

Research Article

Antitumor Activity of *Kielmeyera Coriacea* Leaf Constituents in Experimental Melanoma, Tested *in Vitro* and *in Vivo* in Syngeneic Mice

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Abstract

Purpose: The antitumor activity of *Kielmeyera coriacea* (Clusiaceae), a medicinal plant used in the treatment of parasitic, as well as fungal and bacterial infections by the Brazilian Cerrado population, was investigated.

Methods: A chloroform extract (CE) of *K. coriacea* was tested in the murine melanoma cell line (B16F10-Nex2) and a panel of human tumor cell lines. Tumor cell migration was determined by the wound-healing assay and the *in vivo* antitumor activity of CE was investigated in a melanoma cell metastatic model. ¹H NMR and GC/MS were used to determine CE chemical composition.

Results: We found that CE exhibited strong cytotoxic activity against murine melanoma cells and a panel of human tumor cell lines *in vitro*. CE also inhibited growth of B16F10-Nex2 cells at sub lethal concentrations, inducing cell cycle arrest at S phase, and inhibition of tumor cell migration. Most importantly, administration of CE significantly reduced the number of melanoma metastatic nodules *in vivo*. Chemical analysis of CE indicated the presence of the long chain fatty compounds, 1-eicosanol, 1-docosanol, and 2-nonadecanone as main constituents.

Conclusion: These results indicate that *K. coriacea* is a promising medicinal plant in cancer therapy exhibiting antitumor activity both *in vitro* and *in vivo* against different tumor cell lines.

Introduction

Plant-derived compounds have received considerable attention in recent years because of their pharmacological properties, including cytotoxicity and chemotherapeutic activities in cancer. Brazilian Cerrado, the second largest biome in Brazil,¹ is the source of many species containing bioactive compounds assayed in different experimental models.^{2,3} The Cerrado is one of the 25 important biodiversity hotspots in the World, with an exceptional concentration of rare endemic species.⁴ Recent studies have described the biological activities of several plant extracts or isolated compounds from this tropical savanna ecoregion.⁵⁻⁸

Kielmeyera coriacea Mart. & Zucc. (Clusiaceae) is a medicinal plant derived from the Brazilian Cerrado, used by the native population in the treatment of several tropical diseases, including schistosomiasis, leishmaniasis, malaria, fungal and bacterial infections.⁹ Recent studies have reported on the cytotoxic activity of *K. coriacea* Root

and Bark extracts in tumor cells, such as HCT-8 (human colon carcinoma), HL-60 (human leukemia), SF-295 (glioblastoma) and MDA-MB-435 (melanoma).⁸ Previous studies have shown the low toxicity of a dichloromethane extract of *K. coriacea* stems, suggesting a margin of safety for *in vivo* therapeutic doses.¹⁰ It has also been reported that the dichloromethane fraction from stems of *K. coriacea* can be an important therapeutic alternative in the treatment of anxiety disorders by induction of antidepressant response in rats.^{11,12}

We evaluated the *in vitro* and *in vivo* antitumor effects of different leaf extracts of *K. coriacea* on murine melanoma B16F10-Nex2. We also evaluated *K. coriacea* cytotoxicity in several human cancer cells. Our results indicate that the chloroform extract (CE) from leaves of *K. coriacea* inhibited murine melanoma B16F10-Nex2 cell growth with cell cycle arrest, cell migration *in vitro* and tumor-cell lung colonization *in vivo*. Analysis of CE using ³H-

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NMR (Nuclear magnetic resonance spectroscopy) as well as GC/MS (gas chromatography–mass spectrometry) indicated the presence of long-chain fatty alcohols (C₂₀, C₂₂, C₂₆), ketones (C₁₇, C₁₉) and an alkene (C₂₂) as the main compounds. The length of the alkyl chains may play an important role in eliciting the biological activity of these molecules.¹³ Their unsuspected antitumor activity adds to several other properties of these compounds as insect repellents (USPatent 4774082), antiviral agents, neurotrophic factors, waxes for thermal insulation.¹⁴⁻¹⁶

Materials and Methods

Plant materials and extraction

Leaves of *K. coriacea* were collected in the Cerrado area in the city of Patos de Minas-MG, Brazil (17°30.27'34"S and 45°31.21'17"W) in August 2008, and 2009. The plant was identified by MSc. Alice F. Amaral and a voucher specimen was deposited at the Mandevilla Herbarium, Centro Universitario de Patos de Minas (UNIPAM), No. MGHM0632-7. Hydroalcoholic (HA) and ethyl acetate (EA) extracts were obtained from 50 g of powdered leaves macerated in 250 mL of ethanol (EtOH): H₂O 7:3 (v/v) for 3 h at 45°C. Chloroform (CE), hexane (HE) and heptane (HP) were used to obtain extracts from 20 g of powdered leaves. Triplicate extractions were carried out with 200-mL of each solvent with stirring for 2 h at room temperature. Stock solutions were prepared with the dry residues diluted in dimethylsulfoxide at 10 mg/ml. The yield of extraction of leaf plant compounds with chloroform was higher than with dichloromethane, therefore chloroform was chosen in the subsequent extractions.

Chemical analysis

¹H-NMR spectra were recorded at 300 MHz in a Bruker DPX300 spectrometer, using CDCl₃ as solvent and TMS (tetramethylsilane) as internal standard. GC–MS analysis was performed at 70 eV in a INCOS 50 Finnigan-Mat-quadrupole spectrometer, using a capillary column (DB-5) coated with crosslinked methyl silicone gum (50 m, 0.20 mm i.d., film thickness 0.33 μm). The temperature program was 100°C isothermal for 1 min, then 100–280°C at 10°C/min, and isothermal at 280°C for 20 min. The temperatures of injection and detection were 250 and 280°C, respectively.

Cell lineages and mice

The following cell lineages were used: murine melanoma (B16F10-Nex2), a subclone of B16F10, deposited at BCRJ no. 0342; human colon carcinoma (HCT); human cervical cancer (Siha); human melanoma (A2058, SKmel28 and MeWO) all obtained from the Ludwig Institute for Cancer Research (LICR), São Paulo branch. Cells were cultivated as previously described.¹⁷

In vitro cytotoxicity and proliferation assays

K. coriacea extracts and specifically CE, diluted in RPMI medium with 1% DMSO (10 to 40 μg/ml) were incubated with B16F10-Nex2 cells or human tumor cells (10⁴ cells)

in 96-well plates in a final volume of 100 μL for 24 h for cell cytotoxicity assay. For cell proliferation assay, 5 × 10³ B16F10-Nex2 cells were incubated with 20 and 10 μg/ml of CE in a final volume of 100 μL for 24 h, 48 h and 72 h. Positive controls were carried out with doxorubicin, and negative controls with 1% DMSO supplemented RPMI medium. Cell viability was quantified using the MTT-based Cell Proliferation Kit I (SIGMA). Readings were made in a plate reader at 570 nm. Alternatively, the Trypan Blue exclusion method was also used. All experiments were performed in triplicate.

Wound healing assay

B16F10-Nex2 cells (3 × 10⁵) were seeded in 12-well plates and incubated over-night with a sublethal dose of CE (10 μg/ml). Wounds were made on tumor cell monolayers with a pipette tip, and images were captured at 0, 3, 8 and 24 h during cell migration and gap filling. Images of CE treated cells were compared with control cell images for quantification of cell migration ratio.

Cell cycle analysis

Tumor cells (1 × 10⁶) were incubated with 20 μg/ml of CE for 24 hours. Cells were trypsinized and centrifuged at 1000 rpm for 5 minutes. Pellets were suspended in 1 ml of ethanol 70% for fixation and incubated in ice for 15 minutes. Cells were centrifuged at 1000 rpm for 5 min and suspended in 500 μL of PI (propidium iodide) solution containing 50 μg/ml PI, 0.1 mg/ml RNase A and 0.05% Triton X-100, for 40 min at 4 °C. Cells were pelleted and suspended in 500 μl phosphate-buffered saline (PBS) for analysis in a FACS CantoII flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Inc. Ashland, OR).

Melanoma metastasis assay

Mice were endovenously injected with 5 × 10⁵ B16F10-Nex2 cells in 100 μl of RPMI without bovine fetal serum (SFB) in the tail veins of syngeneic C57Bl6 mice. Three groups of 5 animals were challenged with tumor cells and daily treated with intraperitoneal (i.p.) doses of CE (0.1mg and 0.5 mg/100μl 1% DMSO-PBS) during fourteen consecutive days. After 15 days, the lungs were removed and inspected for metastatic colonization and their masses quantified.

Ethics statement

All necessary permits were obtained for the described field studies, granted by the State of São Paulo Research Support Foundation (FAPESP), Brazil. The Ethics Committee of Federal University of São Paulo approved the main Project submitted by the Experimental Oncology Unit, CEP 1234/2011.

Statistical analysis

The experiments were performed in triplicate and the values are expressed as means ± standard deviations (S.D.). Student's *t*-test was used for significance analyses,

using GraphPad Prism 4.0 software (La Jolla, CA). $p < 0.05$ was considered significant difference.

Results

Cytotoxicity study

The different *K. coriacea* leaf organic extracts were tested in the various tumor cells. The chloroform extract (CE) was the most cytotoxic in B16F10-Nex2 cells with IC_{50} of 10.26 $\mu\text{g/ml}$ (Table 1). CE cytotoxicity was also evaluated in different tumor cells and non-tumorigenic cell lines (Figure 1A), and IC_{50} values are shown in Table 2 after 24 h of incubation. The positive control was run with doxorubicin at IC_{50} 0.03 $\mu\text{g/ml}$ in B16F10-Nex2 cells. We observed that CE induced tumor cell death at 40 $\mu\text{g/ml}$, as shown by Trypan Blue staining, suggesting a cytostatic effect of CE at concentrations lower than 40 $\mu\text{g/ml}$. B16F10-Nex2 cells incubated with subdoses of CE at 10 and 20 $\mu\text{g/ml}$, showed growth inhibition after 48 h and 72 h (Figure 1B), but the cells which showed morphological alterations did not stain with Trypan blue.

Table 1. Cytotoxic activity of different *K. coriacea* leaf extracts in B16F10-Nex2 cells *in vitro*.

<i>K. coriacea</i> leaf extracts	IC_{50} ($\mu\text{g/ml}$)
HA	> 100
EA	> 100
CE	10.26
HE	95.33
HP	> 100
Doxorubicin	0.03

HA – Hydroalcoholic extract / EA – Ethyl acetate extract / CE – Chloroform extract

HE – Hexane extract / HP – Heptane extract

CE interferes in the cell cycle of murine melanoma cells

The CE prolonged the S phase delaying the cell cycle kinetics in melanoma cells. Treated cells showed increased cytoplasmic area (Figure 2). This experiment was run with 1×10^6 B16F10-Nex2 cells incubated with 20 $\mu\text{g/ml}$ of CE for 24 h and cells were processed as described in methods. Interference in the cell cycle at sublethal concentrations explains the growth inhibition shown in Figure 1.

CE inhibits migration of murine melanoma cells

The migration of B16F10-Nex2 cells using the wound-healing assay was significantly inhibited after 8 and 24 h of incubation with 10 $\mu\text{g/ml}$ of CE ($p < 0.05$) (Figure 3).

CE anti-metastatic activity in a syngeneic melanoma system

C57Bl6 mice were endovenously challenged with 1×10^6 B16F10-Nex2 cells and intraperitoneally (i.p) treated with a daily dose of CE during 14 days. We observed that CE induced significant protection in mice treated with 0.5 mg ($p < 0.05$) rather than with 0.1 mg doses of CE (Figure 4A). The tumor mass values were calculated by subtracting the average mass value (20 mg) of normal

lung from the lung mass values of treated animals (Figure 4B). Treated animals had no weight loss or other signs of toxicity during the experiment.

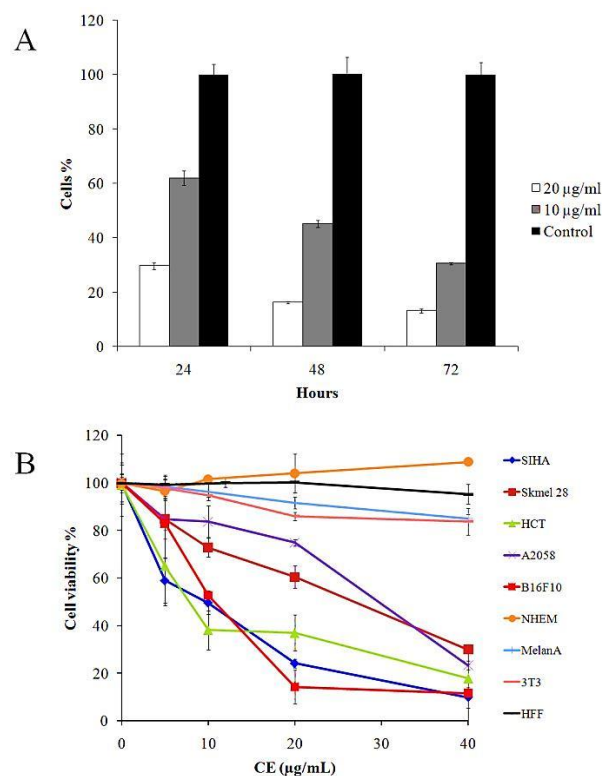


Figure 1. Cytotoxic effects of *K. coriacea* chloroform extract (CE). (A) B16F10-Nex2 cell growth after incubation with low concentrations of CE (10 and 20 $\mu\text{g/ml}$) compared to the untreated control, for 24, 48 and 72 hours; (B) Cytotoxic activity of CE in different cell lines. CE was incubated with 10^4 viable cells at concentrations ranging from 0 to 40 $\mu\text{g/ml}$ for 24 h. Cell viability was determined by the MTT method.

Table 2. IC_{50} values obtained for CE in different cancer cell lines and non-tumorigenic cell lines.

Cell line	-	IC_{50} ($\mu\text{g/ml}$)	SD
Siha	Human cervix carcinoma	6.90	1.21
HCT	Human colon carcinoma	6.88	1.42
SKMel 28	Human melanoma	42.05	0.53
MeWo	Human melanoma	34.26	3.56
A2058	Human melanoma	26.80	3.54
B16F10-Nex2	Murine melanoma	10.26	0.68
3T3	Murine embryo fibroblast	> 100	2.90
Melan A	Murine melanocyte	> 100	3.28
NHEM	Normal human epidermal melanocyte	> 100	3.26
HFF	Human foreskin fibroblast	> 100	2,79

IC_{50} - half maximal inhibitory concentration

SD – Standard deviation

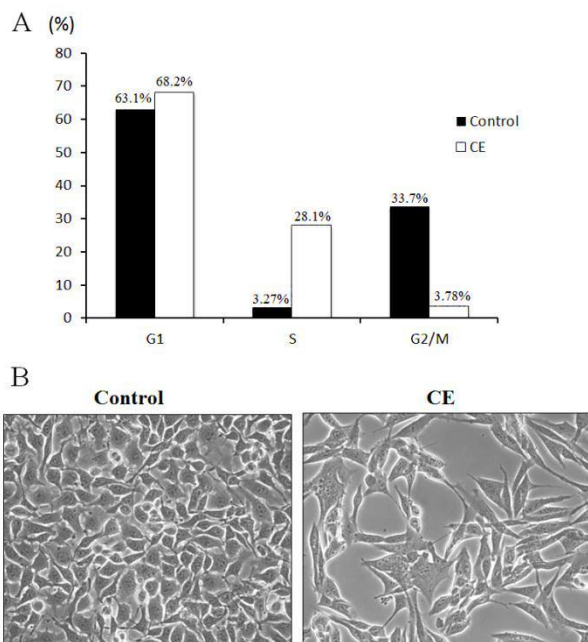


Figure 2. CE effects on melanoma cell cycle. (A) Cell cycle analysis of B16F10-Nex2 cells incubated with 20 µg/ml of CE for 24 h.; (B) Representative images of tumor cell morphology following CE treatment for 24h. Magnification, x200.

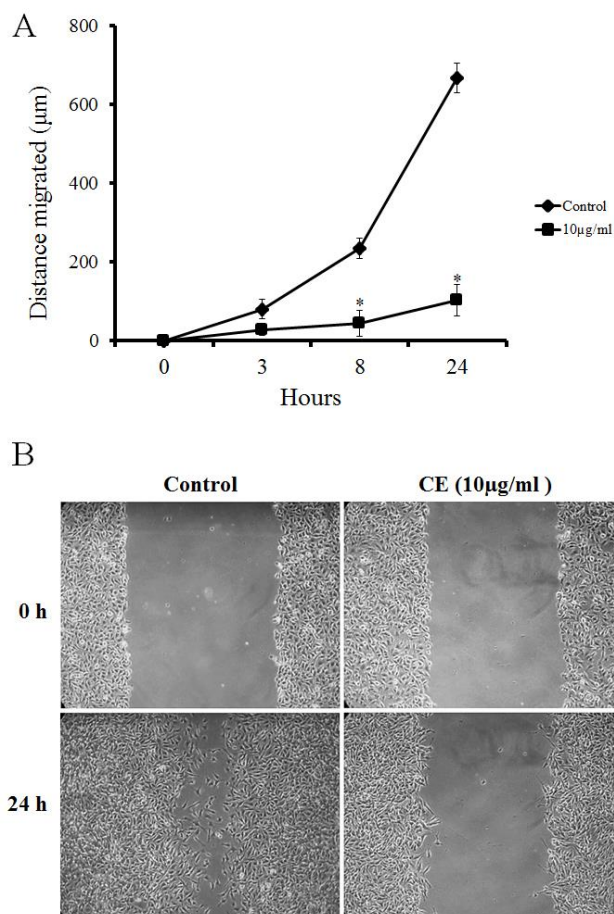


Figure 3. B16F10-Nex2 cell migration during incubation with CE at 10 µg/ml (A) Migration of tumor cells for 24 h. Statistical analysis was performed and data was plotted as the mean ± standard deviation (SD) (**p* < 0.05 vs. control); (B) Migration of CE-treated tumor cells. Magnification, x100.

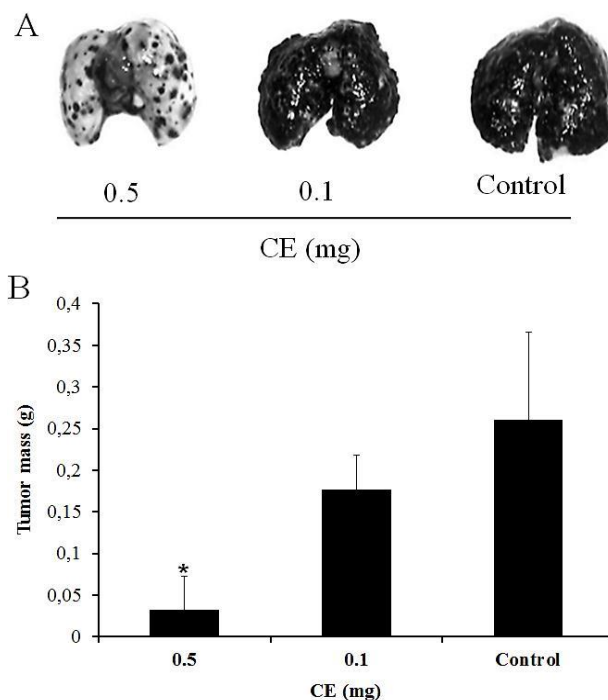


Figure 4. In vivo protection of CE against metastatic melanoma. (A) Representative mouse lungs after treatment with CE; (B) Tumor mass from mice treated with 0.1 mg and 0.5 mg of CE and vehicle (1% dimethylsulfoxide - DMSO in phosphate-buffered saline - PBS) intraperitoneally during 14 consecutive days (**p* < 0.05 vs. control).

Chemical analysis of CE extract

The ¹H NMR spectrum of crude CE extract from *K. coriacea* showed an intense broad singlet at δ 1.2 and a deformed triplet at δ 0.8 (*J* = 7.0 Hz). These signals, associated with the presence of multiplets at δ 5- 6 ppm, indicated the occurrence of long side chain unsaturated hydrocarbon derivatives as major derivatives in this active group.¹⁸ Additionally, several signals at δ 3-4 ppm as well as at δ 2-3 ppm were detected, which suggested the occurrence of alcohol and ketone derivatives as well. Aiming at the identification of these components, the crude extract was analyzed by GC-MS, which allowed the identification of six main fatty compounds (Table 3). ¹H NMR spectrum, GC and MS spectra of CE main compounds are shown in Figure 5. Compounds are grouped in three different classes: alkenes: 1-docosene (16.59%); alcohols: 1-eicosanol (18.46%), 1-docosanol (19.80%), and 1-hexacosanol (13.38%); and ketones: 2-heptadecanone (11.63%) and 2-nonadecanone (20.12%). The characterization of these compounds was based on the mass spectra as well as on the retention times in a DB-5 column.

Discussion

CE cytotoxicity was evaluated since, according to the American National Cancer Institute, the IC₅₀ limit for the cytotoxicity of a crude extract that requires further purification is 30 µg/ml.¹⁹ Previous work has already demonstrated the antitumor potential of the hexane

extract from the root bark of *K. coriacea*, particularly against MDA-MB-435 melanoma cells.⁷⁻⁹ Our present data demonstrate the cytotoxic activity of *K. coriacea* leaf

extracts, showing that the chloroform extract (CE) is the most active *in vivo* and *in vitro* against melanoma and other human tumor cell lines.

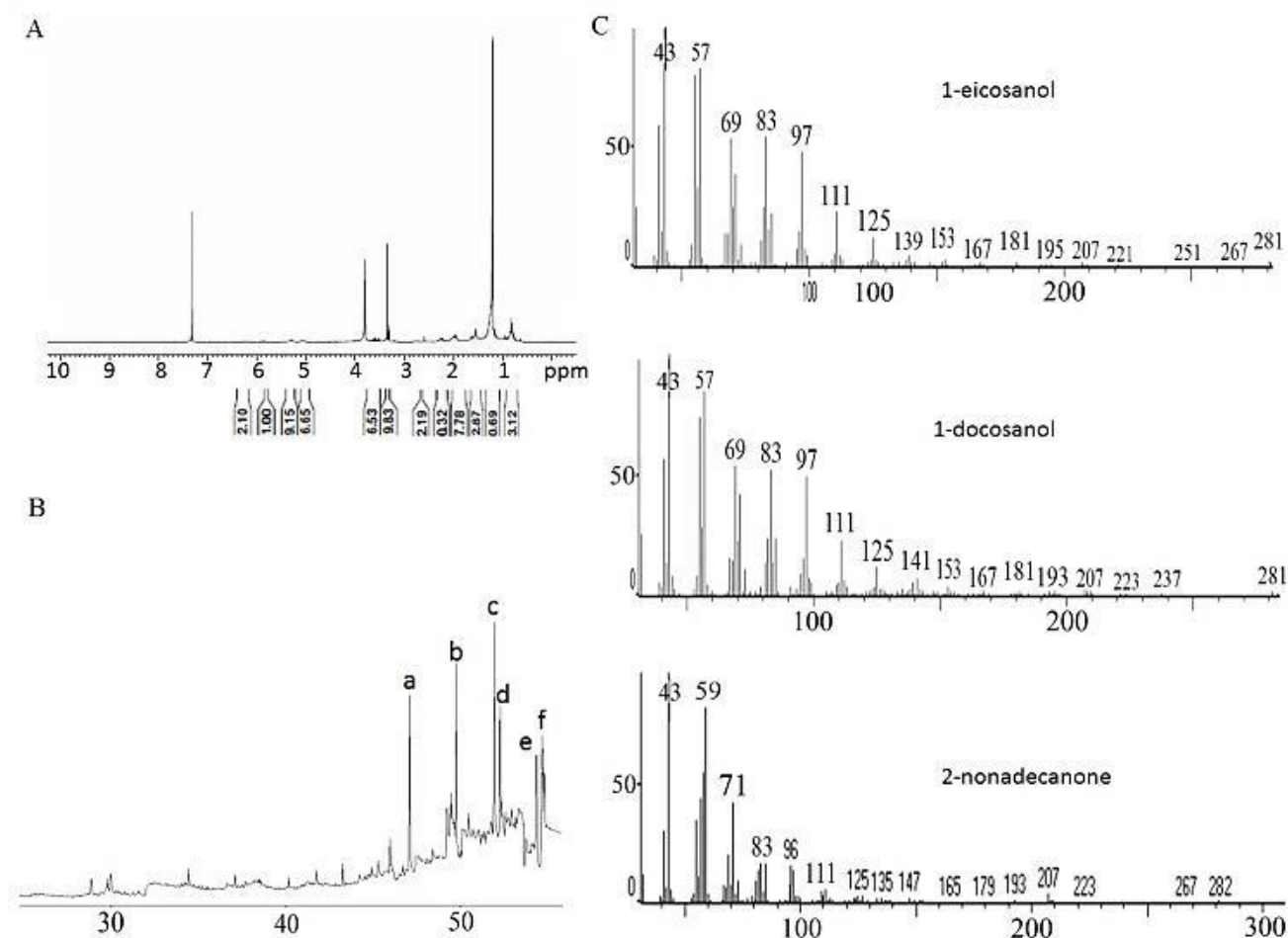


Figure 5. Analyses of CE chemical constituents. (A) ¹H NMR spectrum depicted an intense broad singlet at δ 1.2 and a deformed triplet at δ 0.8 ppm; (B) GC of CE chloroform extract showing peaks a: 1-docosene, b: 1-eicosanol, c: 1-docosanol, d: 2-heptadecanone, e: 2-nonadecanone, f: 1-hexacosanol); (C) MS spectra of the main compounds.

Table 3. Fatty compounds identified in CE.

Rt / min	Molecular formula		Relative amount (%)
47.1	$\text{CH}_3(\text{CH}_2)_{19}\text{CH}=\text{CH}_2$	(1-docosene)	16.59
49.8	$\text{CH}_3(\text{CH}_2)_{18}\text{CH}_2\text{OH}$	(1-eicosanol)	18.46
51.9	$\text{CH}_3(\text{CH}_2)_{20}\text{CH}_2\text{OH}$	(1-docosanol)	19.80
52.3	$\text{CH}_3(\text{CH}_2)_{13}\text{CH}_2\text{C}(=\text{O})\text{CH}_3$	(2-heptadecanone)	11.63
54.4	$\text{CH}_3(\text{CH}_2)_{15}\text{CH}_2\text{C}(=\text{O})\text{CH}_3$	(2-nonadecanone)	20.12
54.7	$\text{CH}_3(\text{CH}_2)_{24}\text{CH}_2\text{OH}$	(1-hexacosanol)	13.4

High concentrations of CE (40 $\mu\text{g}/\text{ml}$) caused cytoplasmic swelling in B16F10-Nex2 melanoma cells, bleb formation and cell lysis thus suggesting both apoptosis and necrosis.²⁰ The genomic DNA in CE treated cells showed degradation in a ladder pattern (data

not shown). Such morphological alterations were not observed at lower CE concentrations. CE was not significantly cytotoxic in non-tumorigenic cells, such as human and murine fibroblasts and melanocytes. The arrest in the S phase of tumor cells as observed in the

present work, led to proliferation inhibition and apoptosis, as previously reported²¹⁻²⁴ Cancer cells lack the growth control of normal cells, exhibiting unlimited self-sufficient replication.^{25,26} For therapeutic effectiveness, drugs are being developed that act as biological modifiers, regulating the cell cycle and promoting cell death.²⁰ Plant derived compounds have been reported to induce cell cycle arrest and cell death in many tumor cell lines.²⁷⁻³² The present study reports on the delay of the cell cycle kinetics at the S phase, significantly reducing the G2/M phase, by *K. coriacea* leaf chloroform extract.

K. coriacea CE inhibited B16F10-Nex2 cell migration *in vitro* and protected against lung metastasis *in vivo*. Migration and invasion are essential steps in cancer cell metastasis.³³ Similar effects have been reported in many plant derived extracts and purified compounds that inhibit cancer cell migration.³⁴⁻³⁸

The previous analysis of hexane extracts from root and bark of *K. coriacea* described δ -tocotrienol, its dimeric derivative,³ and xanthenes in the dichloromethane fraction.¹² *K. coriacea* xanthenes were shown to exert antimicrobial activities against *Staphylococcus aureus* at 50 μ g/ml,³⁹ the plant pathogenic fungus *Cladosporium cucumerinum* and also *Candida albicans*.⁴⁰ A trypanocidal activity has also been described for *K. coriacea* xanthenes.⁴¹

Presently, we show that the chloroform extract from leaves of *K. coriacea* have long-chain fatty compounds. The alkene 1-docosene, the alcohols 1-eicosanol, 1-docosanol and 1-hexacosanol, and the ketones 2-heptadecanone and 2-nonadecanone were identified. With chain lengths of C20 to C36,⁴² these compounds constitute leaf cuticular waxes that may differ widely among species.^{43,44} Genetic studies in *Arabidopsis* clarified the fatty acid elongation steps and the subsequent modification of the elongated products into primary alcohols, wax esters, secondary alcohols, and ketones, disclosing the enzymes involved in these pathways.⁴⁵ Fatty acid derivatives can act as signaling molecules, modulating normal and disease-related phenotypes in animals,⁴⁶ and display antimicrobial and anticarcinogenic activity⁴⁷. One of these long chain hydrocarbon derivatives has already been characterized in plant extracts with antitumor activity, the 1-eicosanol, a component of the acetate fraction of *Leea indica* that inhibits growth of various cancer cell lines.⁴⁸

Regarding ketones (C17, C19) and C22 alkene found in CE, there are still no data on the mechanism of action of these compounds against tumor cells. Apparently, the length of the alkyl group¹³ and the hydrophobicity are related to their biological activities.⁴⁹ Previous work has shown that some long-chain fatty alcohols and their derivatives may act on mitochondria, inhibiting both tumor cell growth *in vitro* and the growth of B16 melanoma *in vivo*.⁵⁰⁻⁵⁴ Regarding the long-chain fatty alcohols found in CE (eicosanol, docosanol and hexacosanol), they were found in plant extracts and fractions cytotoxic to tumor cell lines *in vitro*.⁵⁵

Recently, the activity of mixtures of long-chain alcohols (C26-C32), such as octacosanol, hexacosanol, heptacosanol, eicosanol and many others, derived most commonly from the wax of natural sources⁵⁶ and are similar to CE long-chain alcohols mixture has been studied. Antitumor properties have been described, such as inhibition of angiogenesis and metastasis *in vitro* and *in vivo*, by inhibition of matrix metalloproteinases activity (MMPs) and translocation of Nf-kB to nucleus.⁵⁷ Further studies are needed to clarify the mechanism of action of these compounds on tumor cells.

Conclusion

In the present work we describe the *in vitro* and *in vivo* antitumor activity of the chloroform extract (CE) of leaves from *K. coriacea* containing long-chain fatty alcohols, ketones and an alkene. The CE delayed the melanoma cell cycle with morphological alterations and inhibited tumor cell migration *in vitro*. CE growth inhibition *in vitro* was shown in murine melanoma B16F10-Nex2 and a few human tumor cell lineages. CE exerted *in vivo* protection effect using a syngeneic metastatic melanoma model with a significant reduction in the number of lung tumor nodules. It is still unclear whether any single fatty compound in the mixture, or a combination of constituents may reproduce the antitumor effects of the CE extract.

Acknowledgements

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Conflict of Interest

There is no conflict of interest to be reported.

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