Tropical Journal of Pharmaceutical Research March 2025; 24 (3): 329-337
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v24i3.5

Original Research Article

Selective *in vitro* antitumor and lymphoproliferative activities of *Dioscorea bulbifera* extract

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Sent for review: 13 September 2024 *Revised accepted: 8 March 2025*

Abstract

Purpose: *To assess the anticancer properties of Dioscorea bulbifera extract in vitro.*
Methods: *Murine L5178Y-R lymphoma, MCF-7 and MDA-MB-231 human breast cancer, as well as human HepG2 hepatocarcinoma cells, were treated with varying concentrations of ethanol, methanol and aqueous extracts of D. bulbifera tubers for 48 h to evaluate their in vitro cytotoxic activities. Viability was determined using MTT assay, while the selective cytotoxicity of D. bulbifera extracts against tumor cells was assessed in normal murine splenocytes and thymocytes. Furthermore, 1H-NMR spectra of D. bulbifera extracts were analyzed by nuclear magnetic resonance (NMR) spectroscopy.*
Results: *Significant ($p < 0.05$) cytotoxic activities (ranging from 58 to 72 %) were obtained for ethanol and methanol extracts against L5178Y-R, MCF-7 and MDA-MB-231 tumor cell lines when compared to untreated controls. Ethanol and methanol extracts did not affect the viability of normal murine thymocytes and splenocytes. In addition, the aqueous extract stimulated the proliferation of resting and concanavalin A-activated thymic and splenic cells. The presence of steroidal saponins and clerodane diterpenes in methanol and ethanol extracts, as well as carbohydrates in the aqueous extract, was demonstrated by NMR analysis.*
Conclusion: *Ethanol and methanol extracts of D. bulbifera show selective cytotoxic activity against murine and human tumor cells, without affecting normal cells, whereas the aqueous extract stimulated the proliferation of murine thymus and spleen cells.*

Keywords: *Invasive plants, Cancer, Traditional medicine, Dioscorea bulbifera, Clerodane diterpenes, Air potato*

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INTRODUCTION

Invasive plants are considered a threat to endemic flora and fauna, and their eradication is challenging and costly. However, some species that are considered invasive may have important human uses, such as being a source of food and pharmaceutical agents [1]. *Dioscorea bulbifera*, also known as air potato, potato yam, bitter yam and aerial yam, is a species of the family Dioscoreaceae [2]. This family comprises approximately 630 species, some of which possess health-enhancing properties [2,3]. The medicinal uses [4] and antimicrobial activity [5] of

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Dioscorea metabolites have been reported and their antioxidant, anti-inflammatory [6] and anticancer [7] properties have also been demonstrated.

Although originating in Asia**,** *Dioscorea* species are widely distributed in North America. Whereas the air potato is designated a weed in the United States, it is considered a valuable resource in Mexico [8]. In the southern state of Veracruz, *D. bulbifera* is successfully cultivated and used as food and medicine. According to locals, cancer patients have shown improvement after consuming homemade preparations from the air potato tubers. Therefore, to validate the claim as a sustainable alternative therapy against cancer, the *in vitro* cytotoxic activity of Mexican *D. bulbifera* against murine and human tumor cells was evaluated.

EXPERIMENTAL

Cell lines, culture media and reagents

All tumor cell lines were acquired from the ATCC repository. Complete Roswell Park Memorial Institute (RPMI) 1640 was used for maintenance of the L-5178Y-R cell line (murine lymphoma) while Dulbecco's Eagle Medium (D-MEM) was used to maintain the MCF-7, MDA-MB-231 (human breast cancer) and HEPG2 (human liver carcinoma) cell lines. Reagents were sourced from Sigma-Aldrich (St. Louis, MO, United States), whereas culture media were obtained from Life Technologies (Grand Island, NY, USA). Lysis buffer was prepared as a solution of 20 % SDS in 50 % DMF [9].

Ethical statement

Revision and approval by the Institutional Committee for Research Ethics and Animal Welfare were obtained for all experiments with animals (CEIBA-2018-024). The procedures also complied with mandatory Mexican regulations, specifically the NOM-062-ZOO-1999, which outlines the techniques for the care and handling of laboratory animals in Mexico.

Animals

Male BALB/c mice, 10 to 13 weeks old and weighing 23 – 26 g, were obtained from the Universidad Autónoma de Nuevo León, Bioterium. The animals were kept in a system of individually ventilated cages. Housing complied with standard Bioterium conditions: 22 – 26 ºC temperature, 45 % humidity, and 12/12 h light/dark cycle. Food and water were provided *ad libitum,* while cardboard tubes and PVC pipes were added to the cages to enrich the cage environment.

Collection and preparation of *Dioscorea bulbifera* tubers

Air potato tubers were collected in the municipality of Coxquihui, located in the Sierra de Totonacapan, in the northern region of Veracruz State, Mexico (20 ° 11′ 4.99″ N, 97 ° 35′ 11.05″ W) in February 2017. *Dioscorea bulbifera* was identified by Dr. Deyanira Quistian-Martinez from the Botanical Department of Facultad de Ciencias Biológicas, with voucher specimen no. 25605 and 25606. The tubers were dried over a stove, pulverized and sieved with a no. 200 mesh.

Ethanol and methanol extract preparation

Five grams (5 g) of pulverized and sieved *D. bulbifera* were suspended in 80 mL of ethanol or methanol and stirred for 24 h at 200 rpm in a magnetic hot plate for solubilization (VWR International, Radnor, PA, USA). After centrifugation, 1 mL aliquots of the supernatant were placed in 1.5 mL microtubes and subjected to a vacuum drying process in a Speed Vac concentrator (Labconco, Kansas, MO, USA) at 35 °C for five hours at a pressure of 1.0 mbar and the amount of dried extract was calculated. Dry extracts were then stored at room temperature (28 °C). Ethanol and methanol vehicle controls were also prepared.

Aqueous extract preparation

Five grams (5 g) of pulverized and sieved *D. bulbifera* were suspended in 80 mL of distilled water, boiled for 5 min and allowed to cool under stirring at 200 rpm (VWR International) for 16 h. After centrifugation, 3 mL of the supernatant was transferred to 5 mL vials and frozen at - 80 °C for 48 h. Extracts were vacuum dried for 8 h in a lyophilizer (Labconco) under a pressure of 0.100 mbar and a temperature of - 51 °C. The amount of extract was calculated by weighing the vials after lyophilization and subtracting the weight of the empty vials.

Ethanol, methanol and aqueous extracts were thereafter solubilized in the RPMI-1640 culture medium. For this, 600 µL of RPMI-1640 medium was added to 1.5 mL microtubes containing dried ethanol, methanol, or aqueous extracts, then shaken in a vortex and placed in a sonicator (Laboratory Supplies Co., Hicksville, NY, USA) until solubilized. RPMI-1640 medium was added to adjust the extracts to a concentration of 1 mg/mL, after which the extracts were sterilized

by filtering through 0.22 µm-pore size filters (Whatman International Ltd., Maidstone, England) and stored at 28 °C until use.

Cytotoxic effect of *Dioscorea bulbifera* crude extracts against tumor cell lines

Except for suspension cell line L5178Y-R, tumor cell lines were detached from culture flasks with an EDTA-trypsin solution, washed and collected in 50 mL conical tubes and washed three times. Cell suspensions were adjusted to a 5 x 10^4 cells/mL density in complete RPMI or D-MEM. In 96-well plates with flat bottoms (Becton Dickinson, Cockeysville, MD, USA), 100 µL/well of plant extracts at the concentrations 3.90, 7.8, 15.62, 31.25, 62.5, 125 and 250 µg/mL were added. Wells with complete RPMI-1640 medium were used as negative control and a blank consisting of 200 µL of RPMI-1640, 20 µL of MTT and 100 µL of lysis buffer was also included. Plates were incubated at 37 °C and 5 % CO_2 for 44 h. Subsequently, 20 µL/well of MTT at a concentration of 5 mg/mL were added to the microplates and incubated for a further 4 hours. To solubilize the formazan crystals, 100 µL/well of lysis buffer was added to L5178Y-R suspension cell cultures, whereas for the adherent cells, MDA-MB-231, MCF7 and HepG2 cells, the plates were decanted to add 80 µL/well of DMSO. Next, plates were read in a spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT) to obtain the optical densities at 540 nm. Percentage cytotoxicity of the extracts was calculated as described elsewhere [9].

Thymocyte and splenocyte primary cultures

Healthy male mice (n = 3) were anesthetized via intraperitoneal injection of a ketamine/xylazine cocktail (87.5 mg/kg ketamine + 12.5 mg/kg xylazine) [10] and then euthanized by cervical dislocation [9]. Thymuses and spleens were excised and macerated in RPMI-1640 medium to obtain single-cell suspensions, which were washed three times in the same medium. Cells were resuspended and cell densities were adjusted to 5 x 10^6 cells/mL for thymocytes and 3 x 10^6 cells/mL for splenocytes in complete RPMI-1640 [9].

Lymphocyte proliferation assay

To evaluate the proliferative response of thymocytes and splenocytes treated with *D. bulbifera* extracts, the colorimetric MTT reduction assay was used, as previously described [9]. Thymus or spleen cell suspensions (100 µL/well) were seeded in 96-well plates with flat bottom (Becton Dickinson), volume was brought to 200 µL/well by adding 100 µL/well of complete RPMI medium to the unstimulated control, and 100 µL/well of plant extracts at the concentrations of 3.90, 7.8, 15.62, 31.25, 62.5, 125 and 250 µg/mL, in the presence or absence of 6 µg/mL of concanavalin A. After 44 hours of incubation at 37 °C in a 95 % air and 5 % CO_2 atmosphere, 20 µL/well of MTT (5 mg/mL) was added to the cell cultures and the plates were incubated for an additional 4 hours. Cell cultures were treated with 100 µL/well of lysis buffer and incubated for another 16 hours and culture plates were read at 540 nm to obtain the absorbance (A) resulting from MTT reduction. The proliferation index of lymphocytes (LPI) was calculated as a ratio, dividing the absorbances at 540 nm of cells treated with extracts by the absorbances at 540 nm of cells that were not treated, as previously described [9].

Nuclear Magnetic Resonance (NMR) analysis

The ^{1}H-NMR spectra of *D. bulbifera* crude extracts were analyzed using an Agilent Technologies 400/64 premium instrument (Palo Alto, CA, USA) operating at 400 MHz.

Statistical analysis

Results are presented as mean ± SEM from quadruplicate determinations in a representative experiment. The SPSS software v22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Means for cytotoxicity and LPI were compared between experimental groups applying the ANOVA one-way test, followed by a *post hoc* Tukey's test. Minimal statistical significance was established at $p < 0.05$.

RESULTS

In vitro cytotoxic activity of *D. bulbifera* extracts

The ethanol, methanol and aqueous extracts induced significant cytotoxicity ($p < 0.01$) against L5178Y-R cells, from 26.8 to 63.30 %, 16.34 to 63.07 %, and 21.89 to 23.90 % at the concentrations of 7.8, 15.62, 31.25, 62.5, 125 and 250 µg/mL, respectively, when compared to untreated cells (Figure 1 A). In addition, ethanol and methanol extracts caused significant ($p < 0.01$) cytotoxicity to MDA-MB-231 cells, ranging from 11.90 to 67.95 %, and 15.29 to 67.33 % at concentrations of 7.8, 15.62, 31.25, 62.5, 125 and 250 µg/mL, respectively, when compared to untreated cells (Figure 1 B).

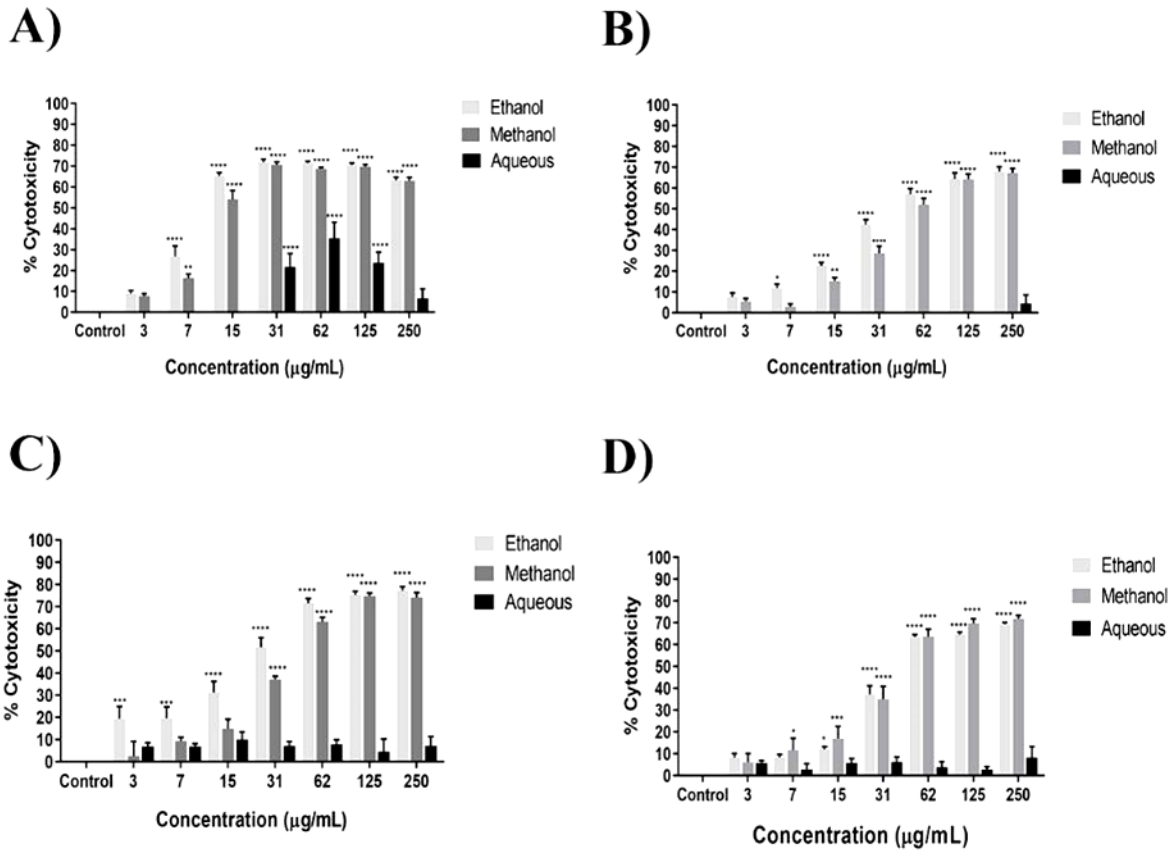

Figure 1: Cytotoxic activity of *D. bulbifera* crude extracts against murine and human tumor cell lines. Cell viability was determined after 48 h of treatments with crude ethanol, methanol and aqueous extracts of *D. bulbifera*. (A) L5178Y-R cells, (B) MDA-MB-231 cells, (C) MCF-7 cells, and (D) HEPG2 cells. *P < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 vs untreated control

Furthermore, ethanol and methanol extracts caused significant (p < 0.01) cytotoxicity to MCF-7 cells, ranging from 19.44 to 77.27 % and 14.90 to 74.11 % at concentrations from 3 to 250 µg/mL (Figure 1 C). Ethanol and methanol extracts also significantly increased cytotoxicity (p < 0.01) in HEPG2 cells, from 12.09 to 69.31 % and 11.73 to 71.79 %, at a range of concentrations from 7 to 250 µg/mL, when compared to untreated cells (Figure 1 D). Vehicle controls for the extracts did not affect the viability of tumor cell lines (Figure 1).

Effect of *D. bulbifera* extracts on thymus and spleen lymphocyte proliferation

To evaluate the selective effect of *D. bulbifera* extracts against tumor cells, their cytotoxic effect on healthy murine thymocytes and splenocytes was determined, using the extracts at the concentrations where they showed the highest cytotoxicity activity against tumor cells (62 µg/mL, 125 µg/mL and 250 µg/mL for the aqueous, ethanol and methanol extracts, respectively). The mitogen, concanavalin A, was used as a positive control. Proliferation of lymphocytes increased by 20 to 25 % and 45 to 72 % in concanavalin A-stimulated thymic and splenic cells, respectively (Figure 2 and Figure 3).

Ethanol, methanol and aqueous extracts from *D. bulbifera* at the concentration of 250 µg/mL for ethanol and methanol extracts, and 62 µg/mL for the aqueous extract significantly (p < 0.001) stimulated thymic lymphocyte proliferation by 5 %, 10 %, and 32 %, respectively, according to LPI data, and splenic lymphocyte proliferation by 5 %, 10 %, and 40 %, respectively, compared with the untreated control (Figure 2 and Figure 3). Furthermore, *D. bulbifera* aqueous extract significantly (p < 0.001) stimulated concanavalin A-activated thymic lymphocyte proliferation by 44 %, whereas the aqueous extract significantly (p < 0.05) stimulated 67 % of the proliferation of concanavalin A-activated splenocytes at 62 µg/mL, compared with the untreated control (Figure 2 and Figure 3). The vehicle controls for the extracts did not affect the viability of thymic or splenic lymphocytes (Figures 2 and 3). For untreated control cells, absorbances at 540 nm were 0.413 ± 0.003, 0.442 ± 0.004, and 0.396 ± 0.004 for thymocytes treated with ethanol, methanol and aqueous extract, respectively.

Whereas for splenocytes, absorbances at 540 nm were 0.406 ± 0.007, 0.402 ± 0.006, and 0.431 ± 0.003 for the same respective treatments.

NMR analysis of *Dioscorea bulbifera* extracts

A comparison of the ^{1}H-NMR spectrum (Deuterium Oxide, DMSO-d_6, 400 MHz) obtained for each *D. bulbifera* extract is shown in Figure 4.

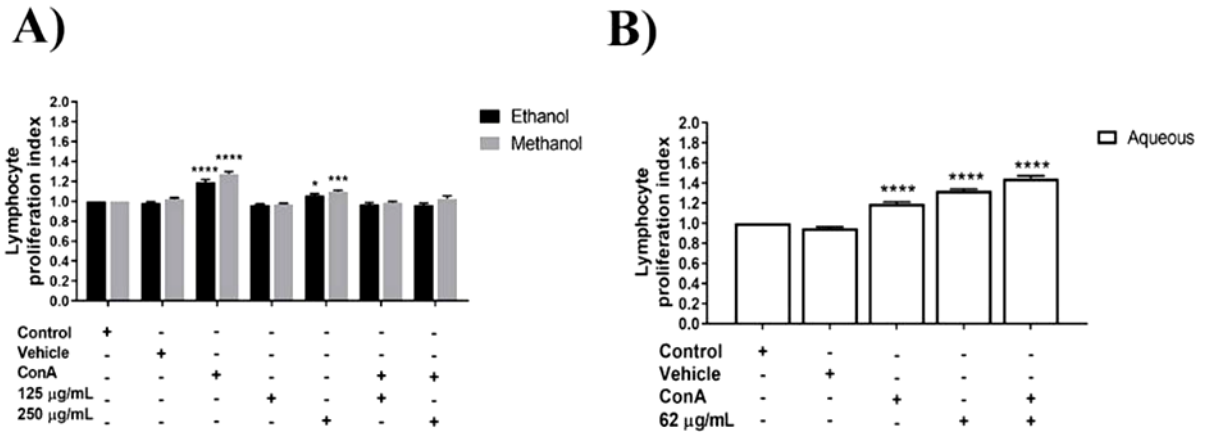

Figure 2: Proliferation of mouse thymocytes. Cells were treated with *Dioscorea bulbifera* crude (A) ethanol and methanol extracts and (B) aqueous extract**.** To determine the selectivity against tumor cells, naive and concanavalin A-activated thymocytes were treated for 48 h with the highest cytotoxic concentration observed in the cytotoxicity assays. **P* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001 vs untreated control

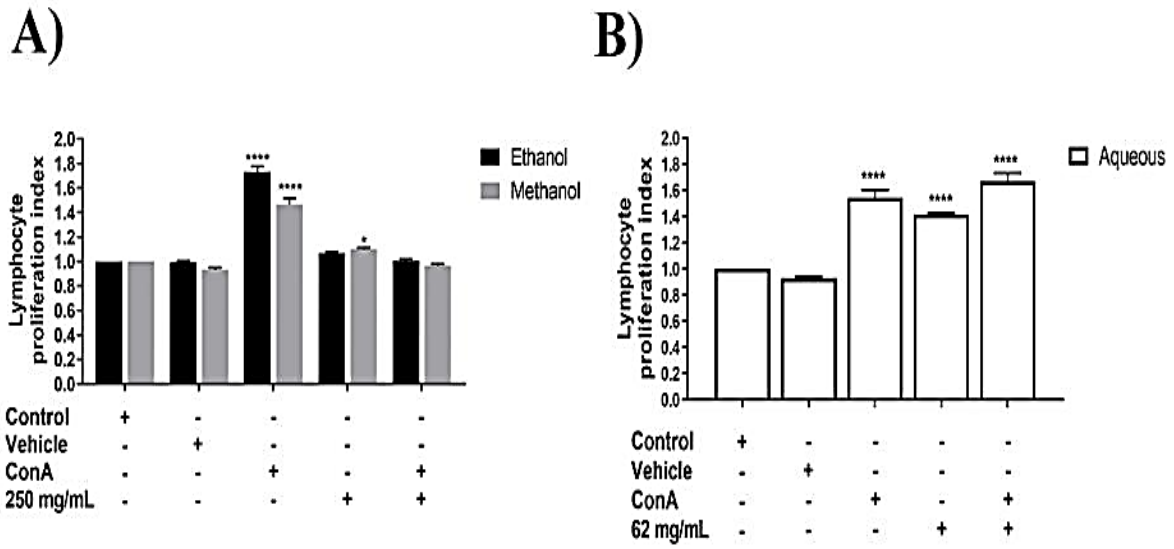

Figure 3: Proliferation of mouse splenocytes. Cells were treated with *D. bulbifera* crude (A) ethanol and methanol extracts and (B) aqueous extract. To evaluate the selectivity against tumor cells, naïve and concanavalin A-activated splenocytes were treated for 48 h with the highest cytotoxic concentration observed in the cytotoxicity assays. **P* < 0.05, ** *p* < 0.01, *** *p* < 0.001, and **** *p* < 0.0001 vs untreated control

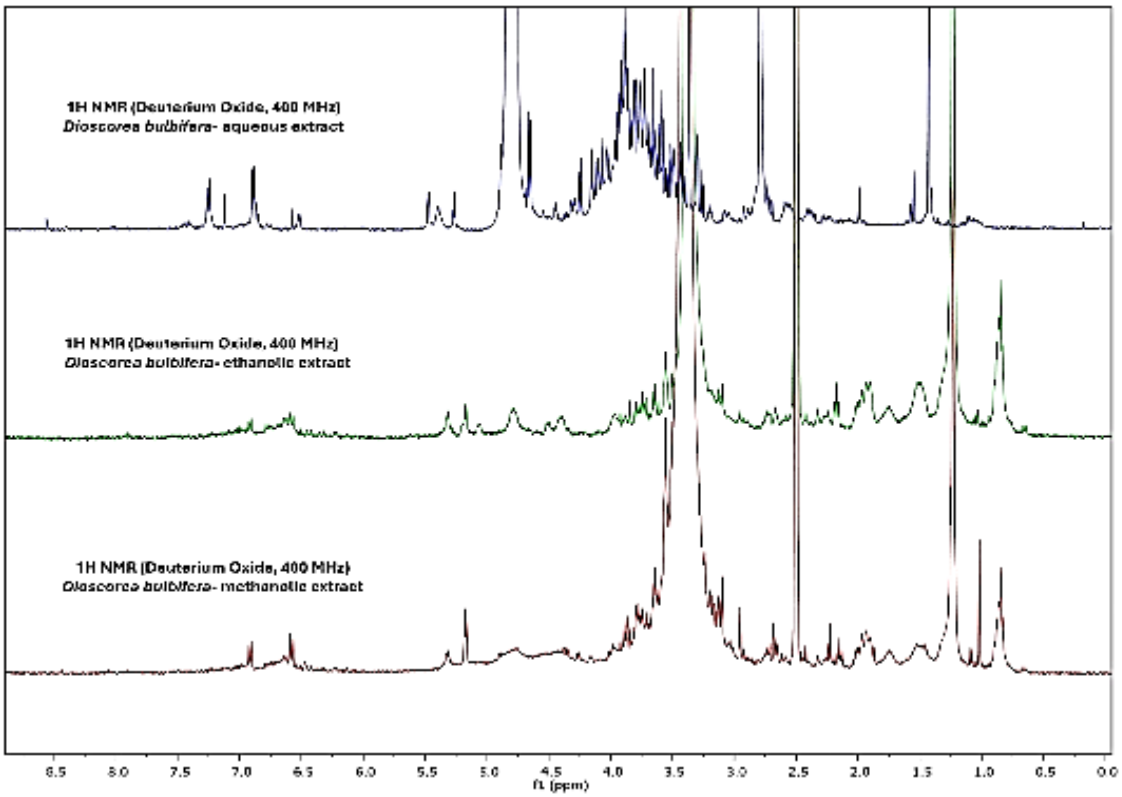

Figure 4: ^{1}H-NMR of *Dioscorea bulbifera* extracts. Comparison of the proton spectra of ethanol, methanol and aqueous *D. bulbifera* extracts

The amount of material available for NMR analysis was insufficient to identify the molecules contained in the extracts. When comparing the spectra of the three extracts, aromatic protons appear in all three cases. For the ethanol and methanol extracts, the chemical shift for these aromatic signals was found to be identical. For the aqueous extract, the chemical shift is different due to the presence of water, but the coupling constants were the same in all three cases. This suggests that the same compound is present in all three extracts. Interestingly, the aromatic proton signals do not correspond to flavonoids or phenolic acids. This is deduced from the absence of characteristic signals for these compounds, which typically resonate between 9 and 12 ppm. Data also suggests the presence of steroidal saponins, clerodane diterpenes, or both, in the ethanol and methanol extracts.

DISCUSSION

The results of the present study demonstrated significantly higher antitumor activity of ethanol and methanol extracts of *D. bulbifera* against L5178Y-R, MCF7, MDA-MB-231 and HepG2 cell lines, as compared with the aqueous extract, at concentrations ranging from 31 µg/mL to 250 µg/mL. Various authors have observed that the antitumor activity of *D. bulbifera* depends on the extract concentration and the part of the plant evaluated [11]. In addition, the antioxidant, antiproliferative and antitumor activities of *D. bulbifera* leaf extracts from Malaysia have been previously reported [4]. Antioxidant and cytotoxic activities were observed for the methanol extract, with an IC_{50} of 8.96 µg/mL against the MCF-7 cell line and 14.29 µg/mL against the MDA-MB-231 cell line. It has also been reported that *D. bulbifera* L. ethanol extracts showed antitumor activity in pathogen-free ICR mice since the intragastrical administration of the extracts decreased tumor size in a dose-dependent manner [7]. Furthermore, studies conducted by Nur and Nugroho [11] assessed the cytotoxic activity of methanol and chloroform fractions of *D. bulbifera* in T47D breast cancer cells. Two fractions of the crude chloroform extract showed high cytotoxicity, with a higher cytotoxic effect observed for extracts isolated from the leaves. This indicates that in the case of *D. bulbifera*, the leaves and tubers are the parts of the plant that contain metabolites with significant antitumor activity.

In this study, the aqueous extracts did not affect the viability of the tumor cell lines evaluated, differing from the findings reported by Steenkamp *et al* [12], where the antitumor effect

of aqueous extracts of medicinal plants from South Africa (*Bidens pilosa*, *Centella asiatica*, *Cnicus benedictus*, *Dicoma capensis*, *Hypoxis hemerocallidea* and *Sutherlandia frutescens*) was evaluated against MCF-7 and MDA-MB-231 cells growth, with *Dicoma capensis* showing the highest cytotoxic effect. Taken together, this indicates that, in the case of *D. bulbifera*, the antitumoral properties observed in traditional medicinal use are present in the ethanol and methanol-soluble fractions of the plant. When evaluating whether the extracts are selective against tumor cells, it was observed that they did not affect the viability of normal splenocytes or thymocytes. On the contrary, extracts, particularly the aqueous extract, significantly increased the proliferation of thymocytes and splenocytes. It has been previously demonstrated that the aqueous extract of *Centella asiatica* stimulates the proliferation of the triple-negative MDA-MB-231 and non-malignant MCF-10A breast cancer cells [12].

It has also been reported that partially purified ethanol fractions from *Dioscorea batatas* tubers alone or in combination with IL-2 stimulate splenocyte proliferation at 250 µg/mL [13]. In the present study, it was shown that all crude extracts, particularly the aqueous extract, stimulated the proliferation of splenocytes and thymocytes, but lymphoproliferation induced by the ethanol and methanol extracts was marginal. This suggests that the proliferation-stimulation properties are present in the aqueous crude extract of *D. bulbifera*. Additional fractionation is required to identify the components responsible for enhanced lymphoproliferation.

In addition, Hidayat *et al* evaluated the cytotoxicity of *D. bulbifera* extracts from Malaysia against tumor and normal cells [14], finding no cytotoxic effects against normal colon cells. This agrees with the findings reported in this study, indicating that the cytotoxic effect of *D. bulbifera* extracts was selective to tumor cells.

The NMR analysis of *D. bulbifera* extracts identified the presence of carbohydrates in the aqueous extract as expected, whereas steroidal saponins and clerodane diterpenes were found in ethanol and methanol extracts. These latter compounds are commonly isolated from *D. bulbifera* [15]. The ^{1}H signals of both extracts are found in regions where these two molecules would be expected. However, additional material for NMR analysis is needed to identify the molecules in the aqueous extract responsible for the increased proliferation of normal thymocytes and splenocytes.

CONCLUSION

Ethanol and methanol extracts of *Dioscorea bulbífera* tubers grown in México show selective cytotoxic activity against breast cancer and hepatocarcinoma tumor cells, whereas the aqueous extract stimulates lymphoproliferation.

DECLARATIONS

Acknowledgement/Funding

The authors thank Dr Javier Hernández-Martínez from Universidad Veracruzana for his support with the NMR analysis. The authors are also grateful to the Laboratorio de Inmunología y Virología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, México, for the financial support provided for this study and to Dr Deyanira Quistian-Martinez for her assistance in the identification of D. bulbifera plant. Authors also recognize the significant contribution of MSc María de la Luz Arriaga Gaona, from Universidad Veracruzana, for identifying the traditional use of air potato among populations in northern Veracruz.

Diana Caballero-Hernández was awarded a PRODEP grant, UANL PTC-835, by Mexico's Secretariat of Public Education. ALCR received a fellowship grant from CONAHCYT with ID 966501.

Ethical approval

As stated in the Experimental section.

Use of Artificial intelligence/Large language models

We also declare that we did not use Generative artificial intelligence (AI) and AI-assisted technologies in writing the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Use of research reporting tools

This manuscript adheres to the ARRIVE guidelines for reporting studies involving animals.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Carolina Solis-Maldonado, Diana Caballero-Hernández, and Cristina Rodríguez-Padilla conceived and designed the experiments; Diana Caballero-Hernández and Ana L. Cantú-Ruiz wrote the original draft, prepared figures, and reviewed and edited the manuscript; Luis J. Perez-Limón and Jemima Rangel-Heredia performed the experiment. Carolina Solis-Maldonado analyzed NMR data. Francisca Sandoval-Reyes, Ricardo Gomez-Flores, and Patricia Tamez-Guerra analyzed data and reviewed and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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