Trabajo Original | Toxicología Experimental

Genotoxic evaluation in vitro of *Erythroxylum minutifolium*, an endemic Cuban plant.

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Abstract

Erythroxylum genus plants have been used like anti-inflammatories in the cure of respiratory diseases. In this work the genotoxic potential of an etanolic extract of *Erythroxylum minutifolium* (ExM) was analized using a modified protocol of the SOS Chromotest and the Comet assay. Results showed that *E. minutifolium* extract no induced primary DNA damage in the cells of *E. coli* PQ37 at neither of the tested concentration using the SOS Chromotest assay. However, at the concentration of 100ug/ml in presence of metabolic fraction and 200ug/ml without and with addition of metabolic activation system, the extract was genotoxic in the Comet assay. Results obtained can be influenced by the extract composition.

Keywords: *Erythroxylum*, genotoxicity, flavonoid, quercetin, SOS assay, comet assay.

Resumen

Evaluación genotóxica *in vitro* de *Erythroxylum minutifolium* una planta endémica cubana.

Las plantas del genero *Erythroxylum* han sido utilizadas como antiinflamatorias en la cura de enfermedades respiratorias. En este trabajo fue analizado el potencial genotóxico de un extracto etanólico de *Erythroxylum minutifolium* (ExM), utilizando una variante fluorescente del ensayo SOS Chromotest y el ensayo Cometa. Los resultados mostraron que el extracto no indujo daño primario en las células de *E. coli* PQ37 a ninguna de las concentraciones testadas usando el ensayo SOS Chromotest. Sin embargo a la concentración de100ug/ml en presencia de fracción metabólica y 200ug/ml con y sin adición de dicha fracción, el extracto fue genotóxico en el ensayo Cometa. Estos resultados pudieran estar influenciados por la composición del extracto.

Palabras claves: *Erythroxylum*, genotoxicidad, flavonoides, quercetina, ensayo SOS, ensayo cometa.

Introduction

The medicinal plants as part of the traditional medicine have played an important part in the treatment of diverse pathologies (Prieto, 2004). The etnomedical properties of the Cuban plants have propitiated an important impulse to satisfy the necessity and to stimulate the convenience to use and to trasform, in sustainable way, our flora (González, 2005). Genus *Erythroxylum* (Erythroxylaceae) is formed of small tree species distributed in tropical America, Africa and Madagascar. In Cuba are described 22 species which 16 are endemics (Bisset, 1988). Some of these plants have been used in the traditional medicine like anti-inflammatories in the treatment of bronchitis, pneumonia, asthma and other respiratory affections (Bisset, 1988). Aqueous and hydroalcoholic extracts from leaves of plants in this genus have shown an important anti-microbial (Álvarez *et al.*, 2005), anti-herpes simplex virus (HSV) type-1 (González, 2006) and anti-human immunodeficiency virus (HIV) infection (Gonzalez, 2004) activity as principal pharmacological activity, besides effects like anestesic, analgesic and stimulates properties for liver, kidney and gall bladder ailments, like diuretic and for the treatment of venereal diseases and muscular problems (Cano and Volpato, 2004).

The majority of these studies have been refers to the flavonoids and alcaloids presents (Srinivasan *et al.*, 2001). Branches and leaves have been the principal parts studied (Alvarez *et al*, 2007). On this genus have been found mainly polyphenols like flavonoids, phenols and tanines and reductive compounds (Jiménez, 2004).The majority flavonoids are flavonols like quercetin, kaemferol and ombuin. They appear mono or diglycolates with sugars like glucose, galactose, arabinose and xilose or with the combination of them (González *et al.*, 2006).

As part of a safety evaluation of any new research product, an evaluation of the genotoxic potential *in vitro* of an *Erythroxylum minutifolium* var Griseb etanolic extract was researched.

Materials and Methods

Plant material.

Leaves of *Erythroxylum minutifolium* var Griseb were collected from Pinar del Rio, Cuba, among the years 2000-2005. Some specimens of the plant were deposited at the Herbarium of the Pedagogic Institute of this province. Leaves of *Erythroxylum minutifolium* were verified by Dr. Armando Urquiola (Botany Garden Manager, Pinar del Rio).The hydroalcoholic extract of *Erythroxylum minutifolium* (ExM) was obtained by the Natural Products Laboratory from Center of Pharmaceutical Chemistry as described Álvarez *et al.*, 2007.

Cytotoxicity studies of ExM.

-Cell survival assay.

Exponential phase cultures of strain PQ37 grown in LB medium at 37°C, were diluted 1:10 in a medium LB(2X). Aliquots of 500ul were distributed into sterile tubes. Final evaluated concentrations of the extract were: 50, 100 and 200ug/ml. Positive controls utilized were 150 Gy of gamma irradiation in the case of tubes in absent of S_9 mix and bleomicin (2mg/ml) in the case of eppendorf tubes in presence of S_9 mix. Negative controls were made with destiled water.

For estimate the cell survival per cent was used the following formula, according to Iwanami and Oda, 1985: $S=N/N_0 \times 100\%$, where: S= Cell survival, N= Number of treated colonies, N_0= Number of colonies of negative control

- Alkaline phosphatase

Like toxicity criterion was used the protein synthesis inhibition in treated *E. coli* PQ37 cells, according to the indicated by Quillardet and Hofnung, 1993.

Genotoxicity studies of ExM.

- SOS Chromotest assay

The original procedure described by Quillardet *et al.*, 1982, was modified in order to avoid interferences in measuring by pigments present in the plant extracts. Basically, the modifications were the use of fluorescent substrates and a modified substrate buffer for the alkaline phosphatasa assay according to Salvo *et al*, 1994.

ExM was considered mutagenic if it produced a dose-response effect and a 2-fold increase in the induction factor (IF) with respect to the control. Induction factor was calculated using the following formula, according to Quillardet *et al*., 1989:

IF= β-galactosidase (induced)/phosphatase (induced)/β-galactosidase (control)/ phosphatase (control)

- Comet assay

Human blood peripheral was obtained by venous puncture from a healthy voluntary, no smoker, between 25-35 years old. Human blood peripheral was incubated with the different concentrations in study of ExM: 5, 50, 100 and 200ug/ml, as in presence as in absence of metabolic activation, at 4°C during 1 hour. The Comet assay was essentially performed as described by Singh *et al*, 1988, with some modifications (Collins *et al.*, 1993).

Statistical Analysis

Were calculated the average values and the corresponding standard mistake of alkaline phosphatase activity, of cell survival per cent and of the SOS Induction Factor. The data normality was proved using the Kolmogorov-Smirnov test. After, the average values were compared respect the controls considered in each case using the Student t ($p<0.05$). Comet data was analyzed with non parametric Kruskal Wallis and Dunnett test.

Results

Cytotoxicity assays.

Results obtained in the cytotoxicity assays indicated that showed an inhibition statistically significant ($p<0.05$, Student t test) of the cell survival percent only at the concentration of 200 µg/ml, in presence and in absence of metabolic activation system compared to control cells (Fig. 1). However, although the extract at this same concentration inhibited the protein synthesis (Table1), it was not statistically significant different to control. Thus, results obtained indicated that the analyzed extract is toxic for the *E. coli* PQ37 cells at this concentration.

SOS Chromotest assay.

E. minutifolium extract did not induce any significant genotoxic activity ($p<0.01$, Student t test) at the tested concentrations (Fig. 2). At neither of the extract concentrations the induction factor was duplicated, nor it depended of the extract concentration according to a simple correlation assay ($r = 0.06$ n.s, $p<0.05$). However, in absence of S_9 mix, the induction factor value at 50µg/ml was 1.57. This value can be clasified like doubtful according to the established criterions by Kevekordes *et al*, 1999.

Comet assay.

Obtained results are showed in Fig. 3. Only at the concentration of 100µg/ml in presence of metabolic fraction and 200µg/ml as in presence as in absence of metabolic fraction, the ExM induced significant differences DNA primary damage in human blood peripheral cells ($p< 0.05$, Dunnett test).

Discussion

In this study a cytotoxic effect of ExM at the concentration of 200µg/ml was detected under our experimental conditions. In the cell survival assay the same survival percent was obtained and it was significantly different ($p< 0.05$ Student t test) respect to the negative control value at the concentration of 200µg/ml either in presence or in absence of metabolic activation. An explaination to these results could be that in presence of S9 reactive metabolites capable of DNA damage do not occur in our experimental conditions. On the

other hand, this significant inhibition of the survival percent of *E. coli* PQ37 cells observed at the concentration of 200µg/ml might not be caused by the antimicrobial activity reported for genus *Erythroxylum* (Álvarez *et al*, 2005), because the results obtained by these authors show that this genus does not have bactericide action versus gram-negative microorganisms like the case of *E. coli* PQ37.

The main components of this mixture are poliphenols like quercetin, kaemferol and ombuin (González *et al*, 2006), and they can act on the cell viability according to different authors (Soares *et al*, 2006). An explaination to our results could be that at the concentration of 200µg/ml any compound of the mixture reached a threshold level is able to interfere with protein synthesis like showed the alkaline phosphatasa results, although they were not statistically significant. These results are consistent with previous reports of flavonoids that have showed cytotoxic potential due to their activity like enzyme inhibitors that participates in replication, transcription and cell expression (Vijayababu *et al*, 2006).

Besides some of poliphenols present in the extract could bind to important macromolecules in the prokariotic cells, making difficult their functions or leading them to desintegration (Kaldas *et al*, 2005).

Conversely, the DNA damage potential of the extract was studied using a modified protocol of the SOS Chromotest and the Comet assay. The bacterial SOS Chromotest with *E. coli* is widely used as a genotoxicity screen. The results obtained in this assay can be compared with those obtained in the Ames test, but the ability of the latter to identify carcinogens is much higher than that of the SOS Chromotest.The SOS Chromotest is based on SOS response induction, which is activated in the presence of DNA damaged and leads to replication blockage (Janion, 2001).

After treatment of bacterial strain PQ37 it was observed that ExM was unable to induce SOS function, since a 2-fold increase in the induction factor (IF) was not observed with respect to the negative control sample, neither correlation was positive, which indicate no genotoxicity according to the established for the natural products evaluation using this assay (Kevekordes *et al*, 1999). However, it is interesting the fact that at concentration of 50µg/ml in absence of metabolic activation, the IF was 1.57, a value considered doubtful in bacterial assays

(Kevekordes *et al*, 1999).These results correlate with those obtained by Vernhes, 2003, which evaluated a *Pinus caribaea* Morelet extract with a high content of tanines using this assay, although this author obtained a doubtful IF value at a more high concentration.

The DNA damage observed by the extract in the Comet assay could be attributed to it phytochemistry composition. The Comet assay was used to determine DNA damage measured as strand breaks and alkali-labile sites on blood peripheral lymphocytes. This assay is based on the individual cells immobilization in agarose gel, their lysis and later electrophoresis of nuclear material. The Comet assay is also widely used as a genotoxicity screen, due to its sensibility, rapid realization, flexibility, low cost and because its application is possible in any nuclei cells population.

It is known that many flavonoids have shown to be genotoxic in a variety of prokariotic and eukariotic cells and in vivo systems (Walle *et al*, 2001; Labiniec *et al*, 2003). The mechanistic basis for this genotoxity has not been fully elucidated, although structure-activity relationship studies have identified as a requisite the flavonoid structural features.

On the other hand, the probably cause of mutagenic effects observed in the Comet assay after exposition to the extract is the formation of ROS associated with the presence of flavonoids inside cells(Labiniec *et al*, 2003). Inside cells phenolic acids molecules (for example) bind with protein and lipids, and they become easily oxidised by O_2, O_2^- or H_2O_2. Free oxygen radicals can be generated during reactions between polyphenol-protein (lipid) complex and oxygen. These radicals may interact with DNA and induce a broad spectrum of DNA damage. It has been demonstrated that oxygen radicals can be generated by the reaction of a phenolic structure in the presence of oxygen (Bors *et al*, 1999).

Differences observed between genotoxicity results obtained in both assays could be due to the different biological models used. No genotoxicity observed in *E. coli* cells at the concentration of 200µg/ml could be related to the cytotoxicity observed at this same concentration.

Future bioassays of fractionation of the etanolic extract of ExM will be necessary to know the components responsible of the cytotoxicity and genotoxicity detected in this study. It is the first report about cytotoxic and genotoxic action of an etanolic extract of ExM. Meanwhile,

other toxicological assays must be performed in different models for a possible use like a terapheutic agent.

Conclusions

In our experimental conditions, the etanolic extract of *Erythroxylum minutifolium* (ExM) was not genotoxic in the *E. coli* PQ37 cells at neither of the evaluated concentrations using the SOS Chromotest assay, however it was able to produce DNA primary damage at the concentrations of 100 and 200ug/ml in human blood peripheral cells throughout the Comet assay.

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Table I. Inhibition percent (%) of the protein synthesis of *E. coli* PQ-37 cells treated with ExM. Data represents the mean ± S.E. of three independent experiments. *p < 0.05 respect to control cells.

Concentration (μg/ml)	X ± S.E. withouth S9	X ± S.E. with S9
0	9.75 ± 2.12	7.96 ± 2.02
50	9.08 ± 2.41	7.98 ± 1.64
100	9.59 ± 1.70	8.18 ± 2.00
200	4.73 ± 0.65	6.36 ± 1.53

Figure 1. Survival percent (%) of *E. coli* PQ-37 cells treated with ExM. Data represents the mean ± S.D. of three independent experiments. *p < 0.05 respect to control cells.

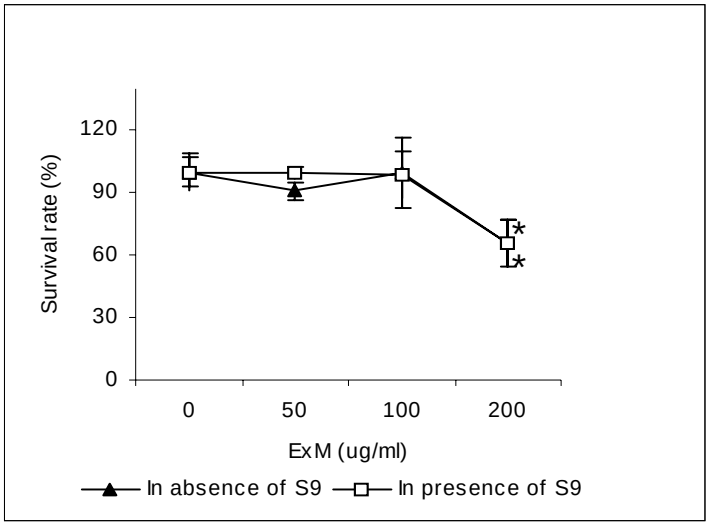


Figure 2. Study of genotoxic potential of *Erytrhoxylum minutifolium* extract. Positive controls used were, in absence of S9: 150 Gy and in presence of S9: Bleomicin 2mg/ml. Data represents the mean ± S.D. of three independent experiments. *p < 0.05 respect to control cells.

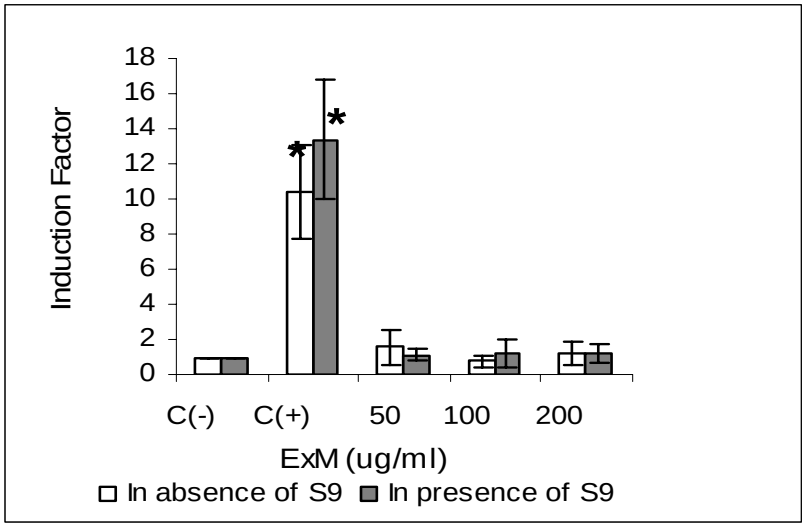
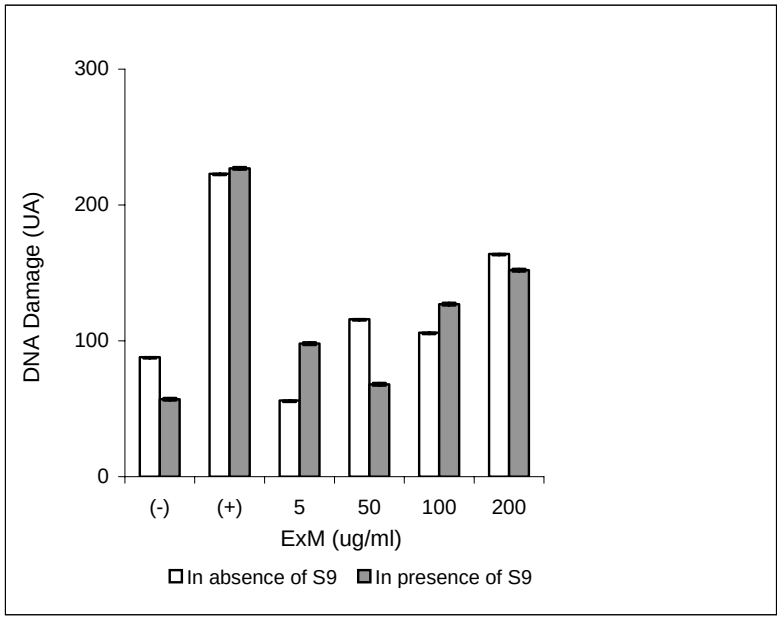


Figure 3. Study of genotoxic potencial of *Erytrhoxylum minutifolium* extract. Positive controls used were, in absence of S9: 150 Gy and in presence of S9: Bleomicin 2mg/ml. Data represents the mean ± S.D. of damage cells of three independent experiments, 100 cells were analyzed by slide in three independent experiments. *p < 0.05 respect to control cells.



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