Original Article

**In vivo** antitumor effect, induction of apoptosis and safety of Remirea maritima Aubl. (Cyperaceae) extracts


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**A R T I C L E   I N F O**

Article history:
Received 15 August 2014
Revised 2 May 2016
Accepted 5 May 2016

**Keywords:**
Remirea maritima
Antitumor
Sarcoma 180
Apoptosis
Toxicity
Tunel

**ABSTRACT**

**Background:** Remirea maritima has been widely used in the treatment of diarrhea, kidney disease, and high fever and for therapeutic purposes, such as an analgesic and anti-inflammatory. However, few scientific research studies on its medicinal properties have been reported.

**Purpose:** The present study aimed to investigate the anticancer potential of aqueous extract (AE), 40% hydroalcoholic extracts (40HA) and 70% (70HA) from *R. maritima* in experimental models and to identify its phytochemical compounds.

**Methods:** The chemical composition of AE, 40HA and 70HA was assessed by HPLC-DAD and ESI-IT-MS/MS. *In vitro* activity was determined on cultured tumor cell, NCI-H385N (Broncho-alveolar carcinoma), OVCAR-8 (Ovarian carcinoma) and PC-3 M (prostate carcinoma) by the MTT assay, and the *in vivo* antitumor activity was assessed in Sarcoma 180-bearing mice. Toxicological parameters were also evaluated as well as the humoral immune response.

**Results:** Among the aqueous and hydroalcoholic extracts of *R. maritima*, only 40HA showed *in vitro* biological effect potential, presenting IC₅₀ values of 27.08, 46.62 and > 50 μg/ml for OVCAR-8, NCI-H385M and PC-3 M cells lines, respectively. Regarding chemical composition, a mixture of isovitexin-2′″-O-β-D-glucopyranoside, vitexin-2′″-O-β-D-glucopyranoside, luteolin-7-O-glucuronide and 1-O-(E)-caffeyl-β-D-glucose were identified as the major phytochemical compounds of the extracts. In the *in vivo* study, the tumor inhibition rates were 57.16–62.57% at doses of 25 mg/kg and 50 mg/kg, respectively, and the tumor morphology presented increasing numbers of apoptotic cells. Additionally, 40HA also demonstrated significantly increased of OVA-specific total Ig.

**Conclusions:** 40HA exhibited *in vitro* and *in vivo* anticancer properties without substantial toxicity that could be associated with its immunostimulating properties.

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**Introduction**

Cancer is a disease highly feared by society because it has become a stigma of death and pain (Almeida et al., 2005). The World Health Organization (WHO) showed that cancer was among the leading causes of morbidity and mortality worldwide, with 14 million new cases, approximately, and 8.2 million cancer related...
deaths in 2012, and over the next 2 decades, the number of new cases is expected to rise by about 70% (WHO, 2015).

Some natural products, such as taxol, vinblastin and vincristine, have been successfully used as anticancer agents (Cragg and Newman, 2013). In addition, topotecan and irinotecan, analogues of the natural camptothecin, which is isolated from the Chinese tree Camptotheca acuminata Decne., (Nyssaceae) are used to treat colon and ovarian cancer (Oberlies and Kroll, 2004; Wall et al., 1966). Another natural compound, homoharringtonine, isolated from Cephalotaxus harringtonia (Knight ex J. Forbes) K. Koch, (Cephalotaxaceae) is used to treat acute and chronic myeloid leukemia (Cragg and Newman, 2005; Kantarjian et al., 1996).

Except for essential oils, non-volatile secondary metabolites from Cyperaceae species have been scarcely studied from a phytochemical and biological points of view in last decades (Pirzada et al., 2015). A previous phytochemical study showed that flavone glycosides are characteristic flavonoids with regular occurrence in the Cyperaceae family as a whole (Williams and Harborne, 1977). Luteolin-7-glycosides and apigenin-C-glycosides are also the important compounds widely found in different genera of Cyperaceae (Harborne et al., 1985).

Remirea maritima Aubl. is a small perennial species belonging to the Cyperaceae family that is commonly found on sea shores in tropical regions (Allan et al., 1969). In Brazil, it is popularly known as “pinheirinha-da-praia” and has been widely used in the treatment of diarrhea, kidney disease, high fever and for analgesic and anti-inflammatory purposes (Siani et al., 2001; Vitta and Prata, 2009).

However to date, only a few studies have examined the chemical and pharmacological properties of R. maritima. So, the phytochemical investigation and evaluation of the antioxidant, anti-inflammatory and antinociceptive activities of the aqueous extract and essential oil of this plant were performed by Rabelo et al. (2013, 2014). The redox-active profile characterization and cytotoxic effect in mouse fibroblasts and melanoma cells of the hydroalcoholic extract of R. maritima was also reported by our group (Dória et al., 2015). Herein, the in vitro and in vivo anticancer effects of R. maritima were investigated.

Materials and methods

Plant material

R. maritima (whole plant) was collected at the beach area of Pirambu City, Sergipe, Brazil (10°55′S, 35°6′W) on February 2011 and identified by Prof. Ana Paula N. Prata, who is a plant taxonomist from the Department of Biology. A voucher specimen (ASE 20166) has been deposited in the Herbarium of the Department of Biology, Federal University of Sergipe, São Cristóvão, Sergipe, Brazil. The authors had authorization from the Chico Mendes Institute for Biodiversity Conservation from the Brazilian Ministry of the Environment for plant collection (# 25,637–1).

Preparation of extracts of R. maritima

The material was cleaned, dried and ground into powder form. Then, the following extracts were prepared (aqueous - AE: 15 g powder/300 ml H2O; 40% hydroalcoholic - 40HA: 15 g powder/300 ml EtOH/H2O 40% v/v; and 70% hydroalcoholic - 70HA: 15 g powder/300 ml EtOH/H2O 70% v/v) under 30 min of heating (77°C) followed by filtration at reduced pressure. After that, AE was submitted to lyophilization. In the 40HA and 70HA, the organic solvent was first removed in a rotatory evaporator at 45°C under reduced pressure (Marconi, MA120) and then hydroalcoholic extracts were lyophilized. Yield: AE - 5.8%, 40HA - 6.2%, 70HA - 8.6%.

Chemical analyses

HPLC-DAD and ESI-IT-MS/MS apparatus and conditions

The HPLC analysis were performed on a Shimadzu system consisting of a degasser DGU-20A3, a SIL-20A autosampler, two LC-20AD pumps, and a SPD20Avp photodiode array detector (DAD), coupled with a CBM20A interface. The chromatographic separation was performed using a Phenomenex Luna C18 analytical column 250 x 4.6 mm (5 μm particle size). The flow rate was 1.0 ml/min and the injection volume was 20 μL. The mobile phase consisted as gradient of water and acetonitrile, starting with 10% of acetonitrile until 60% in 60 min, then returning to initial conditions. Photodiode array detector was set at 320 nm for acquiring chromatograms. Identification was based on comparisons of absorption spectra, ESI-MS/MS spectra and co-injection with standard substances. Samples of AE, 70HE, 40HE, and standard compounds (isovitexin-2’-O-β-glucopyranoside + vitexin-2’-O-β-glucopyranoside, and luteolin-7-O-glucuronide) previously isolated and identified from R. maritima (Rabelo et al., 2013), were dissolved in acetonitrile/water (50:50 v/v) as diluent solution in order to get the final concentration of 1 mg/ml for HPLC analysis. Before injection into the HPLC, the solutions were filtered in a 0.45 μm membrane filter. The data was obtained through the LC Solution software.

For the analysis of the fractions, of R. maritima extracts (AE, 70HE, 40HE) the same HPLC experimental conditions described above were used. So, the fractions corresponding to the major peaks P1, P2 and P3 were collected and then analysed by ESI-IT-MS². The MS/MS experiment in the negative ion mode was performed on a Bruker (Bruker Daltonics, Bremen, Germany) mass spectrometer, equipped with an electrospray ionisation source (ESI) by sample infusion, using a syringe pump at a flow rate 10 μl/min. Conditions: drying gas temperature, 250°C; drying gas flow, 5.0 l/min; nebulising gas pressure, 15 psi (N2); capillary voltage +4500 V; with spectra acquired over a mass range from m/z 100 to 2200; P1, ESI-MS² (m/z 593 [M−H]−; m/z 293 (100), 413; P2, ESI-MS² (m/z 461 [M−H]−; m/z 285 (100); P3, ESI-MS² (m/z 339 [M−H]−; m/z 179 (100), 135. The MS data were processed through Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany).

Quantification of major phenolic constituents

The quantification of the contents of the major phenolic compounds of R. maritima extracts was performed using HPLC-DAD with external standard method, using isovitexin-2’-O-glucopyranoside (~95% pure by HPLC) as standard compound. The HPLC-DAD method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and robustness according to the International Conference on Harmonization (ICH) guidelines (ICH, 2005).

The linearity was obtained through the calibration curve of isovitexin-2’-O-β-D-glucopyranoside (~95% pure by HPLC) by plotting chromatogram peak area at λ=320 nm versus concentrations. The standard solutions were diluted with acetonitrile to obtain five different concentrations: 9.88, 19.76, 29.64, 39.52 and 49.40 μg/ml. Regression equation was calculated after constructing calibration curve. The amount of the phenolic compounds in the extracts was determined according to the peak area of the chromatogram of the extracts with the calibration curve. The LOD and LOQ were calculated based on the standard deviation of the y-intercept (σ) and the slope of the standard calibration curve (S). LOD was calculated by the equation 3.3σ/S and the LOQ also examined by the equation 10σ/S.
The precision of the method was investigated with respect to repeatability (intraday precision) and intermediate precision (interday precision). Six samples of isovitin-2″-O-β-D-glucopyranoside (−95% pure by HPLC) (49.40 μg/mL) were injected in the same day to evaluate repeatability. The intermediate precision was evaluated by analyzing the same solutions used in the repeatability test on different days and by another analyst. The precision was expressed as relative standard deviation (% RSD).

Accuracy was performed by using solutions containing three known concentrations (9.5, 28.5 and 47.5 μg/mL) of the standard compound and then analyzed in triplicate. Accuracy was calculated as percent recoveries of response factor (area/concentration). The ruggedness of the method was evaluated using different analytical columns and changing the flow rate. The results were expressed as mean and relative standard deviation (% RSD).

Cell lines

The in vitro biological effect of the R. maritima extracts was tested against three human tumor cell lines, NCI-H358M (bronchoalveolar lung carcinoma), OVCAR-8 (ovarian adenocarcinoma), and PC-3M (metastatic prostate carcinoma). All tumor cell lines were obtained from the National Cancer Institute of the United States (NCI-US). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL streptomycin, and 100 μ/mL penicillin and incubated at 37°C in a 5% CO2 atmosphere.

Sarcoma 180 tumor cells, which had been maintained in the peritoneal cavity of a Swiss mouse, were obtained from the Laboratory of Experimental Oncology at the Federal University of Ceará.

Animals

Seventy Swiss mice (male, 25–30 g), obtained from the central animal house of Sergipe Federal University, Brazil, were used. The animals were housed in cages with free access to food and water. All animals were kept under a 12h:12h light–dark cycle (lights on at 6:00 a.m.). The animals were treated according to the ethical principles of animal experimentation of CEPA (Comitê de Ética para Pesquisa com Animais), Brazil. The Animal Studies Committee of Sergipe Federal University approved the experimental protocols with number 100/2011.

Determination of the effect of R. maritima extracts on tumor cell lines in vitro

Tumor cell growth was determined as the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to the purple formazan product, as described by Mosmann (1983). For all experiments, tumor cells were seeded in 96-well plates (0.7 × 10⁵ cells/mL in 100 μL of medium). After 24 h, the extracts (0.78 to 50 μg/mL) dissolved in DMSO were added to each well and incubated for 72 h. At the end of the incubation, the plates were centrifuged, and the medium was replaced by fresh medium (150 μL) containing 0.5 mg/mL of MTT. After 3 h, the formazan product was dissolved in 150 μL of pure DMSO, and the absorbance was measured using a multiplate reader (DTX 880 multimode detector, Beckman Coulter Inc., Fullerton, CA, EUA). The drug effect was quantified as the percentage of the control absorbance of the reduced dye at 595 nm. Doxorubicin (purity > 98%; Sigma Chemical Co., St. Louis, MO) was used as the positive control.

Determination of the effect of 40HA on the growth of solid tumors in mice

Ten-day-old sarcoma 180 ascites tumor cells (2 × 10⁶ cells/500 μL) were implanted subcutaneously into the left hind groin of the experimental mice (Bezerra et al., 2006). One day after inoculation, 40HA (25 and 50 mg/kg) was dissolved in saline solution and administered intraperitoneally once a day for 7 consecutive days. The negative control was injected with saline solution and the positive control was injected with 5-fluorouracil (5-FU, purity > 99%; Sigma Chemical Co., 25 mg/kg). On the 8th day, peripheral blood samples were collected from the retro-orbital plexus of the mice while under light ether anesthesia and submitted to further hematological and biochemical analyses. The animals were then sacrificed in a CO2 chamber. The tumors, livers, spleens, and kidneys were excised, weighed, and examined for morphology. Then, they were fixed in 10% formaldehyde for histological analysis. The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = [(A−B)/A−1] × 100, where A is the average tumor weight of the negative control, and B is the tumor weight of the treated group (Bezerra et al., 2006).

Systemic toxicological evaluation

Determination of the effect of 40HA on body and organ weight

The mice were weighed at the beginning and end of the experiment, and the animals were observed for signs of abnormalities throughout the study. Tumor, livers, kidneys and spleens were removed, weighed and observed for any signs of gross lesions or color changes and hemorrhages.

Determination of the effect of 40HA on biochemical and hematological parameters

Biochemical analyses of serum samples were performed using Clinical Chemistry® kits (Abbott; Orlando, U.S.A, Architect C 8000). The biochemical parameters that were measured were aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, globulin and albumin to investigate liver function alterations. Urea, uric acid and creatinine were measured as renal function parameters.

Hematological analyses were performed using optical light microscopy. Hematological parameters, including the total count as well as differential counts of leukocytes, including eosinophils, lymphocytes, neutrophils and monocytes, were measured.

Histopathology and morphological observations

After formaldehyde fixation, the tumors, spleens, liver and kidneys were dehydrated in alcohol, diaphanized in xylene and paraffin-embedded. Subsequently, 5-μm-thick histological sections were obtained and stained with hematoxylin and eosin. Histological analyses were performed under light microscopy.

Terminal deoxynucleotidyl nick-end labeling (TUNEL) staining

To assess the mean number of apoptotic cells by the TUNEL technique, histological sections (5-μm thick, n=5) were obtained from the paraffin-embedded tissue and incubated using an in situ cell death detection kit, POD (Roche Diagnostics, Indianapolis, IN, USA). At first, the sections were deparaffinized in xylene (three changes at 3-min intervals with air-drying in between each change for better section adherence), rehydrated in graded alcohol (99, 95 and 70%) for 3 min each, and washed with deionized water. Then, the samples were treated with proteinase K (20 μg/mL in PBS) to digest the proteins, and endogenous peroxidase activity was quenched with 2% H2O2 in PBS for 10 min at room temperature. Thereafter, sections were washed with 50 μL PBS buffer, diluted TdT enzyme solution was applied, and the sections were incubated at 37°C in a humidified chamber for 1 h. After incubation, the sections were washed again with PBS buffer. Subsequently, 50 μL of antidigoxigenin peroxidase was added, and the sections were incubated in a humidified chamber for 30 min at room temperature.
Once more, the sections were washed with PBS, and dianisobenzidine (DAB)-hydrogen peroxide was used for color development. For negative controls, the TdT enzyme was replaced with PBS on one section on each slide and was processed in parallel. Counterstaining of nuclei was performed with 2% Meyer’s hematoxylin and mounted for examination. Apoptotic cells were identified as cells with brown-stained nuclei or as apoptotic bodies (fragments of apoptotic cells engulfed by neighboring cells). The number of TUNEL-positive cells was determined in 1000 counted cells, and the data are shown at the frequency of brown-stained nuclei in the total cell count.

**Immunomodulatory analysis**

**Subcutaneous immunization**

Three groups of five Swiss mice were immunized subcutaneously with a single dose of ovalbumin (OVA) (2 mg/kg), (OVA) (2 mg/kg) plus 40HA (25 mg/kg) or (OVA) (2 mg/kg) plus 40HA (50 mg/kg). The mice were bled from the retroorbital plexus to obtain serum samples prior to immunization and at 7, 14 and 21 days after immunization.

**Measurement of specific antibody**

Specific antibodies in serum were detected by enzyme linked immunosorbent assays (ELISA). To evaluate the ability of 40HA to increase the response elicited by OVA, the total Ig antibodies against OVA were determined using OVA (10 μg/well)-coated plates. The plates were incubated at 37 °C for 1 h and washed three times with saline solution plus 0.05% Tween 80. The plates were blocked with 5% nonfat milk in saline solution for 1–2 h at 37 °C and washed once, after which 100 μl of the appropriate serum diluted in saline solution was added, and the plates were re-incubated for 1–2 h at 37 °C. The plates were washed again three times with saline solution plus 0.05% Tween 80 and treated with goat anti-mouse IgG/A/M: HRP (100 μl/well: 1:2000 final dilution) for 1–2 h at room temperature. The reaction was developed by the addition of TMB (3.3′-tetramethylbenzidine) solution followed by incubation for 10 min. The intensity of the resulting color was read at 650 nm using a multiplate reader (Synergy Mx, Biotek, USA).

**Statistical analyses**

The data are presented as the mean ± S.E.M. or IC50 values, and their 95% confidence intervals were obtained by nonlinear regression. The differences among the experimental groups were compared using ANOVA followed by the Student-Newman-Keuls test (p<0.05). All statistical analyses were performed using the GraphPad program 5.0®.

**Results**

**Chemical analyses**

**HPLC-DAD and ESI-IT-MS/MS analysis**

The HPLC-DAD analysis of the AE, 40HA and 70HA revealed the presence of three major peaks (P1, P2 and P3) (Fig. 1) which were identified according to retention times, UV and mass spectra, through comparison with authentic samples as isovitexin-2′′-O-β-D-glucopyranoside + vitexin-2′′-O-β-D-glucopyranoside, and luteolin-7-O-glucuronide, respectively (Rabelo et al. 2013). Peak P3 showed [M− H]+ at m/z 339 and its ion of MS/MS were at m/z 179 (base peak) and m/z 135. Based on comparison with ESI-MS data published reports (Shakya and Navarre, 2006; Chen et al., 2012), P3 was tentatively identified as 1-O-(E)-caffeoyl-β-D-glucose (Table 1).

**Quantification of major phenolic constituents of R. maritima**

The method validation was performed using isovitexin-2′′-O-β-D-glucopyranoside as external standard. The method showed good linearity in the concentration range of 9.88 – 49.40 μg/ml, obtaining a regression equation y = 71329x – 17220 and R² = 0.9976 within the parameters of existing legislation (ICH, 2005). The limits of detection (LOD) and the limits of quantification (LOQ) were 0.92 and 1.34 μg/ml, respectively. The repeatability tests showed 0.83% RSD and intermediate precision tests showed 0.72% RSD (day 1) and 0.59% RSD (day 2). These values can be considered excellent for analytical procedures because showed RSD less than 1%. The mean percent accuracy values and % RSD for analyzes in low (9.5 μg/ml), medium (28.5 μg/ml) and high (47.5 μg/ml) concentrations were 104.5%, 0.72%; 104.2%, 0.84%; 102.0%, 0.66%; respectively. The accuracy results were satisfactory, obtaining RSD less than 5%. The robustness was evaluated changing the flow rate (0.9, 1.0, 1.1 ml/min) and using different columns (C18 250 × 4.6 mm and C18 150 × 4.6 mm). The method was considered robust because there wasn’t important modification in the chromatograms.

The content of compounds in the freeze-dried extracts is shown in Table 1. AE presented 2.93 ± 0.04 μg/ml of P1 (isovitexin-2′′-O-β-D-glucopyranoside + vitexin-2′′-O-β-D-glucopyranoside), while 40HA and 70HA showed 25.65 ± 0.04 and 10.68 ± 0.00 μg/ml, respectively. Luteolin-7-O-glucuronide (P2) and 1-O-(E)-caffeoyl-β-D-glucose (P3) were found in major proportion in 40HA, 17.35 ± 0.01 and 17.90 ± 0.02 μg/ml, respectively. AE presented 2.59 ± 0.01 and 2.20 ± 0.00 μg/ml and 70HA showed 10.97 ± 0.06 and 10.05 ± 0.01 μg/ml of luteolin-7-O-glucuronide (P2) and 1-O-(E)-caffeoyl-β-D-glucose (P3), respectively.

In vitro biological evaluation of the R. maritima extracts

The in vitro biological effects of the R. maritima extracts (AE, 40HA and 70HA) against three human tumor cell lines were determined using the MTT method. Only HE40 showed in vitro activity, with IC50 values of 27.08, 46.62 and 50 μg/ml for OVCAR-8, NCI-H385M and PC-3 M, respectively, (Table 2). AE and 70HA did not show any significant in vitro activity at the experimental exposure levels used. Doxorubicin was used as the positive control and showed IC50 values ranging from 0.85 to 1.58 μg/ml in NCI-H385M and PC-3 M cells, respectively.

The criteria of the American National Cancer Institute, which considers promising plant extracts, i.e., an IC50 value that must be less 30 μg/ml, was adopted in our experiment (Suffness and Pezzuto, 1990). In this context, only 40HA showed an IC50 < 30 μg/ml against OVCAR-8 and was submitted to an in vivo test.

In vivo antitumor evaluation of 40HA

The effects of 40HA on mice inoculated with Sarcoma 180 tumors are presented in Fig. 2. A significant reduction in tumor weight in 40HA-treated animals was observed at both doses (25 and 50 mg/kg) (p < 0.05, Fig. 2A). On the 8th day, the average tumor weight of the control mice inoculated with Sarcoma 180 was 1.59 ± 0.14 g. Mice treated intraperitoneally with 40HA presented tumor weights of 0.68 ± 0.09 and 0.59 ± 0.06 g and inhibition ratios of 57.16 and 62.57% at doses of 25 and 50 mg/kg, respectively. The administration of 5-FU reduced the tumor weight to 77.50% within the same period.

Histopathological analysis of the tumors excised from control mice showed intense cellular and nuclear pleomorphisms (Fig. 2B). Muscle invasion, mitosis, coagulation, necrosis, and the presence of tumor cell emboli within blood vessels were also observed in the control group. In the tumors removed from animals treated with 5-FU (25 mg/kg), peripheral nerve sheath invasion by tumor cells was observed.

Apoptotic cell death was also detected in the tumors removed from all experimental groups using the TUNEL assay (Fig. 3).
Fig. 1. HPLC-DAD chromatograms (λ = 320 nm) of the extracts of R. maritima: (A) AE; (B) 70HE; (C) 40HE. Compounds: P1, isovitexin-2′′-O-β-D-glucopyranoside + vitexin-2′′-O-β-D-glucopyranoside; P2, luteolin-7-O-glucuronide; P3, 1-O-(E)-caffeoyl-β-D-glucose.

Table 1
Retention time, mass spectra and content of compounds in the freeze-dried from R. maritima extracts.

<table>
<thead>
<tr>
<th>Peak</th>
<th>RT</th>
<th>Compound</th>
<th>-MS (m/z)</th>
<th>-MS² (m/z)</th>
<th>Content (μg/ml) ± SD (n= 3)</th>
<th>AE</th>
<th>40HA</th>
<th>70HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>19.2</td>
<td>Isovitexin-2′′-O-β-D-glucopyranoside + Vitexin-2′′-O-β-D-glucopyranoside</td>
<td>593</td>
<td>293</td>
<td>2.93 ± 0.04</td>
<td>25.65±0.04</td>
<td>10.68±0.00</td>
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<tr>
<td>P2</td>
<td>25.1</td>
<td>Luteolin-7-O-glucuronide</td>
<td>461</td>
<td>285</td>
<td>2.59 ± 0.01</td>
<td>17.35±0.01</td>
<td>10.97±0.06</td>
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</tr>
<tr>
<td>P3</td>
<td>54.6</td>
<td>1-O-(E)-caffeoyl-β-D-glucose</td>
<td>339</td>
<td>179</td>
<td>2.20 ± 0.00</td>
<td>17.90±0.02</td>
<td>10.05±0.01</td>
<td></td>
</tr>
</tbody>
</table>

RT: Retention Time (minute); SD: Standard Deviation

Fig. 2. Effect of the 40% hydroalcoholic extract (40HA) of Remirea maritima on mice inoculated with Sarcoma 180 tumors. (A) The inhibition rate of 40HA on tumor growth. Data are presented as the mean ± S.E.M. of 10–17 animals. *p < 0.05 compared with the saline group by ANOVA followed by the Student–Newman–Keuls test. (B) Histopathology of Sarcoma 180 tumor cells. The tissue sections were stained with hematoxylin and eosin and analyzed by light microscopy (400x). Circles show the presence of mitosis. 5-Fluorouracil (5-FU, 25 mg/kg) was used as the positive control, and the negative control was treated with saline solution. Horizontal bars = 50 μm.
Fig. 3. The effect of 40% hydroalcoholic extract (40HA) of Remiera maritima on in situ apoptotic cell labeling, as shown by the TUNEL assay. (A) The rate of increase of TUNEL-positive cells in 40HA-treated mice. One thousand cells were counted. *p < 0.05 compared with the saline group by ANOVA followed by the Student–Newman–Keuls test. (B) Immunohistochemistry of apoptotic cells in Sarcoma 180 tumors. The tissue sections were stained by the terminal deoxynucleotidine nick-end labeling (TUNEL) technique and analyzed by light microscopy (400x). 5-Fluorouracil (5-FU, 25 mg/kg) was used as the positive control, and the negative control was treated with saline solution. Horizontal bars = 50 μm.

Table 2

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Histotype</th>
<th>40HA (μg/ml)</th>
<th>Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H385M</td>
<td>Bronchoalveolar lung carcinoma</td>
<td>46.62</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.59 – 53.53</td>
<td>0.56 – 1.30</td>
</tr>
<tr>
<td>OVCAR-8</td>
<td>Ovarian adenocarcinoma</td>
<td>27.08</td>
<td>1.19</td>
</tr>
<tr>
<td>PC-3M</td>
<td>Metastatic prostate carcinoma</td>
<td>&gt;50</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.07 – 2.36</td>
</tr>
</tbody>
</table>

Data are presented as IC_{50} values and their 95% confidence intervals were obtained by nonlinear regression from four independent MTT assays. Doxorubicin was used as the positive control.

TUNEL-positive cells showed brown-stained nuclei and were found in all groups. However, the average number of brown-stained nuclei was significantly increased in the 5-FU group and in both groups treated with 40HA when compared with the control group (p < 0.05).

Systemic toxicological evaluation

There was no significant difference in body weight gain after treatment with 40HA (25 and 50 mg/kg) compared with the control group. After 40HA treatment, no significant changes in the weights of the livers or kidneys were observed in mice inoculated with Sarcoma 180 tumors (Table S1). However, spleen weights were significantly increased when compared to the control group when administered a dose of 25 mg/kg 40HA (p < 0.05). The 5-FU group showed a significant decrease in spleen weight (p < 0.05) (Table S1).

Aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, globulin and albumin were measured to investigate liver function alterations. Urea, uric acid and creatinine were measured to assess the renal parameters. No significant changes in the renal or liver parameters were observed in Sarcoma 180-inoculated mice treated with 40HA (Table S2). On the other hand, aspartate aminotransferase and alanine aminotransferase were significantly decreased in 5-FU-treated animals (p < 0.05).

In the peripheral blood from mice inoculated with Sarcoma 180 tumors, 40HA at both dosages did not induce a significant change (Table S3). The 5-FU group showed a significant reduction in the total leukocytes (p < 0.05).

Histopathological analyses of spleens removed from animals treated with 40HA, showed a moderate increase in the white pulp and nest of megakaryocytes (Fig. 4), which suggests immunomodulatory activity (Mousinho et al., 2011; Vasconcelos et al., 2007). Visible atrophy was observed in the spleens of 5-FU-treated animals. Histopathological analyses of the livers and kidneys removed from all groups showed no remarkable changes in the morphology of their tissues (Fig. S1 and S2).

Immunomodulatory analysis

To investigate the effect of 40HA on induction of the humoral immune response, mice were immunized with a single dose of OVA (2 mg/kg), OVA (2 mg/kg) plus 40HA (25 mg/kg) or OVA (2 mg/kg) plus 40HA (50 mg/kg). The OVA-specific antibody levels, at a dilution of 1:20, in the serum were measured prior to and 7, 14 and 21 days after immunization by ELISA. The amount of OVA-specific total Ig in the sera was significantly increased by 40HA at dose of 50 mg/kg in compared with the OVA control (p < 0.05, fig. S3).

Discussion

An HPLC method was developed, and validated for AE, 40HA and 70HA extracts. The analytical method provided a good separation of the major constituents, afforded adequate calibration curve/linearity, LOD, LOQ, precision, accuracy, and robustness method application. All validation criteria were in the acceptable limits as ICH guidelines.

Herein, a mixture of isovitexin-2″-O-β-D-glucopyranoside and vitexin-2″-O-β-D-glucopyranoside, besides luteolin-7-O-glucuronide were identified in the plant extracts. These compounds were also previously isolated from aqueous extract of R. maritima by Rabelo et al. (2013) Another compound was tentatively identified as 1-O-(E)-caffeoyl-β-D-glucopyranose by comparing their ESI-MS data with those reported in the literature (Shakya and Navarre, 2006; Chen et al., 2012).

Flavones are not widely distributed in nature like other subclass of flavonoids. However, flavone C-glycosides, such as apigenin glycosides, are present in many food stuffs and nutraceuticals and have received much attention recently because of their
antioxidant and anticancer properties (Huang et al., 2005; Zeng et al., 2013). Moreover, flavones such as luteolin and its O-glycosides are widely distributed in several vegetables and fruits, herbs and spices (López-Lázaro, 2009), which have been previously demonstrated to exhibit a variety of pharmacological actions, including anti-inflammatory, antioxidant, antimicrobial and anti-proliferative effects (Chiu and Lin, 2008).

Since flavonoids glycosides are hydrolyzed during the intake, most studies have focused on the biological activity of flavonoid aglycones (Sak, 2014). So, flavones apigenin and luteolin has been shown to be effective against several tumor cell lines (Lin et al., 2008; López-Lázaro, 2009; Shukla and Gupta, 2010). On the other hand, vitexin-glycosides are considered emerging anti-proliferative and pro-apoptotic flavonoids (Papi et al., 2013) shown cytotoxic activity against different cancer cell lines (Gennari et al., 2011; Ninfali et al., 2007).

It is the first time that 1-O-(E)-caffeoyl-β-D-glucose is identified in Cyperaceae species and there has been no previous reports

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It is the first time that 1-O-(E)-caffeoyl-β-D-glucose is identified in Cyperaceae species and there has been no previous reports
on the antitumor effect or pharmacological activities of 1-O-(E)-caffeoyl-β-D-glucose.

We provide herein the first report on the anticancer activity of R. maritima. In our study, 40HA showed in vitro effect against tumor cell lines, with an IC50 value of 27.08 μg/ml for OVCAR-8. According to Suffness and Pezzuto (1990), extracts presenting IC50 values below 30 μg/ml in tumor cell line assays are considered promising for anticancer drug development. As in the current study, 40HA was able to inhibit the growth of carcinoma180 tumor cells, and these effects might be related to its chemical constituents, particularly flavone C/O-glycosides (P1, isovitexin-2′-O-β-D-glucopyranosyl + vitexin-2′-O-β-D-glucopyranoside; and P2, luteolin-7- O-glucuronide), since 40HA have higher amounts of these constituents (25.65 and 17.35 μg/ml, respectively) compared with AE (2.93 and 2.59 μg/ml) and 70HA (10.68 and 10.97 μg/ml). However, 40HA also presented high content of 1-O-(E)-caffeoyl-β-D-glucose (17.90 μg/ml) compared AE (2.20 μg/ml) and 70HA (10.05 μg/ml), which could be beneficial or more likely their association.

Apoptosis is a genetically regulated programmed cell death phenomenon, and it is important in host defense and cancer suppression (Khan et al., 2008). Many anticancer therapies work by inducing apoptosis in cancer cells as a result of a cascade of biochemical events that promote the typical changes in cell morphology, such as membrane blebbing, DNA fragmentation and formation of apoptotic bodies, resulting in cell death (Chen et al., 2010; Elmore, 2007). The TUNEL assay is a widely accepted technique for detecting DNA fragmentation in apoptotic cells (Dutta et al., 2012). In our experimental model, we observed that 40HA significantly increased the number of TUNEL-positive cells in carcinoma 180 tumors. Therefore, these results suggest that the antitumor effect of 40HA may be related, at least in part, to its pro-apoptotic effect. One of the biggest challenges regarding the development of new chemotherapeutics against cancer is the minimization of the adverse side effects of these drugs. Therefore, the systemic toxicological parameters were also examined in 40HA-treated animals. None of the analyzed systemic toxicological parameters were affected by 40HA treatment. On the other hand, S-FU, a clinically useful chemotherapeutic agent, induced a significant decrease in body weight, relative spleen weight and total leukocytes. In addition, to the atrophy of the spleen white pulp, these findings are indicative of the immunotoxicity of this drug, as demonstrated in previous reports (Gonzaga et al., 2009). However, the serum levels of transaminases (ascpartate aminotransferase and alanine aminotransferase) were decreased in this study. These findings might be explained by the lower growth of the tumors in S-FU-treated animals in comparison with the saline-treated animals, which would ultimately result in less invasion-derived tissue damage and consequent lower release of these transaminases into the serum.

In contrast, 40HA seemed to act as an immunostimulatory agent, as suggested by the increased relative spleen weight, the number of megakaryocytic nests and the production of OVA-specific antibodies in 40HA-treated mice.

In conclusion, 40HA showed in vitro and in vivo antitumor effects without presenting substantial toxicity. This activity seemed to be related to its in vitro biological effect and immunostimulant properties.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgments

The authors are grateful to CAPES, CNPq, FINEP and PAPIPEC/SE for financial support and fellowships. We also thank the Prof. Silvana Maria Zucolotto Langassner for donation of material. This work was performed according to the special authorization for access to genetic resources in Brazil # 010240/2013-6, issued by CENpq/MCTI.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.phymed.2016.05.001.

References


