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Agave mapisaga aqueous extract shows *in vitro* and *in vivo* activity on murine prostate cancer cells

[El extracto acuoso del *Agave mapisaga* presenta actividad *in vitro* e *in vivo* sobre células de cáncer de próstata murino]

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Abstract: Plants are a source of multiple antineoplastic treatments. However, the effect of many species used in traditional medicine has yet to be demonstrated. In this work, the taxonomic identification of *Agave mapisaga* was made and a high-performance liquid chromatography-mass spectrometry (HPLC-MS) study suggested the presence of the aglycone hecogenin, which is part of compounds such as agavoside C and cantalasaponin 4. The antineoplastic activity of an aqueous extract was tested *in vitro* and *in vivo* on PEC-Src epithelial murine prostate cancer cells. *In vitro* study revelead a significant chemosensivity at 0.125 mg/100 µL (p=0.0001). Also, in *in vivo*, using an isotransplantation model with $1x10^6$ cells subcutaneously, it was observed that the group treated with 50 mg/kg presented a lower tumor implantation compared with the control without treatment (p=0.04).

Keywords: Agavacceae; Isotransplantation; Murine model; Cytotoxicity assay; Prostate cancer.

Resumen: Las plantas son fuente de múltiples tratamientos antineoplásicos. Sin embargo, aún falta demostrar el efecto de muchas especies usadas en la medicina tradicional. En este trabajo se realizó la identificación taxonómica del *Agave mapisaga* y un estudio de cromatografía líquida de alta definición–masas (HPLC-MS) que sugirió la presencia de la aglicona hecogenina, que forma parte de compuestos como el agavósido C y la cantalasaponina 4. Se probó la actividad antineoplásica de un extracto acuoso *in vitro* e *in vivo* sobre células de cáncer de próstata murino epitelial PEC-Src. En el estudio *in vitro* se observó una actividad citotóxica significativa a partir de 0.125 mg/100 µL (p=0.0001). Mientras que, en los experimentos *in vivo*, se isotransplantaron $1x10^6$ células por vía subcutánea, se observó que el grupo tratado con 50 mg/kg presentó una menor implantación tumoral con respecto del testigo sin tratamiento (p=0.04).

Palabras clave: Agavaceae; Isotrasplante; Modelo murino; Ensayo de citotoxicidad; Cancer de prostata

INTRODUCTION

Cancer is one of the most relevant public health problems to face in this century. It has increased considerably in recent years, from 6.7 million deaths in 2002 to 9.6 million in 2018 (Bray *et al*., 2018). Several treatments are available, depending on the type of cancer and its stage, the most used are surgery, chemo-, radiation-, immune-, and hormone-therapy. These tools represent an important development because their use has decreased the lethality in 1.8% for men, and 1.4% for women, in the period from 2012 to 2016 (Ward *et al*., 2019). Nevertheless, the incidence and the mortality of cancer on a global scale show a growing trend. Related to the above, the search for new treatments is still a priority for biomedical sciences. Multiple antineoplastic treatments have been isolated from plants, vincristine, vinblastine, and procarbazine, comes from *Vinca rosea* or *cantharanthus roseus* (Devita & Schein, 1973); camptothecine from the *Camptotheca acuminata* tree (Hitt, 2002); paclitaxcel, docetaxel, and ortataxel from *Taxus brevifolia* (Biganzoli *et al*., 2009); as well as podophyllotoxin and etoposide isolated from species of the genus *Podophyllum*. Although in recent years the search for anti-tumor activity in compounds or in crude extracts of different plant families has increased in depth, its effects have not been proven yet. The *Agavaceae* family is widely distributed in the American continent, having its highest concentration and diversity in Mexico. It has been used with medical purposes by indigenous cultures of north-central Mexico. There are studies that demonstrate that agave extracts have antimycotic (Yang *et al*., 2006), antibacterial (Ade-Ajayi *et al*., 2011), anti-inflammatory, and analgesic activity in murine models, by decreasing cyclo-oxygenase 1 and 2 expression (Dunder *et al*., 2013). Also, extracts from species such as *A. macroacantha*, *A. americana*, *A. barbadensis*, and *A. utahensis* have shown cytotoxic activity in human colon cancer cells (HCT-116, LoVo, CaCo-2, SW620 and LS) and breast cancer cells (Yokosuka & Mimaki, 2009, Eskander *et al*., 2010; Chen *et al*., 2014). The activity is dependent on the presence of steroidal saponins (Yokosuka & Mimaki, 2009; Chen *et al*., 2011) and is mediated by the stimulation of nitric oxide (NO) production and other reactive oxygen species (ROS) (Eskander *et al*., 2010), as well as by increases in the intracellular Ca^{2+} concentration. *Agave mapisaga* had been used by the Hñähñu communities of the El Cardonal region in the State of Hidalgo, Mexico who uses it as an infusion for trauma treatment (Reyes-Agüero *et al*., 2019), and when tumors suggesting neoplastic processes appear. (Interview to traditional doctors, data not published). *Agave mapisaga* research had been principally focused in the composition of its sap ("agua miel") in a nutritional point of view (Ortiz-Basurto *et al*., 2008), in the characteristics of its fibers for craft products (Parra-Negrete *et al*., 2010, Reyes-Samilpa *et al*., 2020) and in ethnobotanical studies (Reyes Samilpa, 2016; Reyes-Agüero *et al*., 2019).

The aim of this study was to analyze:

a) the cytotoxic activity of an aqueous extract of *A. mapisaga* in murine prostate cancer cells *in vitro*, and b) its effect *in vivo* in mice isotransplanted with prostate cancer.

MATERIALS AND METHODS

Plant collection and taxonomic identification

The collection of the *A. mapisaga* was performed in San Andrés Daboxtha, in the el Cardonal municipality of the State of Hidalgo in laderas pedregosas at these coordinates 20°52'41.6"N, 99°06'38.8"W. A single specimen of the agave was selected, both the leaves and the inflorescences were taken by cutting them in the place.

The processing of the samples of leaves and inflorescences needed for identification of the agave were carried out in the facilities National Herbarium (MEXU) of the Department of Botany of the Institute of Biology UNAM, México.

Extract

The extract was obtained from the leaves of *A. mapisaga*, by removing the epidermis to obtain the pulp, this was cut into fragments of 1 to 2 cm^3, one kilogram of pulp was put in a container and added two liters of distilled water at boiling point and let it rest for 24 h. Finally, the aqueous solution was collected and concentrated with a rotoevaporator at 70 RPM, 87 mbar, and 58°C for 24 hours, 95 ml of final concentrated solution where obtained. HPLC was performed to determine the compounds present in the extracts. This was done in the facilities of the Research and Industry Support Services Unit (USAII), Faculty of Chemistry, UNAM.

Stability of the extract

In order to know if low pH condition related to oral administration could affect the general characteristics of extract, a protonation test was performed. After exposure to a hydrochloric acid solution at pH of 2.5,

crude extract absorbance patterns were evaluated by UV spectrophotometry (Labomed, UVS-2700) in a wave length range of 200-300 nm. Spectra were compared to assess possible changes.

In vitro effect of A. mapisaga extract in cell amount

The aim of this experiment was focused on evaluate the effect of *A. mapisaga* crude extract on murine prostate cancer cell line (PEC-Src) (Hernández-Esquivel *et al*., 2018). Cells were cultured in DMEM (Gibco®) supplemented with 10% fetal bovine serum (Gibco® FBS) and incubated at 37°C with 5% of CO_2. As an internal control of non-transformed cells required for *in vitro* study, a primary culture of murine splenocytes was used, spleens were obtained from two 9-11 week-old male ICR mice from the Department of Microbiology and Immunology of the School of Veterinary Medicine and Zootechnics of the UNAM. Splenectomy was performed and organs were disaggregated with a nylon cell retractor. Cells were collected using the Histopaque®-1077 (SIGMA®) gradient solution, by centrifugation at 200 gravities for 10 min, obtained cells were harvested in Gibco® RPMI culture media supplemented with 10% Gibco® FBS and incubated at 37°C with 5% of CO_2.

PEC-Src cells were seeded in a 96-well polystyrene microplate at a 10000 cells per well. They were incubated with 100 µL culture medium per well during 24 h. Medium was substituted by 90 µL DMEM Gibco® added with 10 µl of the extract of *A. mapisaga* to get a final concentration of 1.25, 2.5, 5, 10 and 20 mg/mL. Each condition was placed in quadruplicate. The negative controls received 10 µL of distilled water and the positive controls received *Cis*-diamine dichloroplatinum CDDP(II) at 3.4, 7.8, 15.6, 31.5 and 62.5 µg/mL concentrations and were incubated for 24 hours. After the incubation period, the supernatant was removed, and cells were fixed with 4% paraformaldehyde for 7 min at room temperature. Afterwards, 3 washes with phosphate buffered solution (PBS) were performed and stained with diaminophenilindole (DAPI) at a concentration of 1 µg/mL, for 15 minutes and washed three times with PBS. The reading of the cell count was made with the Imagexpress® equipment of Molecular Device®(Pierozan et al., 2018). The results were analyzed with the IBM SPSS Estatistics 20® and JMP 5.1® statistical programs.

Murine splenocytes culture used as controls were seeded in 96-well polystyrene microplates at an 8 x 10^4 cells per well concentration. The challenge was carried out 120 hours after the seeding, adding the concentrations of the water-soluble extract of *A. mapisaga*, distilled water, and CDDP (II) in the same concentrations described above. The reading was made 24 hours after adding the treatments.

Assessment of Agave mapisaga extract toxicity

Take into consideration that none information about *in vivo* toxicity of Agave extracts was found in literature, a limit test was performed. Six Hsd:ICR mice consisting of three females and three males, acquired from Harlan Laboratories®, were used. They were placed in polysulfone cages with the following dimensions: 27 cm long x 16 cm wide x 12 cm tall, and distributed in two groups by sex. Treatment was administered orally trough oral gavage needle, each group received of 3000 mg/kg in a 0.5 mL volume, according to the principle of the limit test (OECD, 2008). Animals were individually observed for 2 h after dosing, and periodically for 24 hours, recording signs of toxicity as increased motor activity, tremors, arching, rolling, convulsions, tonic extension, lacrimation, pilo-erection, salivation, sedation, cyanosis (Akhila *et al*., 2007). After that, animals where sacrificed and necropsy where performed to assess organ damage in liver, kidneys and spleen (OECD, 1994).

Isotransplantation test

The aim of isotransplantation test is to know about the effect of *A. mapisaga* against prostate cancer cells in an *in vivo* model. Thus, forty-eight FVB line male mice, 28 ± 3 grams and 9-10 weeks old were used (Harlan Laboratories®). They were placed in polysulfone cages with the following dimensions: 27 cm x 16 cm x 12 cm, and distributed in an individual manner to avoid aggressions. The sample size was determined with Tang´s test scenario method (power curves for F test) (Tang, 1938) obtaining a test power ¶=0.84 (β = 0.16).

1 x 10^6 PEC-Src neoplastic cells were inoculated subcutaneously, in a volume of 100 µL with 20% of matrigel (BD biosience), under the skin of sacral-lumbar region. (Sicoli *et al*., 2014) The day of cell inoculation was considered the day zero of the experiment. Mice were randomized and split into four sets, groups one and two received oral administration of 50 mg/kg and 100 mg/kg respectively in a 4 mL volume, daily from day 1 to 34. Group three was positive antitumoral treatment control, which receive intraperitoneal CDDP(II) on days 1, 3 and 9, at a rate of 2.5 mg/kg. And group four was negative treatment control, which received drinking water, that was used

as diluent of *A. mapisaga* extract treatment.

Mice were observed daily in an individual manner for a total of 35 days, tumor size measurement was taken on days 17, 24 and 31 of experiment to calculate the tumor volume value. Mice were weighed on days 3, 10, 17, 24, 31 and 35 of the study. At the end of the study all mice were sacrificed using a carbon dioxide chamber and necropsy were performed to assess organ alterations in liver, kidneys and spleen. The dependency of values was assessed by Pearson´s χ^2 test.

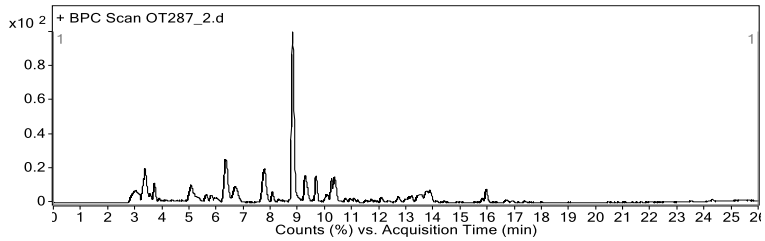
All procedures were endorsed by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the Faculty of Chemistry (OFICIO/FQ/CICUAL/097/15), UNAM.

RESULTS

Agave mapisaga extract has hecogenin aglycone as one of its components

The HPLC of the aqueous extract of *A. mapisaga* showed approximately 30 different compounds between minutes 3 and 18 of the test (Figure No. 1). From period 13.629 to 13.984 min, an isotopic pattern compatible with hecogenin aglycone was found, corresponding to what has previously been described by Leal-Diaz *et al*. (2015).

A)



B)

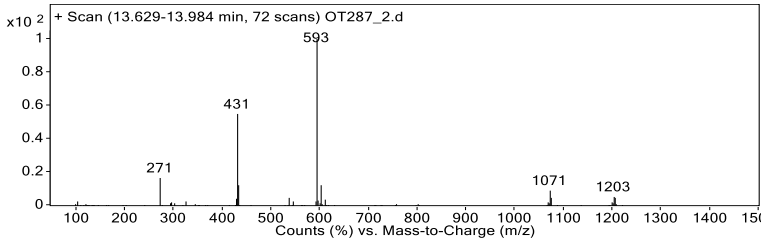


Figure No. 1
A) Curve of the high performance liquid chromatography (HPLC) of the aqueous extract of *A. mapisaga*
B) Isotopic pattern comparable to that of hecogenin aglycone found in period from min. 13.629 to 13.984 min (Leal-Díaz *et al*., 2015)

The extract is stable in conditions of acid pH

UV spectrophotometries were conducted to the aqueous extract to determine changes in its general structure under conditions of 2.5 pH in hydrochloric acid. Three peaks were observed: the first at a 211-214 nm wavelength, the second at 219-220 nm, and the third at 272 nm. (Figure No. 2). No differences in the general patterns were observed, indicating that the extract is stable at an acid pH.

Agave mapisaga extract had an effect on cell amount in vitro for murine prostate cancer cells

A significant decrease (p<0.001) in PEC-Src cells amount was observed when they were exposed to the extract of *A. mapisaga* in 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL concentrations, as compared to the control without treatment. In the primary culture of murine splenocytes, significant decrease of cell amount was observed at 20 mg/mL concentration in comparison with non-treated control. A difference in PEC-Src cells and murine splenocytes survival was observed at 10 mg/mL, 5 mg/mL, and 2.5 mg/mL concentrations (Figure No. 3), which suggests a selective effect over cancer cells. At 1.25 mg/mL concentration no effect on cell amount in the PEC-Src or in the murine splenocytes was observed, while the 20 mg/mL dose was toxic to both cell types (Figure No. 3).

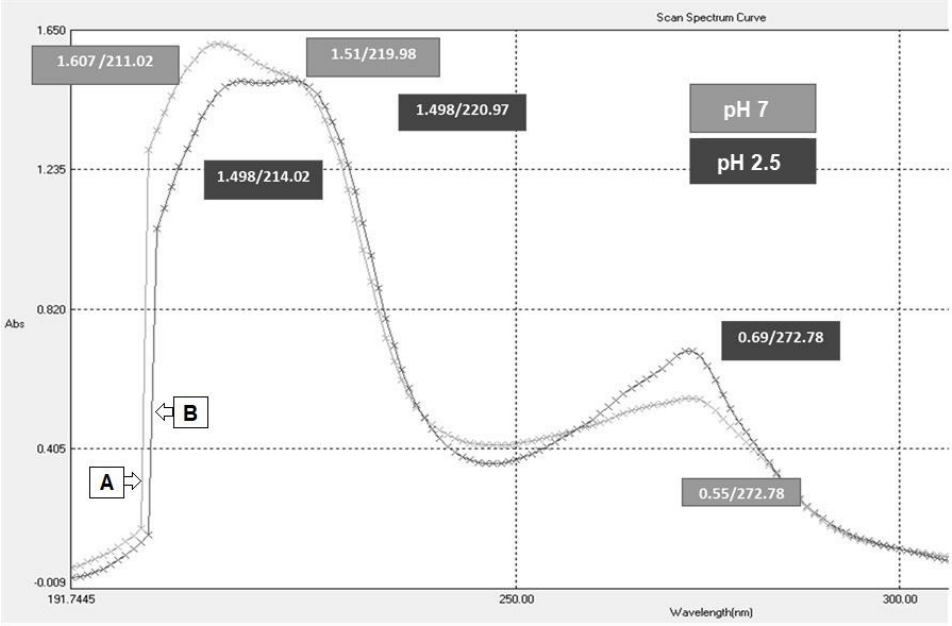


Figure No. 2

Stability of the extract under acidic pH conditions. Comparative curve of UV spectrophotometry of the aqueous extract of *Agave mapisaga* between A) extract in H_2O under pH 7 conditions, and B) extract under pH 2.5 protonation in hydrochloric acid conditions

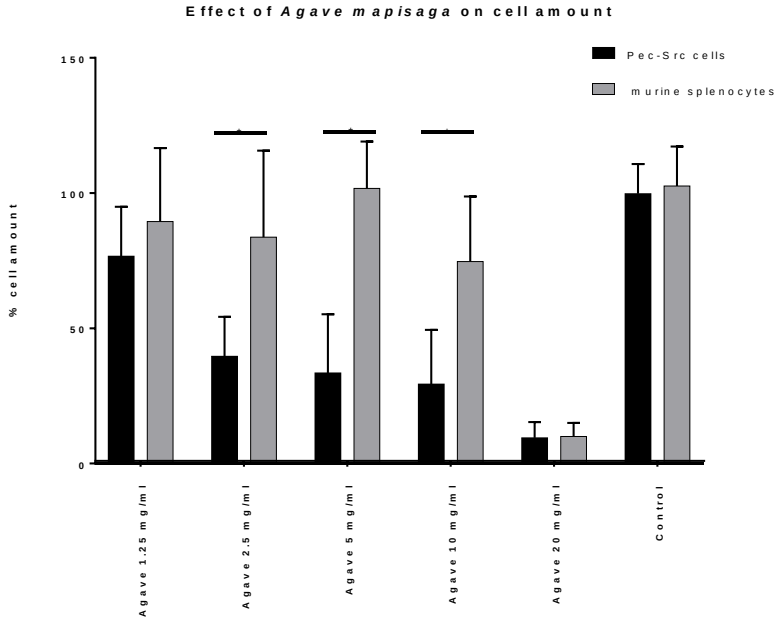


Figure No. 3

Effect of each concentration 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL of the water-soluble extract of *A. mapisaga* on PEC-Src cells and murine splenocytes amount. Comparison between the epithelial prostate cancer cells, and the murine splenocytes primary culture. *p<0.001

Agave mapisaga* extract shows no toxicity effects *in vivo

The toxicity test was performed to ensure that the maximum concentration of *A. mapisaga* administered was not toxic. None of the animals showed signs of toxicity derived from toxicity test of the *A. mapisaga* extract. No signs were observed in the clinical daily check-up or in the histopathological studies in liver, kidneys and spleen (data not shown). The foregoing indicates the safety of the extract at the maximum concentration used (100 mg/kg) for 35 days in the *in vivo* study.

Agave mapisaga extract decreases implantation of PEC-Src prostate cancer tumor cells on FVB mice

All tumors were detected between day 15 to 17 after implantation, no tumors appear besides those days. The group that received 50 mg/kg of the *A. mapisaga* extract showed less tumor implantation (4 mice with tumor out of 12 implanted) (Figure No. 4A), which represents a significant difference (p=0.0404), (Figure No. 4E), with respect to the control that received the diluent (7 with tumor out of 8 implanted), (Figure No. 4C). In the control group with treatment that received CDDP(II), one mouse showed tumor and 8 did not (Figure No. 4D). The group that received 100 mg/kg did not show any difference to the control with no treatment, showing 2 mice without tumor, and 10 with tumor (Figure No. 4B) There was no difference in tumor growth between *A. mapisaga* extract treated groups and negative control. CDDP(II) control display low tumor growing compared with control group as expected. No significant differences were observed in body weight between groups, with a mean of 27 grams (data not shown). None of mice died during the experiment.

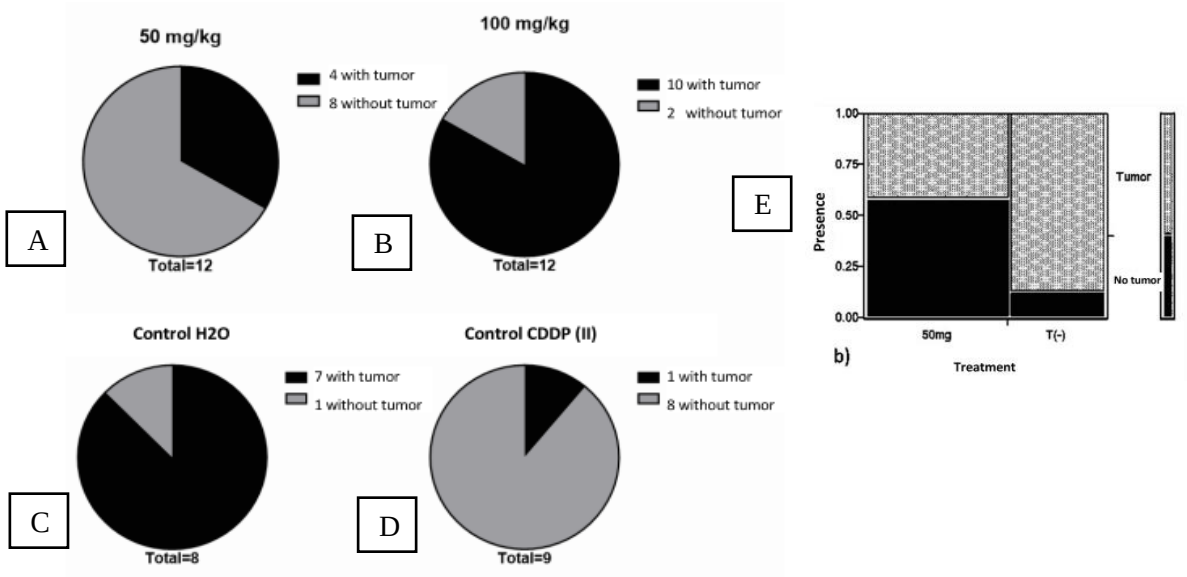


Figure No. 4

A. Number of mice in group 50 mg/kg that developed tumors after implantation B. Number of mice in group 100 mg/kg that developed tumors after implantation C. Number of mice in negative control group H_2O that developed tumors after implantation D. Number of mice in positive control group CDDP(II) that developed tumors after implantation E. Pearson χ^2 statistical test of the group that received 50 mg/kg of the aqueous extract of *Agave mapisaga* in relation to the control group that received H_2O (p=0.0404)

DISCUSSION

The *A. mapisaga* extract had effect on cell amount in *vitro*, in epithelial prostate cancer cells at a 2.5 mg/100 µL concentration. This activity corresponds with several authors, who found that crude extracts obtained from different species of agave are cytotoxic to colon cancer cell lines as HCT-116, LoVo, CaCo-2, SW620 and LS (Yokosuka & Mimaki, 2009), liver, breast, glioblastoma, and lung tumor cells (Chen *et al*., 2011; Gutierrez-Uribe & Serna-Saldivar, 2013; Chen *et al*., 2014, Santos-Zea *et al*., 2016). Santos-Zea *et al*. (2016), evaluated *A. salmiana* extract and found its capacity to reduce cell viability by generating cytostatic, apoptotic and necrotic responses in HT29 colon adenocarcinoma cells. It is important to point that in the culture of murine splenocytes exposed to the extract in 2.5, 5, and 10 mg/mL concentrations no effect was observed, which suggests a selective effect at those concentrations upon transformed prostate cancer cells but none over none-transformed ones. Selectivity effect has been observed in extracts from other species of agavaceae family, like *A. utahensis*, on HL-60 cells at a 4,9 mg/ml concentration, these compounds induced cell death by apoptosis related to the activation of caspase-3 (Yokosuka & Mimaki, 2009).

A. mapisaga extract does not produce toxicity in mice at 3000 mg/kg dose, therefore it was considered safe to proceed to *in vivo* test. In the murine model a significant decrease in tumor implantation was observed in the group which received 50 mg/kg/day treatment with *A. mapisaga* extract. Nevertheless, no effect was observed at a concentration of 100 mg/kg. This indicates a range of therapeutic activity, which is consistent with Shao *et al*. (1996), reports, who demonstrated that the antineoplastic activity of steroidal saponins present in the extracts of other plants, has a cytostatic effect in intermediate concentration levels, and in case of exceeding the cytostatic concentrations, the effect is no longer observed due to some mechanisms of apoptosis resistance. In addition, there are reports of antitumoral activity in A549 and LA795 lung cancer cell mice model in which saponins from other plant species at a range of 2.5, 5 and 7.5 mg/kg decreases tumor development (Eskander *et al*., 2010; Shuli *et al*., 2011; Tong *et al*., 2011; Li *et al*., 2013). In most of them, a purified steroidal saponin was employed, this also lead us to the hypothesis of other compounds present in different proportion in a crude extract could interfere with steroidal saponins effects when dose increases.

It is also possible that the inhibition of the tumor implantation observed is due to an anti-inflammatory effect. El-Hawary *et al*. (2020), report a total of 25 steroidal saponins and sapogenins isolate from *A. americana*, *A. americana* var. *marginata*, *A. angustifolia var. marginata*, *A. pygmaea* and *A. desmettiana*, with anti-inflammatory effect in a carrageenan induced acute inflammatory rat model at a doses of 200 mg/kg. As well, some elements of Agave sp. extract have an anti-inflammatory activity equal to or even more potent than the one produced by dexamethasone 21-phosphate, without adverse effects such as gastric damage and immunosuppression (Peana *et al*., 1997). Is well known that cellular migration induced by inflammatory process is fundamental for tumor establishment and development, several cell types as immune cells, stromal cells and cancer cells configures the tumor immune microenviroment, which is necessary for cancer cells to acquire cellular characteristics and continue the disease process (Mantovani & Sica, 2010; Corrêa *et al*., 2017; Binnewies *et al*., 2018; Vitale *et al*., 2019). To prove it, we would have to determine the relevance of the inflammatory process in our tumor implantation model, as well as the inflammatory contribution of the immune system cells and the extract effect on them.

It has been shown that plants of the Agavaceae family have a wide range of compounds among which highlight fructanes (Lopez *et al*., 2003; Mancilla-Margalli & López, 2006), flavonoids, terpenes (López-Romero *et al*., 2018), saponins and steroidal sapogenins (Pérez *et al*., 2013; Sidana *et al*., 2016; Puente-Garza *et al*., 2018; Cortés *et al*., 2020). The latter two have a wide pharmacological activity, as commented above. Currently, more than 50 species of Agave are being investigated for their saponin and sapogenin constituents which structural variations, depending on the specie (Tinto *et al*., 2005; Pérez *et al*., 2013).

The isotopic pattern found in HPLC at 13.629 to 13.984 min, is compatible with hecogenin aglycone, which partially matches with those corresponding to agavoside C, cantalasaponin 4, gentrogenin, magueyoside H, and kamogenin saponins analyzed in other studies (Yokosuka & Mimaki, 2009; Leal-Díaz *et al*., 2015; Santos-Zea *et al*., 2016). although it is possible that in *A. mapisaga* compounds presents a singular molecular structure or location changes in the functional groups. A purification procedure of our extract must be carried

out in order to obtain the elements which could be responsible of the effects observed, and then use other techniques of molecular description, as crystallography or magnetic resonance imaging, because in steroidal compounds, minimal structural differences translate into significant changes in pharmacological effect (Serra *et al*., 2012). The effect *in vitro* and *in vivo* observed in this study, could be explained by cytotoxic and cytostatic function related to steroidal saponins and sapogenins which are composed by hecogenin aglycone founded in our HPLC test. However, is also wide reported the anti-inflammatory effect (Sidana *et al*., 2016; Garcıa *et al*., 2000), which may be involved in decrease of tumor implantation, considering the tumor promoting and progression inflammatory process (Bui & Schreiber, 2007; Hanahan & Weinberg, 2011; Fouad & Aanei, 2017).

Neither the cell amount effect observed *in vitro* nor the effect in the tumor implantation can be attributed to a single element of the extract, since it is a complex mixture of compounds. However, even starting from a heterogeneous and unpurified mixture there were significant results, and it can be assumed that, when the active compounds are isolated and concentrated, the effect could be enhanced and have demonstrable anti-tumor activity.

Phytomedicine is widely used around the world. The use of plants and their components is one of the healthcare´s most common therapeutic tools, for millions of people it is the main source and , in many cases, the only one (WHO, 2002). It is crucial to encourage the study of possible sources of treatments accessible and affordable to most people, in order to contribute to the current protocols for cancer and other diseases.

CONCLUSIONS

The *A. mapisaga* extract has activity *in vitro* over prostate cancer cell culture, decreasing cell amounts at 2.5, 5, and 10 mg/mL concentration. No effect was observed in non-transformed splenocytes primary cultures which suggests a selective effect.

In *in vivo* test, the effect of the treatment was observed in the significant decrease in tumor implantation in the animals which received 50 mg/kg/day. Also extract shows no toxicity effects in *vivo*.

The isotopic pattern found in HPLC is compatible with hecogenin aglycone, which partially coincides with those corresponding to agavoside C, cantalasaponin 4, gentrogenin, magueyoside H, and kamogenin.

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