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SHORT COMMUNICATION

Comet assay application in evaluation the safe use of medicinal plants

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ABSTRACT

Genotoxicity of three aqueous extracts obtained of *Acacia cavens* (espinillo), *Acacia furcatispina* (garabato) and *Prosopis torquata* (tintitaco), was evaluated by comet assay. Three Concentrations (0.1, 10 and 20 mg/mL to *A. cavens* and *A. furcatispina*; 0.2, 2 and 20 mg/mL to *P. torquata*) were tested and the damage index for each was calculated. Based on the results, it was determined that both aqueous extracts of *A. cavens* and *A. furcatispina* showed no damage DNA at 0.1 and 10 mg/mL but, at 20 mg/mL induced DNA damage ($p < 0.05$), showing moderate level damage (category 2). On the other hand, *P. torquata* extract was strongly genotoxic at all concentrations tested.

Keywords: *Acacia cavens*; *Acacia furcatispina*; Comet assay; Genotoxicity; *Prosopis torquata*

INTRODUCTION

Growing resistance of bacteria to antibiotics is a global public health problem (Mahmoud et al., 2016). Among the factors contributing to the development of resistance, misdiagnosis, unnecessary prescriptions and the misuse of antibiotics by patients are included (Baos et al., 2006). In this connection, the search for alternative antimicrobial agents with new pharmaceutical properties from natural sources, is the growing interest of researchers. Furthermore, the trend globally promotes the declining use of synthetic additives or products with significant environmental impact through the development of new compounds with biological activities safe from natural sources that offer effective and promising alternative, innocuous to humans and the environment. Plants and chemical derivatives compounds could be used as antibacterial agents promising. Many phytochemicals have antioxidant and antibacterial activity beneficial for human health (Tan et al., 2015). The Argentinean flora presents a great diversity of species which are used with commercial purposes, among these the aromatic and medicinal plants being the most required (Ordoñez et al., 2006, Barboza et al., 2009). *Acacia cavens* (espinillo), *Acacia furcatispina* (garabato) and *Prosopis torquata* (tintitaco) are plants used in folkloric medicine in the treatment of gastrointestinal disorders, antiseptic or wound healing (Del Vitto et al., 1997). Although since ancient times plants they have been used as medicines, few studies to determine whether these have toxic activity for the body. Toxicity is the ability of a chemical to produce adverse effects on a living being, when in contact with him and toxicological principles include the degree of exposure or dose, exposure time and sublethal effects (Sanchez-Bayo, 2011). In relation to this, genotoxicity is the ability to cause damage to genetic material by physical, chemical or biological agents; damage to the genetic material includes not only DNA, but also to all those cellular components that are related to the functionality and behavior of chromosomes within the cell. Genotoxicity assays are designed to detect compounds that induce directly or indirectly damage the genetic material by different mechanisms, being a fundamental requirement for the assessment mutagenicity toxicological characterization of a chemical (Repetto et al., 2009). In these tests, the comet assay is useful for detecting the genotoxic effect induced by chemical, physical or biological agents on the cells of a living tool. Quantify allows sensitive and rapid damage cellular DNA. Cells with increased DNA damage have greater DNA migration towards the anode (Alvarez et al., 2013).

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In this context, the aim of this study was to evaluate the safe use of aqueous extracts obtained from plants native to our region by comet assay technique.

MATERIALS AND METHODS

Plant materials and preparation of aqueous extracts

Plant materials were collected in the province of San Luis, Argentina. Leaves of *A. cavens, A. furcatispina* and *P. torquata,* were used for the study. Plant species were deposited in the herbarium of the National University of San Luis. Plant materials were dried in air flow oven at not more than 50° C and pulverized using a grinder to blade. Decoctions were prepared according to Pharmacopeia Argentina (1978).

Genotoxicity assay

Comet assay

A mixture of human blood obtained by venipuncture of young healthy non-smokers volunteers was performed (with prior consent). Briefly, 50 µL of heparinised whole blood was mixed with 950 µL of RPMI-1640 medium (Sigma) and 50 µL extract of espinillo and garabato at 0.1, 10 and 20 mg/mL concentrations; and tintitaco at 0.2, 2 and 20 mg/mL. Then, the cell suspensions were incubated at 37 ºC during 2 h and centrifuged at 2000g for 5 min at room temperature. Negative (whole blood and RPMI-1640) and positive controls (whole blood, 50 µM H_2O_2 and RPMI-1640), were included. Cellular viability was determined by exclusion method with Trypan Blue.

Comet Assay technique was performed as described by Singh et al. (1988) with a few modifications: On slides previously covered with a film of 1% agarose normal melting point, cell suspensions embedded in agarose low melting (LMPA) were added and incubated at 4° C for 10 min. Two slides were made for each sample. The slides were transferred into lysis solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris buffer, 10% DMSO, Triton X-100 0.8%, pH 10 at 4 ° C 1 h) and protected from light until neutralization. The slides were placed in an electrophoresis chamber for 20 min to allow DNA unwinding. Then, electrophoresis (20 min at 25 V/300 mA), neutralization and staining GelRed Nucleic Acid Gel (Biotium) was performed.

Valuation of comets by image analysis

Analysis of comets was carried out employing fluorescence microscope and images were taken using the camera attached to the microscope. The comet images were visualized and captured at 400X magnification. Comets were analysed on the basis of four categories according to the average queue length (comet) ± standard deviation, as follows: Category 0 (no damage): 0 to 27 µm; Category 1 (low damage): 28 to 31 µm; Category 2 (medium damage): 32 to 35 µm; Category 3 (high damage): Greater than 36 µm (Brugés et al., 2007). The rate of DNA damage for each sample was calculated using the following formula: damage index, $DI = n_1 + 2n_2 + 3n_3 + 4n_4$, where n_1 are cells included in Category 1, n_2 in Category 2, n_3 in Category 3 and n_4 in Category 4. Bioassays were performed in duplicate and 200 cells were analyzed per treatment.

Statistical data analysis

The results were analyzed using no parametric *Kruskal Wallis* test, at the 95% confidence.

RESULTS AND DISCUSSION

Genetic mutations influence the process of carcinogenesis and some assays, such as Comet Assay, identify substances with genotoxic agents and potential risks to human health (Repetto Jiménez et al., 2009). The comet assay was performed to determine DNA damage in peripheral blood lymphocytes. This assay is based on the immobilisation of cells in agarose, electrophoresis and lysis of nuclear material (Singh et al., 1988, Alvarez Moya et al., 2013).

The comet assay is also widely used as a screening genotoxicity, due to its sensitivity, speed and low cost.

Based on previous results, extracts of *A. furcastipina, A. cavens* and *P. torquata* showed antibacterial activity, mainly against *Staphylococcus* (250-4000 µg/mL) (Martínez et al., 2014) but was not found scientific information on safe use of these decoctions. In this sense, our study allowed us to determine the presence or absence of genotoxic potential risk of products derived from these plants.

The Table 1 show no damage DNA by the *A. cavens* extract at the 0.1 and 10 mg/mL but, at 20 mg/mL induced significant differences DNA damage on human blood lymphocytes ($p < 0.05$) (Fig. 1A). Similarly, *A. furcatispina* showed no genotoxicity at lowest concentrations tested (0.1mg/mL and 10 mg/mL), however at 20 mg/mL was genotoxic showing moderate level damage (category 2; Fig. 1B). The various species of *Acacia* have low or no toxicity and may even be antigenotoxic (Bouhlel et al., 2008; Bouhlel et al., 2009). Previous studies indicated that aqueous extracts of *A. aroma* were neither cytotoxic nor genotoxic while ethanolic extracts showed genotoxicity at concentrations above 5 mg/mL (Mattana et al., 2012).

On the other hand, *P. torquata* was strongly genotoxic at all concentrations tested. In Fig. 2 may be different levels of genotoxicity with increasing concentration. The Table 1 shows the values of damage index of all concentrations of extracts evaluated. As can be seen, *P. torquata* extract at 20 mg/mL the DI was greater even than the positive

Table 1: Damage index (DI) value at increasing concentrations of aqueous extracts of *A. cavens*, *A. furcatispina* and *P. torquata*

Extract	Concentration (mg/mL)	Damage Index (DI)
A. cavens	0.1	120±1.0
	10	150±1.1
	20	170±1.0
A. furcatispina	0.1	130±1.2
	10	135±1.3
	20	165±1.2
P. torquata	0.2	170±1.1
	2	230±1.3
	20	310±1.0
Negative control	-	110±1.1
Positive control	50	290±1.2

NC: Negative control. PC: Positive control (*μM)

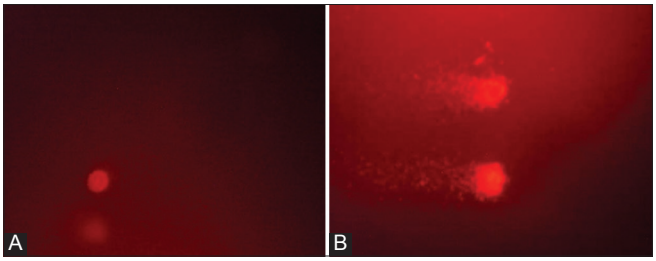

Fig 1. Comet Assay. Nucleoids without and with genotoxic damage. A) Nucleoids without genotoxic damage (*A. cavens* extract at 10 mg/mL). B) Nucleoids degraded and formation of comet (*A. furcatispina* extract at 20 mg/mL).

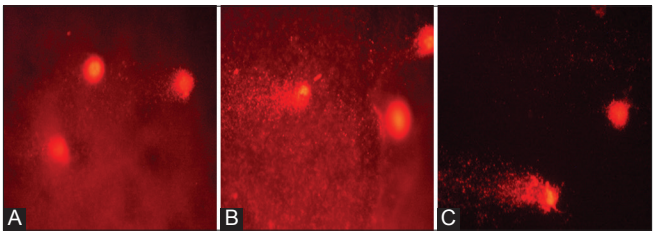

Fig 2. Comet Assay. Evaluation of genotoxic effect at different concentrations of *P. torquata*. Nucleoids with genotoxic damage in A) *P. torquata* extract at 0.2 mg/mL. B) *P. torquata* extract at 2 mg/mL and C) *P. torquata* extract at 20 mg/mL. Note the long tail of the comet at higher concentration.

control (DI=310±1.0), displayed clear degraded cores forming a queue (category 3) (Fig. 2).

Tannins, flavonoids and saponins were the major metabolites present in *A. Cavens, A. furcatispina* and *P. torquata* previously determined in a phytochemical study by our research group (Martínez et al., 2014). They have reported evidence on the double role of flavonoids as antioxidants or as a pro-oxidant (Perez Trueba et al., 2003). The mechanisms by which they exert their antioxidant action resulting from a combination of chelating properties of transition metals and sequestering free radicals and the inhibition of oxidases and other enzymes action. However, these compounds can act as pro-oxidants, feature probably responsible for the mutagenicity and genotoxicity, also found for some of these metabolites in prokariotic and eukariotic cells *in vivo* systems (Walle et al., 2001; Labiniec et al., 2003). Some of the mechanisms through which they exert their pro-oxidant actions include copper reduction, generation of reactive oxygen species (ROS) and participation antioxidant nuclear defense system: Glutathione and glutathione-S-transferase (Pérez Trueba et al., 2003). Probably the cause of mutagenic effects observed in the comet assay after exposure to genotoxic extracts, is the formation of ROS associated with the presence of flavonoids (Labiniec et al., 2003) where molecules of phenolic acids bind with proteins and lipids, and they are oxidized by O_2, O_2- or H_2O_2. Bors et al (1999) attributes the DNA damage free radicals generated oxygen during the reactions between protein-polyphenol complex and oxygen. ROS can be generated by the reaction of a phenol structure in the presence of oxygen. The three plants tested in this work contain flavonoids and sapogenins but not accurately known proportions of each compound. The two species of *Acacia* were genotoxic at high concentrations (20 mg/mL) while *P. torquata* presented genotoxic effect at all concentrations tested (0.2, 2 and 20 mg/mL). One possible cause of this behavior most genotoxicity presented by *P. torquata* may be the largest percentage composition of flavonoids as well as structural characteristics of flavonoids that favor a more pronounced pro-oxidant effect. Our results indicate that under the experimental conditions tested, *A. cavens* and *A. furcatispina* are not genotoxic below 10 mg/mL concentrations, so their use in folk medicine would be safe. Conversely, the use of *P. torquata* is not recommended because of its high genotoxicity presented at low concentrations (0.2 mg/mL). However, bioavailability studies and *in vivo* toxicity tests are necessary to ensure the safe use of these decoctions.

CONCLUSIONS

The aqueous extracts of the three tested plants showed varying degrees of genotoxicity by Comet Assay. *A. cavens* and *A. furcatispina* decoctions were not genotoxic at 10 mg/mL but they presented genotoxic activity at 20 mg/mL while *P. torquata* presented genotoxic effect from 0.2 mg/mL. They must be subjected to other rigorous tests conducted *in vivo* to ensure their safe use for health.

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Author's contributions

AM Mohamed participated in the experiments and data analysis, and also contributed to the writing of the

manuscript. CM Mattana, the corresponding author designed the research plan, organized the study, participated in experiments, coordinated the data analysis, and contributed to the writing of the manuscript. MA Cangiano participated in data analysis of comet assay. SE Satorres and LE Alcaráz participated in the experiments. AL Laciar contributed to the writing of the manuscript.

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