

EVALUATION OF THE TOXICITY OF EXTRACTS FROM GRAPE MARC POLAR

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ABSTRACT

Argentina is the major wine producer of south hemisphere, much of that production causes mainly two types of solids wastes, called marc and stems. Two of principal varieties of vine used in this region to the production of fine wine are Malbec and Syrah. The most important secondary metabolites in both wine and their by-products are the tannins and others phenolic compounds [anthocyanin, flavonoids, flavonols, stilbenoids (resveratrol) and phenolic acids] responsible for benefits attributed to human health. The extracts, to be used for raw material with pharmaceutics and/or food industrial propous, first must be verified their potencial toxicity. In this work, we presented the evaluation of toxicity of six marc polar extracts to various

experimental models (crustaceans, fishes, and a cellular line) at concentrations up 1000 $\mu\text{g}/\text{cm}^3$, demonstrating that they do not cause toxicity to experimental models used.

KEYWORDS: Wine, Fish, Crustaceans, RAW cells, Viability.

INTRODUCTION

According to International Organization of Vine and Wine, Argentina is the major wine producer of south hemisphere with average production of $14.98 \times 10^5 \text{ m}^3$ per year; most of this production is generated in the Cuyo region (94% of total, according National Institute of Viticulture - 2013). The winemaking causes mainly two types of solids wastes, called marc and stems, obtaining respectively 4kg and 15kg of each for 0.1 m^3 of wine. Although

there are a lot of vine varieties cultured as Malbec, Merlot, Cabernet Sauvignon, Lambrusco Maestri, Syrah, Chardonnay, Riesling, Torrontés, Tocai, Chenin and Suavignon blanc; two of principal vine varieties used to produce fine wine in the Cuyo region of Argentina are Malbec and Syrah.

The most important secondary metabolites in both wine and their by-products are the tannins and others phenolic compounds. The polyphenols are responsible of very biological activities and benefits attributed to human health.^[1,2,3] Phenolic compounds are anthocyanin, flavonoids, flavonols, stilbenoids (resveratrol) and phenolic acids.^[4,5,6]

There are multiple studies to assume that polyphenols would be responsible of beneficial effects on degenerative and cardiovascular disease,^[7,8,9] and certain cancers^[10,11] due to act reducing oxidative stress in plasma and slow-down aging.^[12,13]

Some raw material can be used to pharmaceuticals and/or food industrial purposes, but first must be verified the potential toxicity of them. This evaluation can be performed at different levels of complexity, thus there are *in vivo* assays in different experimental models, among which highlights, by its simplicity, economy and speed, indicative assays that use fishes and crustaceans very used for our working group,^[14,15,16] as studies in mammal cell lines, which may be considered more conclusive.

The murine macrophages, involved in inflammatory response and oxidative stress;^[17,18] are an experimental model widely used in research because handling technique and its culture are standardized.

The objective of this work was to perform a series of toxicity testing of extracts from grape marc polar of two varieties of *Vitis vinifera*, Malbec and Syrah to experimental models of different complexity levels among them crustaceans, fishes and cell line murine macrophages RAW 264.7 to analyze their potential use due to important bioactivities previously reported.^[19, 20, 21, 22, 23, 24]

MATERIALS AND METHODS

Samples

Marc samples of strains mentioned, are recollected in Rodeo del Medio (Latitude: -32,983°, Longitude -68,650°), Mendoza, Argentina during the months of February and March 2011.

Obtaining of the different extracts

Infusion was prepared adding 100 cm³ of boiling distilled water to 30 g of marc and was sonicated for 0.12 ks. It was filtered by Buchner and the process was repeated with the residual material, and it was placed in vials to cool and lyophilizing. This extract was denominated Extract-Aqueous Infusion (EAI). Later, with the residual material, a decoction is made during 0.9 ks with 150 cm³ distilled water; this operation was repeated once more. It was placed in vials to cool and lyophilizing. This extract was denominated Extract-Aqueous Decoction (EAD).

For the methanolic extract, 30 g of dried plant material (marc) was macerated in 100 cm³ of methanol at room temperature for 0.9 ks subjected to ultrasound. The liquid was filtered. This process was performed in duplicate. The solvent was evaporated under reduced pressure on rotavap, obtaining the dry extract named Extract-Methanolic (EM).

The extracts obtained, were dissolved in distilled water and sterilized with filters of 0.22µm. They were named according to the variety and the method of production

- A. MAD (Malbec-Aqueous Decoction)
- B. MAI (Malbec-Aqueous Infusion)
- C. MM (Malbec-Methanolic)
- D. SAD (Syrah-Aqueous Decoction)
- E. SAI (Syrah-Aqueous Infusion)
- F. SM (Syrah-Methanolic Syrah)

Acute toxicity to fish and crustaceans

The technique recommended by the U.S. Fish and Wildlife Service^[25] has been modified in order to employ a smaller amount of testing compounds as it was already reported by our group.^[15] The animals and the solutions were placed in a 2 dm³ capacity container, at controlled temperature (296.15 K) without aeration; thereby they were maintained during the measurements.

The assay begins with an initial exposure to extract at 500 µg/cm³ and continues for 3.456 ms. Every 86.4 ks the number of dead specimens in each container were counted and then removed. The toxicity effect of extracts was evaluated in fish of the specie *Danio rerio* of 0.7 to 1 cm of size approximately. Ten organisms were exposed to each extract. This assay was

already reported by our group for berberine,^[26] antifungal peptic novel and antifungal drugs commercial use.^[15, 16]

For crustaceans, the solution volume used was 100 cm³ and the exposure time 86.4 ks; with the initial exposure to extract at 500 µg/cm³.

Citotoxicity to RAW 264.7 cell line

The murine macrophage cell line (RAW 264.7) were cultured in DMEM (Sigma-Aldrich®-St. Louis, MO 63178; USA) containing 10% fetal bovine serum (Natocor®-Tokio 415; Villa Carlos Paz; Argentina) (complete medium), and were grown at 310.15 K, 5% CO₂ in fully humidified air.

Citotoxicity of extracts to cell line was determined by assaying mitochondrial reduction of a yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to purple formazan crystals.^[27]

1x10⁵ cells/well were seeded in 96 well-plates and they were incubated with increasing concentrations of each extract during 86.4 ks. The extracts were evaluated at 10, 100 and 1000 µg/cm³ in complete medium. A parallel control incubating the cells with complete medium was performed.

The results were expressed as viability percentage (Mean ± SEM) relative to control considered the latter generator 100 % viability.

Statistical analysis

For the results of acute toxicity in fish and crustaceans Chi Square method was applied to analyze and compare the frequencies among mortality rates from acute toxicity of the extracts evaluated.

For studies to cell line, we determined the significance of differences between treatments and control by one-way ANOVA (p<0.001) with Dunnett's post hoc testing using Graphpad 5.00 program for windows. The quantitative data are reported as the mean values ± standard error of the mean (SEM) from three independent experiments (n=6).

RESULTS AND DISCUSSION

The results obtained from toxicity assay to fish and crustaceans are shown in the **Table 1**.

Table 1: Results of toxicity effects of extracts to crustacean and fish

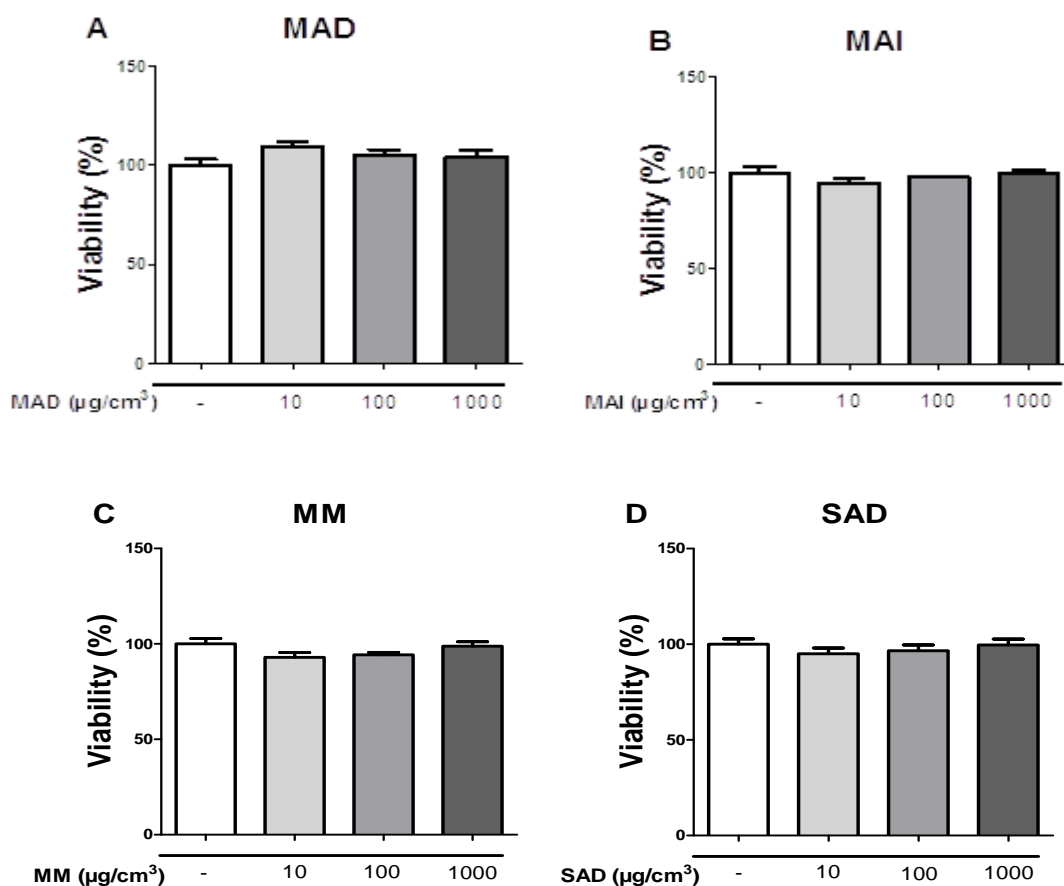
Extract	Mortality Percentage at 500 $\mu\text{g}/\text{cm}^3$	
	Crustacean	Fish
MAD	0	0
MAI	0	0
MM	0	0
SAD	0	0
SAI	0	0
SM	0	0

The toxicity effects of grape marc extracts to fish and crustaceans are represented as mortality percentage.

None of the tested extracts generated acute toxicity to fish (*Danio rerio*) and crustaceans (*Artemia salina*) at the concentration tested ($500 \mu\text{g}/\text{cm}^3$).

In the second stage of this work, and searching more conclusive experimental models, the extracts were subjected to studies of their toxicity on a murine macrophage cell line following the methodology described.

The Figure 1 represents the results obtained.



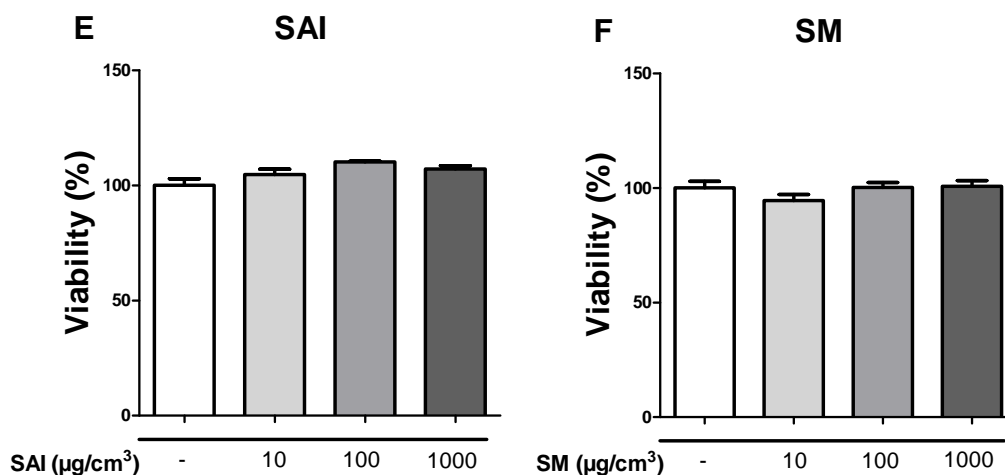


Figure 1: Cytotoxicity effects of grape marc extracts. The extracts were assayed at different concentrations (0, 10, 100 and 1000 $\mu\text{g}/\text{cm}^3$) on RAW 264.7 cell line, expressed as viability percentage (Mean \pm SEM) of triplicate.

The results show that all tested extracts do not exhibit toxicity on experimental models tested at concentrations up to 500 $\mu\text{g}/\text{cm}^3$, it is very encouraging that observed in murine macrophage cultures where decrease in cell viability was not detected even at concentrations up to 1000 $\mu\text{g}/\text{cm}^3$. There is a correlation among the results of toxicity to fish, crustaceans and cell lines.

CONCLUSIONS

Given that the extracts evaluated come from waste products in the wine industry, so it could be very advantageous to provide useful primarily in relation to health; and because no accused acute toxicity to these three experimental models, even at concentrations of 1000 $\mu\text{g}/\text{cm}^3$, they can be considered as potential resources for use in the pharmaceutical and/or food industry. Additionally it could be suggested as range limit in the evaluation of potential bioactivities a value of 1000 $\mu\text{g}/\text{cm}^3$, mainly due to the assays on cell line.

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