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Genotoxic Potentials of Natural Products Detected by a Short-Term Test Using Diploid Strains of *Aspergillus nidulans*

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Abstract: Main aim is to evaluate the genotoxic potentials of three classes of natural products, a sesquiterpene lactone (cynaropicrin), a saponin (stigmast-7en-3β-ol-glucopyranoside) and a steroid (stigmasterol), at low doses, using the Homozygotization Index test (HI). This short-term test, deals with the determination of alterations in mitotic crossing-over frequencies eventually involving heterozygous auxotrophic genes in diploid strains of *Aspergillus nidulans*. In this way, treatments of UT448//UT184 and *Dp* II-I//UT184 diploid strains with the natural products showed that saponin and lactone significantly increased the homozygotization index (HI) of both strains, especially for the diploid Dp II-I//UT184. No effect was observed with the steroid. On this account it presents remarkable potentials to detect the genotoxic/carcinogenic effects of bioactive natural products. Furthermore, this is the first time the low doses effects of bioactive natural products were detected using *A. nidulans*.

Keywords: Natural products, Sesquiterpene lactone (cynaropicrin), Saponin (stigmast-7en-3β-ol-glucopyranoside), Steroid (stigmasterol), Genotoxic potential, Mitotic crossing-over, *Aspergillus nidulans* short-term test.

INTRODUCTION

Even in the lack of adequate scientific basis, natural products have long been used in human therapeutics. Increased use of plant extracts by the pharmaceutical industry and the appearance of so-called nutraceutic foods indicate the need for systematic studies on medicinal plants mainly to evidence eventual hazardous effects, and to disclose efficient methods involved into such aimed at detection.

Plants provide an extraordinary reservoir of new molecules. Chemical compounds commonly isolated from plant species include steroids, terpenes, flavonoids, lactones, saponins, alkaloids, etc. These compounds are biosynthesized by the plant as a primary defense against their potential enemies, as bacteria, fungi, insects and other predator animals. Consequently they show a variety of biological effects, as anti-inflammatory [1], trypanocidal [2], antibacterial [3, 4], and anti-arteriosclerotic properties [5]. In addition, either direct, or indirectly they often integrate the composition of human daily food stuffs [5, 6]. Therefore, studies to evidence chemical, toxicological and pharmacological properties of plant products (extracts, fractions and pure substances) are under urgent needs and should be strongly encouraged.

Evidently, before using a given product a crucial aspect should rely on its safe use. For such, determination of genotoxic potentials and allied genotoxicity interval [7] under submutagenic doses is fundamental for searching new safe drugs and medicines originating from plant products. In addition, mitotic recombination is a process participating in numerous important biological events, such as DNA repair mechanisms [8], human genetic diseases, and cancer risks [9, 10]. It also plays a role in the rearrangements occurring during immunoglobulin synthesis and it possibly also responds for the integration of the exogenous genetic material during cell transformation processes [9].

Physical and chemical agents can enhance DNA damage and induce mitotic crossing-over. Lesions caused by these agents can induce mitotic recombination that, on its turn, can indicate DNA damage [11]. On account of its well-understood classical genetics background the filamentous fungus *Aspergillus nidulans* is the ideal organism to provide information on mitotic recombination and related aspects. Because occurrence of diploid cells, mitotic recombination and haploidization take place during fungal parasexual and sexual cycles [12], produced haploid cells express the mitotic rearrangements occurring during the diploid phase. So, they can be used to determine the genotoxicity of a substance. On such grounds, Pires and Zucchi [13], developed a short-term test (Homozygotization Index, HI) that uses two *A. nidulans* diploid strains: UT184//UT448, with normal DNA repair mechanisms; and *Dp* II-I//UT184, presenting recombinational repair mechanisms, only. Both diploids bear several genetic markers scattered all over the eight fungal chromosomes, and the strain *Dp* II-I presents high sensibility

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for any kind of DNA damage, as reflected by increased recombination frequencies. Furthermore, Zucchi *et al*. [14] showed that mentioned short-term test was sensible enough to detect DNA alterations even after using submutagenic doses of a putative genotoxic compound.

Therefore, aim of present study was to evaluate natural plant products eventual genotoxicity by determining the frequency of mitotic crossing-over alterations in diploid strains of *A. nidulans*. The following three metabolite classes were studied: the sesquiterpene lactone (cynaropicrin), the saponin (stigmast-7en-3β-ol-glucopyranoside) and the steroid (stigmasterol).

MATERIALS AND METHODS

Haploid and diploid *Aspergillus nidulans* Strains

Aspergillus nidulans haploid strains were obtained from Utrecht (Holland) stocks (UT448 and UT184) and have been described by Zucchi [15]. The strain (*Dp* II-I) was derived from UT448 after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment [16-18]. Partial maps of haploid strains are shown in Clutterbuck [19] and Pires and Zucchi [20]. The mutant *loci* are as described: UT448- *w*A2 (II), white conidia; *ribo*A1, *paba*A124, biA1 (I), with requirements for riboflavine, *p*-aminobenzoic acid and biotine, respectively; *Acr*A1 (II), resistant to acriflavine. UT184- *cha*A1 (VIII), “chartreuse” conidia; *pyro*A4 (IV); *s*B3 (VI); *nic*B8 (VII); *ribo*B2 (VIII), with requirements for pyridoxine, sodium thiosulphate, nicotinamyde and riboflavin, respectively; *gal*A1 (III); *fac*A303 (V); *lac*A1 (VI), unable to utilize galactose, acetate and lactose as the sole carbon source, respectively; *sul*A1 (I); *Acr*A1 (II), resistant to sulphanylamide and acriflavine, respectively. *Dp* II-I- *w*A2 (II), white conidia; *ribo*A1, *paba*A124, *bio*A1 (I), with requirements for riboflavin, p-aminobenzoic acid and biotin, respectively; *Acr*A1 (II), resistant to acriflavine; *uvs* (II), sensitive to UV-light [21]; *Dp* II-I, duplication of a segment from chromosome II transposed to chromosome I, inserted into *paba-y* genetic interval [15-18].

The UT448//UT184 and *Dp* II-I//UT184 diploid strains were prepared following Roper’s methodology [12]. The mutant alleles were allocated to their linkage groups by mitotic haploidization [22] facilitated by treatment with *p*-fluorophenylalanine (*p*FA) [23, 24].

Media and Culture Conditions

Complete medium (CM) and minimal medium (MM) were as described by Van de Vate and Jansen [25]. CM was used to maintain stock cultures. Selective medium (SM) was MM supplemented according to the requirements of each strain. Solid medium contained 1.2% agar. The strains were incubated at 37°C and kept at 4 °C.

Plant Material

Cynaropicrin (sesquiterpene lactone-guaianolide) [26] was isolated from *Moquinia kingii* (H. Robinson) Gamerro (Vernonieae, Asteraceae) [27, 28]. Stigmast-7en-3β-ol-glucopyranoside (saponin) and stigmasterol (steroid) [29] were isolated from *Alternanthera maritima* (Gomphreneae, Amaranthaceae) [30-31]. The compounds were isolated and purified by chromatographic methods and the structures were characterized using spectroscopic methods (nuclear magnetic resonance, ^{1}H and ^{13}C NMR and electrospray mass spectrum, ESI-MS). In previous study this compounds showed *in vitro* antimicrobial and trypanocidal activity against human pathogenic microorganisms [27, 30]. The isolated compounds were dissolved in propylene glycol (Merck) and diluted in MM for analysis in genotoxic assay.

The Homozygotization Index (HI) Short-Term Test [13]

Mitotic crossing-over promotes homozygotization of genes present in heterozygous condition in diploids and, if the treatment induces mitotic crossing-over in the heterozygous diploids, +//+, +//-, -//+ and -//-, diploids are formed irrespective of each marker. Since the treatment is applied in MM, only heterozygous (+//- and -//+) or homozygous (+//+) diploids can develop. After haploidization when no mitotic crossing-over occurs, the ratio of haploid segregants is 4(+) : 4(-). As described above, selection against recessive homozygous classes after recombination between any marker and centromere reveals that HI, (i.e. the ratio between percentages of + and – segregants colonies) is changed to 2.0, instead of expected 1.0, corresponding to 4(+) : 2(-) and 4(+) : 4(-) haploid segregant colonies, respectively. Therefore an HI value higher than 2.0 indicates the occurrence of more than one mitotic crossing-over event for the given marker. Conversely, an HI value lower than 2.0 indicates, either the absence of crossing-over for the marker, or that it was not selected on MM, e.g., markers for conidia color.

Following a method recently devised for *A. nidulans* genetic analyses, or Delphi 4 (PLACAS, Copyright 2000 cbonato), results from genetic mitotic analyses were presented into Latin Square Tables Format (not shown). HI results estimated from those data presented in Latin Square Tables and were analysed by cluster analysis, principal component, and a modification of the χ^2 test known as a contingency table with 1% significance.

Assays Carried Out with Conidia Obtained from Each Diploid *Aspergillus nidulans* Strain (UT448//UT184 and *Dp* II-I //UT184)

Since preliminary trials have shown that 50 µg/mL drug concentration inhibited *A. nidulans* development (data not shown), 25 µg/mL concentration was used for the three compounds in the subsequent experiments. This concentration was established as a parameter to compare the genotoxic potential of the tested drugs, and to evaluate the methods response to different chemical skeletons. Propylene glycol (2.5 µL propylene glycol/mL MM, final concentration 0.025%) was used as solvent and negative control. Stock solutions of each test drug were prepared in propylene glycol and aliquots (2.5µL/mL MM) were mixed with MM at 50°C and poured into Petri dishes (final concentration of the test drugs, 25 µg/mL). After incubation, conidia of the diploid strains UT448//UT184 and *Dp* II-I//UT184 were inoculated at four equidistant inoculation points and the plates were incubated at 37°C for 7 days. Various still diploid sectors that supposedly suffered mitotic crossing-over were observed. For each tested drug, and from each strain, six sectors of which morphology differed from the original diploid strain (green) were obtained.

Subsequently, the mitotic analysis was carried out. For haploidization induction, each of the six isolated sectors was

inoculated onto 10 CM+*p*FA plates (at 4 equidistant points). After 5 days of incubation at 37°C, formation of likely haploid sectors was observed: either conidia showing same color as the parental ("chartreuse" and white) or, recombinant strain (green). Each *Dp* II-I//UT184 colony originated approximately 8 haploid sectors per plate, whereas the UT448//UT184 colonies originated a mean of 6 haploid sectors per plate. Conidia from the edge of each diploid sector, is isolated in order to be phenotypically analyzed. Purified sectors were individually inoculated at 25 defined positions (5 x 5 patterns) on a CM plate (master plate) and incubated at 37°C for 48 h. Colonies from Master Plates were replicated to SM plates using a multi-wired replicator. Colonies development was recorded after 24, 48 and 72 h at 37°C.

RESULTS

Mitotic Analyses of Untreated UT448/UT184 and *Dp* II-I//UT184 Strains

This kind of analyses allowed observing the segregation of the genetic markers in all the eight fungal chromosomes altogether. During mitotic divisions a typical Mendelian segregation (1+ : 1-) is usually reported for the involved genetic markers. Genetic disturbances on this typical behavior can be attributed to the effects of the chemicals used under low doses, and allied selection against homozygous recombinant diploids for the recessive alleles, in MM. So, distortions in the allelic segregation rates indicate efficiency of the genotoxic tested drug as inducing DNA recombinogenic lesions, and subsequent HI increase. Mitotic analyses of untreated strains are reported in Table **1**.

Diploid sectors analyses were performed independently. Table **1A** encompasses analyzed data of all UT448//UT184 altogether. The used propylene glycol as diluent at 2.5 µL/mL MM, final concentration of 0.025%, did not alter the frequencies of mitotic crossing-over either in UT448//UT184, or *Dp* II-I//UT184 (Table **1**). In the negative control experiment (diluent solution alone) diploids present a subtle allelic segregation rates distortion (different from 1:1), indicating that the allelic segregation rate results from DNA damage originating from the proper self-replication process. Since this diploid presents normal DNA repair mechanisms, such minor alterations were hardly noticed. In similar proportions, the segregation rate alterations affect the pattern of all focused gene markers scattered all over the eight fungal chromosomes [13]. In the untreated diploid strain *Dp* II-I//UT184 it can be seen that significant allelic segregation rate distortions arise due to low frequency of auxotrophic haploid alleles (Table **1B**) in MM. Even in the absence of any recombinogenic specific inducer such results evidence that this diploid presents constitutive recombination potentials.

Table 1. Mean Homozygotization Index (HI) Obtained for the Several Genetic Markers of the Diploid *Aspergillus nidulans* Strains UT448//UT184 (A) and *Dp* II-I//UT184 (B) Exposed to Lactone, Saponin and Steroid at a Concentration of 25 µg/mL and Negative Experimental and Literature Controls

(A)

Linkage Group	I	I	II	IV	VI	VII	V	III	I	VIII	Numbers of Tested Colonies	
Genetic Markers	***paba***	***bi***	***w***	***pyro***	***lac***	***nic***	***fac***	***gal***	***ribo***	***cha***	**Diploid**	**Haploid**
Literature positive control* (80 s UV)	13.3	11.4	2.1	15.8	20.2	13.4	29.4	-	-	-	6	241
Literature negative control* (0 s UV)	2.5	2.7	0.8	3.0	1.5	1.2	2.5	-	-	-	7	262
Experimental negative control	2.0a	1.7a	1.0a	0.8a	0.8a	0.3a	0.3a	1.7a	1.5a	2.0a	6	175
Lactone	8.9b	1.4a	2.1b	2.0a	1.3a	0.8a	0.6a	2.0a	2.0a	2.4a	6	149
Saponin	86.5b	18.4b	0.43b	42.8b	24.0b	34.0b	1.9b	0.5b	2.3a	2.1a	6	175
Steroid	1.8a	0.5a	0.8a	1.9a	2.1a	2.3b	1.3b	0.3b	1.1a	1.1a	6	150

(B)

Linkage Group	I	I	II	IV	VI	VII	V	III	I	VIII	Numbers of Tested Colonies	
Genetic markers	***paba***	***bi***	***w***	***pyro***	***lac***	***nic***	***fac***	***gal***	***ribo***	***cha***	**Diploid**	**Haploid**
Literature positive control* (80 s UV)	35.7	37.6	2.5	37.6	29.7	27.0	26.0	-	-	-	6	236
Literature negative control* (0 s UV)	21.8	21.7	1.2	21.7	11.7	18.2	18.2	-	-	-	9	184
Experimental negative control	18.4a	1.9a	2.1a	10.7a	18.4a	14.9a	3.7a	1.6a	2.8a	1.7a	6	175
Lactone	87.0b	1.6a	2.4a	86.5b	174b	86.5b	18.4b	1.5a	0.5b	0.7b	6	175
Saponin	149b	7.3b	0.5b	149.0b	36.5b	150a	3.8a	2.5a	2.8a	0.7b	6	150
Steroid	13.4a	1.1a	2.8a	9.0a	4.6a	3.4b	2.4a	0.9a	2.6a	0.7b	6	150

- not done.
*PIRES and ZUCCHI, 1994.
Note: same letter, X^2 non-significant differences. Contingency table corrected after Yates at 1% (p<0.01).

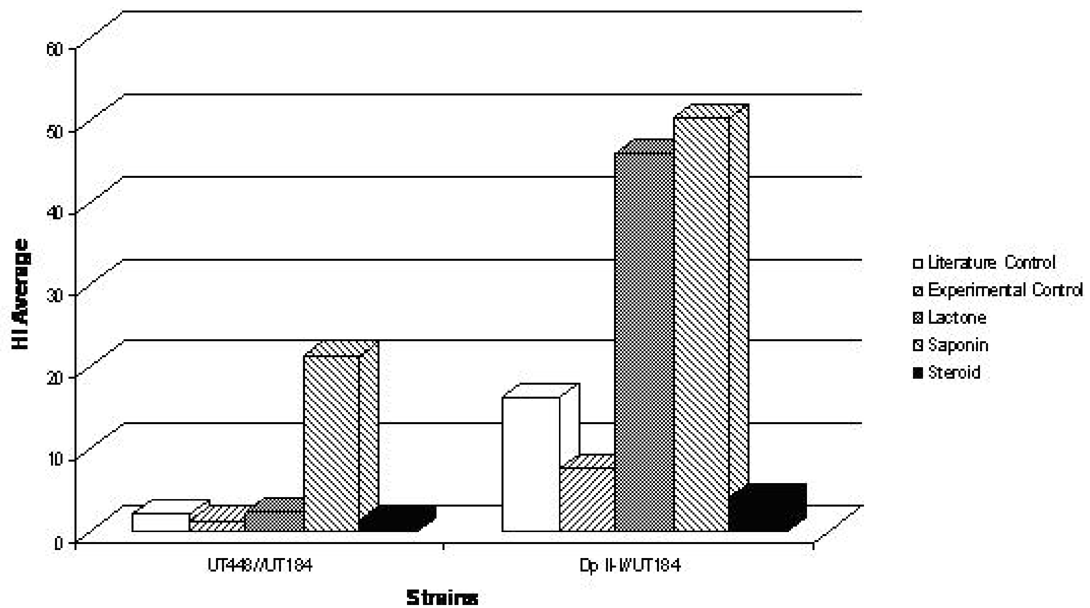

Fig. (1). Homozygotization Index (HI) average in UT448//UT184 e *D*p II-I//UT184 diploids. Note: HI average values correspond to those presented in Tables **1A** and **1B**.

Natural Products Genotoxic Effects

The genotoxic effects of natural products (lactone, saponin and steroid) have been evaluated (Table **1** and Figs. (**1**) and (**2**) in both used diploid strains (UT448//UT184 and *Dp* II-I//UT184). Conidial induction of mitotic recombination showed mean HI differences in the haploid UT448//UT184 cells exposed to the different test drugs at a concentration of 25µg/mL. Such cells showed a variable, undefined response pattern (Table **1A**).

In the case of lactone, eight markers (exceptions *paba* and *cha* markers) among of ten showed HI ≤ 2, while HI ≤ 2 was observed for all markers in the case of the steroid. For the saponin, HI observed for the markers *paba*, *bi*, *pyro*, *lac* and *nic* was higher than that observed for the control (HI > 2), suggesting that for these markers more than one recombinational event occurs.

For the *Dp* II-I//UT184, saponin and lactone increased the mean HI of five among ten analyzed genetic as markers compared to controls (except for *bi* in the case of lactone of which obtained value was lower than the control), indicating a possible recombinational event (Table **1B**). Fig. (**1**) shows average HI values for all studied nutritional markers. In-

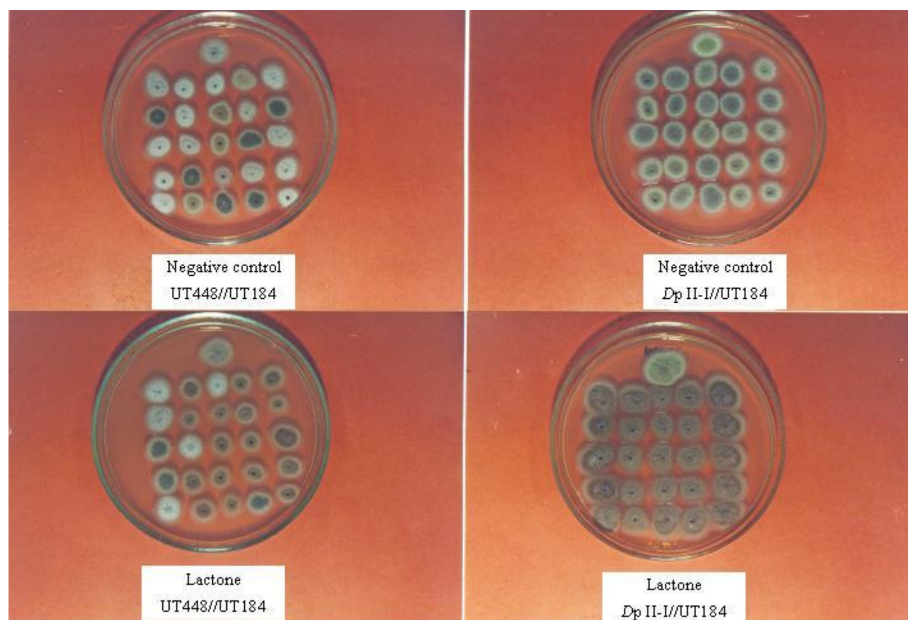

Fig. (2). Master plate containing 25 colonies of segregants growing on CM for experimental negative control and treated with lactone (25µg/mL) for two strains of *A. nidulans* (UT448//UT184 e *D*p II-I//UT184) used.

creased distortions occurring after several treatments are easily seen.

For the steroid, all markers showed HI values similar to or lower than those obtained for the control, indicating the absence of recombinational events. In both strains HI values obtained for the *w*, *fac*, *gal*, *ribo* and *cha* markers of both strains were equal to or lower than the control value, with the *w* marker (color marker) being not selectable on MM. The two studied strains are known to differ in their genetic characteristics [13]. The UT448//UT184 strain is control normal for all known repair mechanisms, while the *Dp* II-I//UT184 strain is deficient in photoreactivation and excision repair, with recombination being the main form of DNA damage repair. Since HI exactly expresses recombinational events occurring during mitosis, one can assume that drugs inducing HI higher than the control possess genotoxic and recombinogenic potential.

Statistical Analysis

Statistical analysis [4, 32], cluster analysis and principal component (Fig. **3**) were performed to determine similarity degree in terms of genotoxic potential for the tested drugs and for the experimental control and their distribution between the two strains. The isolated compounds were used as variables, and genetic marker (*paba, bi, w, pyro, lac, nic,* and *ac*) as characters.

For the UT448//UT184 strain, the experimental control, lactone and steroid formed one group and saponin another group up to 65% of the mean Euclidean distance (ED). Based on the principal component analysis, traits responsible for this distribution were: X axis, the genetic marker *nic* (λ= 6.89230; 68.92%); Y axis, the genetic marker *cha* (λ= 2.75960; 27.60%); Z axis, the genetic marker *w* (λ= 0.34810; 3.48%). This analysis mainly indicates that the lactone and steroid did not induce recombination events since they formed a group similar to the negative control, whereas an increased number of recombination events were observed for saponin, which therefore constituted a separate group.

For the *Dp* II-I//UT184 strain, the control and steroid formed one group up to 65% of the mean ED, followed by the lactone (85.78% ED), which formed a group with the two first substances and saponin. The traits responsible for this distribution as determined by analysis of the main components were: X axis, the genetic marker *paba* (λ= 5.71232; 57.12%); Y axis, the genetic marker *paba* (λ= 3.41744; 34.17%); Z axis, the genetic marker *cha* (λ= 0.87024; 8.70%). These results indicate a steroid behavior similar to the control, suggesting that the steroid did not induce any recombinational event; while lactone and saponin formed groups separate from the control and steroid, indicating a genotoxic and recombinogenic potential for the former.

DISCUSSION

Human diet usually contains about 200 to 300 mg/day of steroids [33]. Plant materials and especially vegetable oils contain free, esterified and glycosylated steroids [34]. These compounds are incorporated by the cell membrane, playing an important role in the treatment of hypercholesterolemia. According to Piironen *et al.* [5], diets rich in these compounds are recommended. However, it should be noted that oral saponins ingestion is submitted to metabolization, undergoing hydrolysis, resulting into free aglycon (steroid) and sugar. Besides, the steroidal saponins shown surfactant prop-

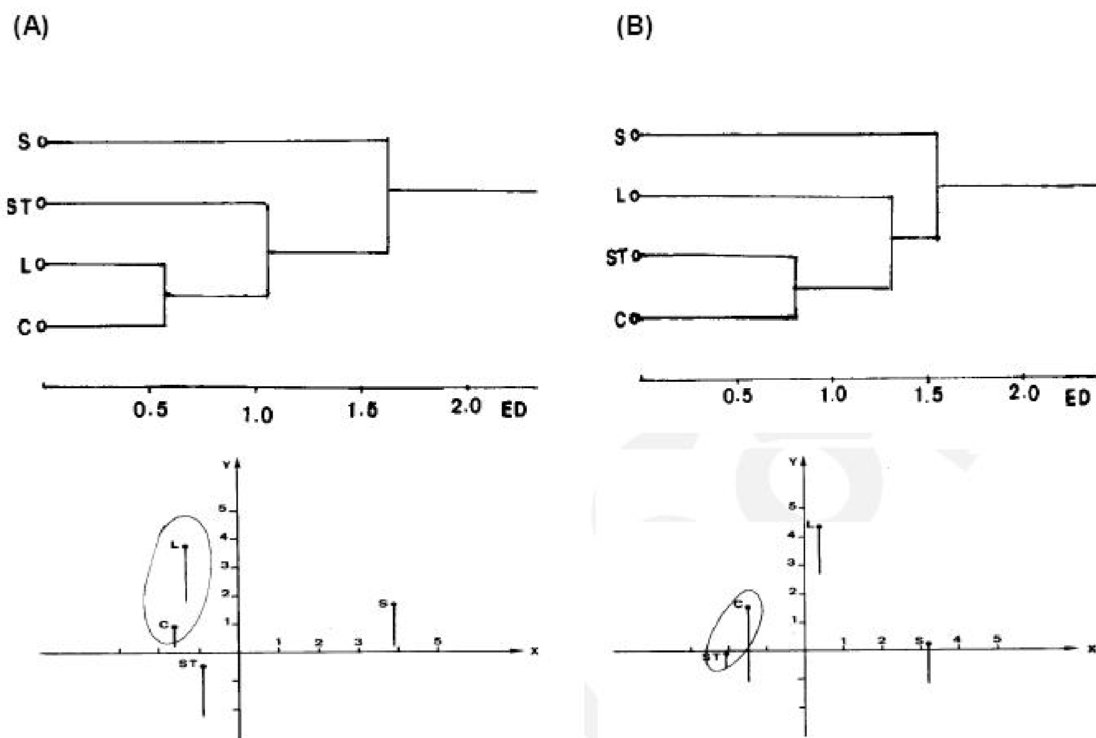

Fig. (3). Dendrogram and principal component analysis of the mean homozygotization indices (HI) obtained for the different genetic markers of the diploid *Aspergillus nidulans* strains (**A**) UT448//184 and (**B**) *Dp* II-I//UT184 induced by (L) lactone, (S) saponin and (ST) steroid (25µg/mL) and (C) negative control.

erties and induce hemolysis by erythrocytic membrane instability [35].

Lactones are terpenoids consisting of various skeletons are frequently present in species of the family Asteraceae. Studies determining *in vitro* genotoxic activity of lactones on human lymphocytes showed that skeletons such as erematholide-C and 15-deoxygoyazensolide presented no genotoxic effects [36]. Similar results were obtained for pseudoguaianolide, goyazensolide [37] and eremanthine [38]. However, according to Burim *et al.* [39], glaucolide Bt presents no clastogenic action on *in vivo* mammalian cells but, on the other hand, it showed *in vitro* cytotoxic and clastogenic effects.

Since there are considerable dispute about natural products genotoxicity (mainly due to a lack of adequate methodologies), a short-term test which assesses specifically for genotoxic events could play a crucial role in related matters [13, 14]. In this ways, used *A. nidulans* versatile eukaryotic *in vivo* system, was useful to clarify the biological action of eventual genotoxic compounds, and to help understanding the possible modulation of its action by a holistic interaction of many cellular systems. Of course, it seems likely that there are limitations towards using *A. nidulans* as a mammal surrogate due to differences in the molecular environment, and to the more complex genetic interactions in mammals.

Comparing obtained results for the two studied strains, the steroid did not induce recombinational alterations in both strains, while saponin led to significant HI increase as compared to the control despite presence of normal repair mechanisms in the UT448//UT184 strain. Lactone showed an interesting profile, with no recombinational activity detected in the UT448//UT184 strain, while in the *Dp* II-I//UT184 strain recombinogenic potential was observed in five of the analyzed markers (Table **1B**).

Furthermore, because tested drugs at a concentration of 25 μg/mL exerted different effects on the ten analyzed genetic markers, used methodology permits detecting the genotoxic potential of plant secondary metabolites using even small samples (10 mg). Despite such trends present short-term test has been used to disclose suspected genotoxicity rapidly and efficiently. Besides, the test is very sensitive as genotoxicity can be detected even after application of low doses of a given agent, such as UV [13], benzene fumes [14], sodium nitroprusside [40], benznidazole [41], cisplatin and cytosine arabinoside [42] and X-ray [43]. These characters altogether allied to test inexpensiveness, suggest involved eukaryote microorganism (*A. nidulans*) can be proposed as a cell model to guide early approaches towards rapid screening of genotoxic potential and biological activities of many chemicals.

In conclusion, above results and comments suggest that employed short-term test used bears remarkable potentials to evaluate the genotoxic/carcinogenic effects of bioactive natural products, since needed HI can be easily calculated for any heterozygous marker of *A. nidulans* diploid strains.

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