), and the slides were stained with 3% Giemsa for 5 minutes.

The criteria established by Fenech (2000) were used to analyze micronuclei and binucleated cells. A total of 3000 binucleated cells were scored per treatment, corresponding to 1000 cells/treatment/repetition. For the evaluation of cytotoxicity of the treatments was analyzed 1500 cells analyzed per treatment, for a total of 500 cells per repetition, and the nuclear division index (NDI) was calculated. Cells with well-preserved cytoplasm containing 1-4 nuclei were scored. The NDI was calculated using the following formula (Kirsch-Volders et al. 2004):

$$NDI = (M1 + 2 \times M2 + 3 \times M3 + 4 \times M4) / N$$

Where; *M1-M4* is the number of cells with 1, 2, 3, and 4 nuclei, respectively, and *N* is the total number of viable cells.

Additionally, the cytotoxicity index (CI) of KAMg and KA was calculated as described by Kirsch-Volders et al. (2004):

$$CI = 100 - 100 \times [(NDI_t - 1) / (NDI_c - 1)]$$

Where; *NDI_t* is the NDI value found for the different treatments, and *NDI_c* is the NDI value of the negative control.

Data analysis

Data were statistically analyzed by analysis of variance (ANOVA) for completely randomized experiments; the statistical F and its respective "*p-value*" were calculated. The Tukey method compared the treatment means in cases where *p* < 0.05, and the minimum significant difference was calculated for $\alpha = 0.05$. All statistical analyses were performed using the GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Cytotoxicity assessment

Dose-dependent changes in the viability of KAMg-treated cells were evaluated using the clonogenic efficiency assay. Each survival curve represents the mean of three independent experiments, and the bars indicate the standard error of the mean (Figure 2). Cultures treated with KAMg concentrations above 20.0 µg/mL showed significant reductions in cell viability compared to the control cultures, indicating a cytotoxic effect. Therefore, KAMg concentrations of 2.5, 5.0, 10.0, and 15.0 µg/mL were chosen for further analysis in the genotoxic assay.

Figure 2. Cell survival means percentages obtained after exposure of V79 cells to *ent*-kaurenoic acid-rich extract from *M. glomerata* Sprengel (KAMg) concentrations ranging from 0.3 to 160.0 µg/mL. MeOH: methanol (0.4%); MMS: methyl methanesulfonate (110 µg/mL). *Significantly different from the control group (*P* < 0.05).

Table 1. Mean frequencies of micronuclei (MN), nuclear division index (NDI), and cytotoxicity index observed in V79 cell cultures treated with different concentrations of *ent*-kaurenoic acid-rich extract from *M. glomerata* Sprengel, and their respective controls

Treatment ($\mu\text{g/mL}$)	MN frequency	NDI ^b	Cytotoxicity index (%)
	Mean $\pm$ SD	Mean $\pm$ SD	
Control	5.33 $\pm$ 1.15	1.74 $\pm$ 0.04	-
MeOH	4.33 $\pm$ 1.15	1.69 $\pm$ 0.08	6.76
2.5	9.00 $\pm$ 2.00	1.66 $\pm$ 0.09	10.82
5.0	11.33 $\pm$ 5.86	1.63 $\pm$ 0.10	14.87
10.0	16.00 $\pm$ 2.65 ^c	1.60 $\pm$ 0.05	18.92
15.0	17.33 $\pm$ 2.52 ^c	1.35 $\pm$ 0.03 ^c	52.71
MMS	62.33 $\pm$ 1.53 ^c	1.60 $\pm$ 0.07	18.92

Note: MeOH: methanol (0.4%); MMS: methyl methanesulfonate (44 $\mu\text{g/mL}$). ^aA total of 3000 binucleated cells was scored per treatment, corresponding to 1000 cells/treatment/repetition for the determination of micronucleus frequency. ^bA total of 1500 cells was analyzed per treatment group, corresponding to 500 cells/treatment/repetition for calculation of NDI. ^cSignificantly different from the control group ($P < 0.05$).

Table 2. Mean frequencies of micronuclei (MN) and nuclear division index (NDI) were observed in V79 cell cultures treated with different concentrations of kaurenoic acid and their respective controls.

Treatments ($\mu\text{g/mL}$)	MN frequency	NDI ^b
	Mean $\pm$ SD	Mean $\pm$ SD
Control	6.67 $\pm$ 2.08	1.66 $\pm$ 0.02
DMSO	6.33 $\pm$ 1.15	1.70 $\pm$ 0.12
1.25	14.67 $\pm$ 2.08	1.64 $\pm$ 0.06
2.5	20.00 $\pm$ 5.29 ^c	1.65 $\pm$ 0.09
5.0	17.67 $\pm$ 2.31 ^c	1.76 $\pm$ 0.14
7.5	17.67 $\pm$ 4.04 ^c	1.72 $\pm$ 0.13
MMS	62.33 $\pm$ 1.53 ^c	1.68 $\pm$ 0.08

Note: DMSO: dimethylsulfoxide (1%); MMS: methyl methanesulfonate (44 $\mu\text{g/mL}$). ^aA total of 3000 binucleated cells was scored per treatment, corresponding to 1000 cells/treatment/repetition for micronucleus frequency determination. ^bA total of 1500 cells was analyzed per treatment group, corresponding to 500 cells/treatment/repetition for NDI calculation. ^cSignificantly different from the control group ($P < 0.05$).

Genotoxicity assessment

Table 1 shows the mean frequencies of binucleated cells with micronuclei and the mean standard deviation after exposure to KAMg. There was no significant difference in micronuclei induction observed between cultures treated with KAMg concentrations of 2.5 and 5.0 $\mu\text{g/mL}$ and the control group. However, a significant micronuclei frequency increase was observed in cultures treated with 10.0 and 15.0 $\mu\text{g/mL}$ of KAMg compared to the control, demonstrating genotoxicity for these concentrations. In relation to NDI, the cultures treated with the highest concentration of KAMg tested (15.0 $\mu\text{g/mL}$) showed significantly lower indexes when compared to the cultures of the control group, with a cytotoxicity index equal to 52.71%. In this way, KA (in a percentage corresponding to KAMg concentrations) treatment groups were included to assess whether the genotoxic effect of KAMg at the highest concentrations was due to the presence of KA.

Table 2 shows the mean frequencies of binucleated cells with micronuclei and NDI after exposure to KA. The lowest tested concentration of KA did not significantly increase the micronuclei frequency compared to the control group. On the other hand, the results showed a significant increase in micronuclei frequency at concentrations of 2.5, 5.0, and 7.5 $\mu\text{g/mL}$, compared to the control group, demonstrating genotoxic activity. The results of NDI (nuclear division index) showed no significant differences for any KA concentration compared to the control group, revealing the absence of cytotoxicity.

Discussion

The present study aimed to investigate the cytotoxic and genotoxic potential of KAMg in V79 cells. The results demonstrated cytotoxic and genotoxic effects of KAMg at the highest concentrations tested through the clonogenic efficiency assay (above 20 $\mu\text{g/mL}$) and the micronucleus

assay (10 and 15 $\mu\text{g/mL}$), respectively. In order to investigate the influence of KA on the genotoxicity of the extract, the genotoxic potential of this diterpene was evaluated in the concentrations present in KAMg. Except for the lowest KA tested concentration, all others revealed genotoxic effects. These results demonstrate that KA should be responsible, at least in part, for the genotoxicity of KAMg.

Some studies have already demonstrated the genotoxic potential of *M. glomerata* extract and KA. Dalla Nora et al. (2010) observed the genotoxic and antiproliferative effects of *M. glomerata* leaves extract on *Allium cepa* cells. The genotoxicity of the *M. glomerata* leaves extract was also reported by Costa et al. (2008) using murine hepatoma cells (HTC).

Cavalcanti et al. (2006) demonstrated the genotoxic effect of KA at concentrations of 30 and 60 $\mu\text{g/mL}$ in V79 cell lines using the comet and micronucleus assays. However, Cano et al. (2017) investigated the genotoxicity of KA in the CHO-K1 (Chinese hamster ovary) cell line using the micronucleus assay, and this diterpene showed no genotoxicity at concentrations ranging from 7.36 to 30.25 $\mu\text{g/mL}$. O'Donovan (1990) and Erexson et al. (2001) reported that CHO and V79 cell lines vary significantly in their ability to metabolize endotoxins. This difference in cellular metabolic capacity may explain the results difference obtained in relation to the genotoxicity of KA.

In relation to the genotoxicity of KAMg, it is important to note that extracts are complex mixtures of biologically active substances that may act synergistically or antagonistically. All these effects may lead to attenuation or accentuation of genotoxic and or cytotoxic effects caused by some specific substance. The enrichment of *M. glomerata* extract with KA may have contributed to the genotoxicity of the KAMg, since KA showed to be genotoxic at the same concentrations present in the enriched extract.

Cavalcanti et al. (2010) demonstrated that the genotoxic and mutagenic properties of KA are due to clastogenic damage, probably derived from DNA double-strand breaks and/or inhibition of topoisomerase I. In addition, the authors confirmed that the exocyclic double bond (C16) in the chemical structure of KA is an important element in its genotoxicity and mutagenicity.

Despite the genotoxicity of the extract observed in the in vitro test system, KAMg did not show genotoxic or cytotoxic activity in Swiss mice bone marrow by the micronucleus test (Moreira et al. 2016). Factors such as metabolism, pharmacokinetics, and pharmacodynamics alter compounds' distribution and concentration in more complex organisms than cellular units (Singulani et al., 2018).

The oral bioavailability of numerous drugs is greatly reduced before reaching systemic circulation (Kuppens et al. 2005). The first pass effect reduces the concentration of a drug to such an extent that only a small amount of active drug emerges from the liver to the systemic circulation and the target tissue, thus impacting its biological effect on in vivo systems (Sousa & Bernkop-Schnürch 2014).

Jiang et al. (2019) carried out the pharmacokinetic analysis of KA after oral administration of 10, 20, and 40 mg/kg in rats to 36 h after the treatment. The pharmacokinetic parameters indicated that KA was widely distributed in body tissues with high Vz/F values (apparent volume of distribution during terminal phase after non-intravenous administration) and experienced slow in vivo elimination after oral administration with terminal phase half-life ($t_{1/2z}$) > 10 h values for all doses.

The absence of genotoxicity of KAMg in in vivo test system may be related to the high liposolubility of KA. The high value in the partition coefficient of KA (5.4) may influence a higher concentration in adipose tissues and a slow concentration released into the bloodstream that exerts genotoxic effects on the bone marrow.

Under the experimental conditions used, KAMg demonstrated cytotoxicity as well as genotoxicity at the highest concentrations tested. At the same time, KA showed to be genotoxic at the same concentrations present in KAMg in V79 cells. These results demonstrate that KA should be responsible, at least in part, for genotoxicity KAMg.

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