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In vitro evalutation of cytotoxic, genotoxic and apoptotic properties of herbal products from leaves of *Gymnema sylvestre*

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Abstract
Gymnema sylvestre is a woody vine common in tropical areas of Asia, Africa and Australia and it is believed that this plant possesses antiviral, antibacterial, anti-allergic, anti-oxidants and hypoglycemic properties. Many studies conducted on this plant have actually shown that it has remarkable hypoglycemic and antioxidants properties. However, data in the scientific literature are scarce to definitively assess its safety for humans. The purpose of this study was to evaluate *in vitro*, in human-derived cancer cells (HepG2 and K562), the cytotoxic, genotoxic and pro-apoptotic activities of three extracts of *Gymnema sylvestre*. The cytotoxicity was assessed by the lactate dehydrogenase assay (LDH assay), genotoxicity was evaluated by the comet assay and the ability to induce apoptosis was assessed by the mitochondrial potential assay and the DNA fragmentation assay. *Gymnema sylvestre* was found to induce a strong hyperpolarization of mitochondrial membrane in both cell lines; furthermore, *Gymnema sylvestre* caused DNA damage in HepG2 cells.

Keywords: *Gymnema sylvestre*; Safety assessment; Herbal product; Genotoxicity; Mitochondrial potential, DNA fragmentation

1. Introduction
Gymnema sylvestre is a woody vine plant belonging to the subfamily of *Asclepiadaceae*, widespread in tropical areas of Asia, Africa and Australia. In Ayurvedic medicine the leaves and the dried roots of this plant are used because it is believed that they possess antiviral, diuretic, digestive, anti-allergic, hypoglycemic and hypolipidemic properties [1]. The major constituents of *Gymnema sylvestre* are represented by 9 different gymnemic acids, saponins of gymnemic acids (gymnemasin A, B, C and D), gymnemagenins, gurmarin, condurithol A (tetraoxicycloexene), some phytosterols and some pectins [2]. The hypoglycaemic and antioxidant activity of *Gymnema sylvestre* results from different molecules that take place in different levels [3]. Glucidic molecules of *Gymnema sylvestre* have been reported to be able to reduce the absorption of glucose in intestine due by competition processes [4]. It has also been observed that this plant is able to improve glycemic control, by promoting the insulin's release by the islets of Langerhans [5, 6]. In streptozotocin induced diabetic rats the administration of *Gymnema sylvestre* leaves ethanol extract has led to an increase of 30% of the total weight of pancreas, of the number of the islets and the number of cells per islet, with the consequent recovery of blood glucose level control [7]. Furthermore, radical scavenging activity and cytoprotective effect of *Gymnema sylvestre*have been evaluated with reported positive effects of ethanolic leaves extracts on lesions of the gastric mucosa of rats, associated to a decrease of gastric secretion's volume and of acidity in a concentration-dependent relationship [8]. As regards the safety for humans of *Gymnema sylvestre*, currently it is reported in literature, only a single case of hepatotoxicity induced by the administration of this plant in a 60 years old patient with type 2 diabetes [9]. The aim of the present study was to evaluate *in vitro* the cytotoxic, genotoxic and pro-apoptotic properties of three different extracts of *Gymnema sylvestre* leaves purchased by herbalists shops in Italy.

2. Material and methods.
2.1 Gymnema sylvestre extracts
Herbal products containing *Gymnema sylvestre*were purchased from herbalist's shops in Italy. Two samples (samples A and B) consisted in capsules containing *Gymnema sylvestre* leaves dry extract and leaves powder, whereas a third sample (sample C) consisted of dried *Gymnema sylvestre* leaves (raw material) that was subjected to lyophilization; briefly, dried cutted leaves

were subjected to extraction with ethanol 70% (v/v) for 6 –8 h, at 50°C. After removal of exhausted herb by filtration, the extract volume was reduced under vacuum and finally subjected to freeze-drying for 72 hours. For active principles dissolution, samples were extracted following the protocol of the standardized approach NICEATM [10].For all the samples of *Gymnema sylvestre* the complete extraction was obtained in DMSO at a concentration of 150 mg/ml; coarse and insoluble parts were removed by centrifugation.

2.2 Cell lines

For testing *Gymnema sylvestre,* two different cell lines were used: HepG2 and K562.HepG2 cell line originates from a human tumor cell clone isolated in 1975 from the liver of a 15 years-old Argentine boy with hepatocellular carcinoma diagnosis [11, 12]. HepG2 cells grow forming epithelial monolayer and are maintained in culture at 37 °C in a humidified atmosphere and 5% CO_2 on MEM medium (Minimum Essential Medium) supplemented with Earle's salts, inactivated FBS (fetal bovine serum), (10%), l-glutamine (2mM) and antibiotics (penicillin 100 µg/ml, streptomycin 100 µg/ml). This cell line has retained most of the morphological characteristics of hepatic parenchyma cells and it has maintained the main phase I and II enzyme activities involved in the activation and/or detoxification of xenobiotics [13, 14]. K562 cell line originates from a human tumor cell clone isolated in 1979 from the bone marrow of a 53 years-old woman suffering from chronic myelogenous leukemia [15] and grow in suspension in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with inactivated FBS (10%), l-glutamine (2 mM) and antibiotics (penicillin 100 µg/ml and streptomycin 100 µg/ml) in flasks from 75 cm^2, at 37° C in a humidified atmosphere of 5% CO_2.

2.3 Cell treatments

The same protocols were applied to treatment of both HepG2 and K562 cells, with minor procedural differences due to the different growth pattern (adhesion and suspension). For cytotoxicity testing, from the exponential growth phase, cells were sub-cultured in 96 well plates (2.5×10^4 cells/well, assay volume 200 µl) in culture medium with reduced FBS concentration (1 %, to avoid interference with reagents used in the assay). After overnight incubation, cells were exposed for 24 hours with 5 serial dilutions of 3 samples under examination. The highest tested concentration was 1.5 mg/ml which represents the maximum analyzable quantities to avoid false positives due to the presence of DMSO higher than 1%. Positive control (Triton X-100 1%), a solvent control (DMSO 1%) and a negative control (culture medium) were also set up. For genotoxicity testing, cells in exponential growth phase were seeded in 6-well plates (5×10^5 cells/well, assay volume 5 ml) in complete medium. The cells were cultured in plates for 48 hours and then treated for 4 hours with samples. Five scalar concentrations of the three samples were tested. Positives control (1 µM 4-nitro-quinoline n-oxide (4NQO) for HepG2 and 100mM 1, 2, 4benzentriol (BT) for K526 cells), solvent control (1% DMSO) and a negative control (complete medium) were also set up. For the assessment of pro-apoptotic potential, cells were seeded in 6-well plates (1×10^6 cells/well, volume assay 5 ml) in complete medium and, 48 hours later, the cells were treated for 4 and 24 hours with 5 scalar concentrations of the 3 extracts. Positive controls were also set up (0.5 µM valinomycin for mitochondrial potential; 1 µM staurosporine for DNA fragmentation); 1% DMSO served as solvent control and complete medium as negative control.

2.4 Cytotoxicity Testing

For the evaluation of cytotoxicity potential of *Gymnema sylvestre,* LDH assay was performed using a LDH assay kit (Cat. # MK401 - Takara Biochemicals, Japan), according to manufacturer's instructions. Briefly, at the end of treatments, plates were centrifuged for 10 minutes at 250×g and 100 µl of supernatants from each well were recovered. The recovered supernatants were incubated for 30 minutes at 37 °C in the dark with 100 µl of reaction mixture. The absorbance of formazan produced was measured spectrophoto metrically at 517 nm with the Sunrise™ microplate reader (Tecan Trading AG, Switzerland). The results were expressed as the percentage of cytotoxicity relating to the experimental data of LDH released from negative controls (untreated cells) and positive controls (cells treated with 1% Triton-X100), with ones released by the samples at different concentrations.

2.5 Genotoxicity Testing

Evaluation of genotoxicity was assessed by the Comet Assay. At the end of the treatments, cells were harvested from wells. HepG2 cells: cells were washed twice with PBS, and subjected to trypsinization for 5 minutes at 37 °C. Complete MEM was then added to each well to block trypsin's action. Cell suspensions were transferred to 1.5 ml microtubes and centrifuged at 1000×g. K526 cells: the entire volume of each well was transferred to a 15 ml tube and centrifuged at 1000×g. Each pellet was recovered, washed, resuspended in 1 ml of PSB, transferred in micro tubes and centrifuged at 67×g. Thereafter, HepG2 and K526 cells were processed in the comet assay under alkaline conditions (alkalineun winding/alkaline electro phoresis, pH>13), basically following the original procedure [16, 17]. Briefly, cells were embedded in agarose microgels and lysedinice-cold freshly prepared high-salt solution with detergents. The resulting nucleoids were subjected toelectro phoresisas already described elsewhere [18, 19]. For computerized evaluation of DNA damage, slides were examined by fluorescence microscopy using a specialized analysis system ("Comet Assay III", Perceptive Instruments, UK) [20, 21]. The extent of induced DNA damage was measured as the percent of fluorescence migrated in the comet tail (i.e., tail intensity), which is considered to be the most informative parameter [22]. One hundred cells (50 cells per duplicate slide) were analyzed for each experimental point.

2.6 Early apoptosis evaluation.

In order to evaluate the induction of early apoptosis, the mitochondrial potential assay was performed using the lipophilic cationic dye 1H-Benzimidazolium, 5,6-dichloro-2-[3-(5,6-dichloro-1,3-diethyl-1,3-dihydro-2H-benzimidazol-2-ylidene)-1-propenyl]-1,3-diethyl-, iodide (JC-1),that allows to distinguish healthy and apoptotic cells [23]. The assay was performed using a Nucleo Counter® NC-3000™ analysis system (Chemo Metec A/S, Denmark) according to the manufacturer's instructions. Briefly, after the treatment, cells were suspended in PBS in order to obtain an approximate final cell concentration of $1x10^6$ cells/ml. Subsequently samples were added with 12.5 µl of JC-1 (200 µg/ml) and incubated for 10 min at 37 °C. Cells were then washed twice in PBS, and finally pellets were resuspended in 0.25 ml of 4', 6-diamidino-2-phenylindole (DAPI) (1 µg/ml in PBS) and the samples were analyzed with the Nucleo Counter® NC-3000 ™ analysis system [19].

2.7 Late apoptosis evaluation.

The evaluation of late apoptosis was performed by the DNA

fragmentation assay. In late apoptosis DNA is degraded by nucleases [24, 25] and it is possible to determinate late apoptosis's phase, quantifying the residual DNA in cells [26]. The assay was performed using a Nucleo Counter® NC-3000 ™ analysis system according to the manufacturer's instructions. Briefly, cells were collected after the treatments and then they were washed and resuspended in 0.5 ml of PBS. The cell suspensions were then transferred into 15 ml centrifuge tubes and were added 4.5 ml of 70% ice-cold ethanol was added to each sample. Samples were vortexed vigorously and left at + 4 °C for at least 12 hours. After that, samples were centrifuged (500g for 5 minutes) and pellets were washed with 5 ml of PBS and then resuspended in 0.5 ml of DAPI solution (1 µg/ml and 0.1% Triton X-100 in PBS). Samples were then analyzed with the Nucleo Counter® NC-3000 ™ analysis system [19].

2.8 Statistical analysis.

In vitro tests were repeated in triplicate and the results are presented as the mean ± SE (standard error). For each sample, data distribution was verified using the Kolmogorov-Smirnov test. Non-parametric comparisons treated vs control were then conducted by running separate Mann-Whitney *U*-tests (multiple pairwise comparisons); Bonferroni correction of the α was applied in order to maintain the overall probability of a type I error at 0.05. For correlation analysis, the Spearman's rho coefficient was evaluated. All statistical evaluations were performed using the SPSS package for Windows version 12.0. Values of *p* less than 0.05 were considered to indicate statistically significant differences compared to controls.

3. Results and Discussion

3.1 Cytotoxicity: LDH assay

Results of LDH assay are shown in Table 1. Any of the three *Gymnema sylvestre* extracts shows cytotoxic effects at tested concentrations, with the exception of the highest concentrations (1.5 mg/ml). In samples A and B there is a slight cytotoxic effect on both cell lines while the highest concentration of sample C results very cytotoxic in both cell lines. This difference is probably caused by the composition of the samples. Samples A and B are in fact herbal formulations, which contain numerous excipients respectively equal to 24% and 10% of total weight. These excipients, for the same weight, have reduced the absolute concentration of the biologically active molecules, compared to sample C, but they may also have played an antagonist role respect to the analyzed properties.

3.2 Genotoxicity: Comet Assay

Results of comet assay are expressed as mean percentage of tail intensity (± standard error) in figures 1-3. All the tested samples induced primary DNA damage, with a dose-response trend in HepG2 cells (sample A: Rho = 0.969, p = 0.000; sample B: Rho = 0.916, p = 0.000; sample C: Rho = 0.941, p = 0.000). As for cytotoxicity, even in this case, the greater effect was observed in cells treated with sample C. On the other hand, in K562 cells, samples A and B did not significantly induce DNA damage extent, while a significant increase in DNA migration respect to the negative control was observed at two highest tested concentrations of sample C. In K562 cells, only for sample C percentage of tail intensity was linearly related to sample concentrations (Rho = 0.835, p = 0.000). Unlike the K562, HepG2 cells have the ability to metabolize many xenobiotics, therefore it can be deduced that the primary DNA damage is caused only in small part from the active compounds present in *Gymnema sylvestre* (genotoxicity of sample C also in K562) but, the high damage in HepG2 could be caused mainly by the metabolites of such compounds, which are formed directly in cells. The comet assay showed DNA damage as the presence of fragments of single or double-strand of the DNA; the damage observed could therefore be the result of an interaction of the active principles of *Gymnema sylvestre* or metabolites with DNA (direct damage), or the result of induction of an apoptotic process.

3.3 Induction of apoptosis: Mitochondrial Potential Assay and DNA fragmentation

Regarding the induction of apoptosis, the results of assays are reported in Tables 2-3.

In HepG2 cells, samples A and B exhibited an increase in % of hyperpolarized cells (both at 4 and 24 hours) in a concentration-dependent manner (sample A 4h: Rho = 0.919, p = 0.000; sample C 4h: Rho = 0.933, p = 0.000; sample A 24h: Rho = 0.622, p = 0.031; sample B 24h: Rho = 0.602, p = 0.038; sample C 24h: Rho = 0.876, p = 0.000). Whereas sample B caused at 4 hours a concentration-dependent increase only in the percentage of hyperpolarized cells. About mitochondrial membrane depolarization, we found that HepG2 cells treated with *Gymnema sylvestre* did not emit green fluorescence, and percentage of depolarized cells observed for all samples resulted comparable to that of negative control. In contrast, we readily observed an increase in % of DNA fragmentation which follows the same trend (sample A 4h: Rho = 0.777, p = 0.003; sample B 4h: Rho = 0.989, p = 0.000; sample C 4h: Rho = 0.961, p = 0.000; sample A 24h: Rho = 0.933, p = 0.000; sample B 24h: Rho = 0.989, p = 0.000) also observed in the Comet assay. This fact suggests that DNA damage observed in HepG2 cells could derive from a DNA fragmentation caused by an induction of apoptosis, which however do not involve the depolarization of mitochondrial membrane or because this induction of apoptosis is so quick to not allow the observation of mitochondria depolarization.

We observed an increase in % of cells with hyperpolarized mitochondria also in K562 cells, with a concentration-dependent trend only for the sample A, both at 4 and 24 hours (sample A 4h: Rho = 0.777, p = 0.003; sample A 24h: Rho = 0.777, p = 0.003). For sample B we did not observe any correlation; however at the highest treated concentrations (1.5 mg/ml at 4 hours; 0.75 mg/ml and 1.5 mg/ml at 24 hours) we observed a marked statistically significant increase in the percentage of hyperpolarized cells. In sample C, as shown in Table 2, at the highest concentrations the percentage of cells with hyperpolarized mitochondria, diminishes in favor of those with depolarized mitochondria and this is probably due to the fact that the sample C is so strong that push forward the apoptosis process. Hyperpolarization in fact leads cells to a state of reversible awareness, subsequently followed by a depolarization of mitochondrial membrane that leads to apoptosis [27, 28]. If the stimulus that caused the hyperpolarization ends, cells return to their normal state, but if the stimulus is strong enough, the hyperpolarization will be followed by a depolarization that will trigger the apoptotic process. This could be the explanation for which is observed depolarization only in K562 cells treated with the sample C. This fact is further confirmed by results of DNA fragmentation assay. In K562 cells we observed no DNA fragmentation in samples A and B, only sample C caused a statistically significant DNA fragmentation at the highest concentration after 24 hours of treatment.

3.4 Tables an Figures

Table 1: Results of LDH assay HepG2 and K562 cells (percentage of cytotoxicity ± SE) treated with different concentrations of the three extracts of *Gymnema sylvestre*at 24 hours. *=p<0.05

			HepG2	K562
Samples (mg/ml)	A	CTRL	2.43 ± 2.54	3.25 ± 0.45
		0,075	1.44 ± 4.94	4.45 ± 0.35
		0,15	1.95 ± 1.52	3.65 ± 0.15
		0,375	1.37 ± 2.46	3.20 ± 0.40
		0,75	2.31 ± 3.21	4.20 ± 1.2
		1,50	10.00 ± 3.31	12.55 ± 4.85
	B	CTRL	2.10 ± 2.23	3.25 ± 0.45
		0,075	2.39 ± 0.38	3.65 ± 0.65
		0,15	1.73 ± 0.52	3.30 ± 1.00
		0,375	1.45 ± 1.16	1.85 ± 0.05
		0,75	3.73 ± 0.85	5.05 ± 0.75
		1,50	11.21 ± 3.54	9.30 ± 0.7
	C	CTRL	1.94 ± 3.41	3.25 ± 0.45
		0,075	2.23 ± 1.58	2.90 ± 0.50
		0,15	11.08 ± 3.17	2.65 ± 0.05
		0,375	4.45 ± 0.95	3.55 ± 0.25
		0,75	2.68 ± 4.12	5.25 ± 0.85
		1,50	79.38* ± 11.65	69.55* ± 13.35

Table 2: Results of the mitochondrial potential assay and DNA fragmentation assay on HepG2 cells (percentage ± SE) treated with different concentrations of the three extracts of *Gymnema sylvestre*at 4h and 24 hours. *=p<0.05

HepG2 cell line			**% of hyperpolarized cells (mean ± SE)**		**% of depolarized cells (mean ± SE)**		**% of DNA fragmentation (mean ± SE)**	
			4h	**24h**	**4h**	**24h**	**4h**	**24h**
CTRL			1.39 ± 0.40	0.67 ± 0.07	2.17 ± 0.87	1.27 ± 0.20	3.08 ± 0.30	3.87 ± 0.51
Samples (mg/ml)	A	0,075	1.87 ± 0.44	0.32 ± 0.04	0.13 ± 0.05	1.30 ± 0.18	24.91* ± 0.87	9.48 ± 0.16
		0,15	2.05 ± 0.37	0.57 ± 0.10	0.73 ± 0.13	0.65 ± 0.14	36.53* ± 1.13	11.47 ± 0.22
		0,375	2.80 ± 0.43	0.49 ± 0.04	1.03 ± 0.09	1.04 ± 0.14	33.14* ± 1.68	23.28* ± 0.67
		0,75	4.11 ± 0.49	2.87 ± 0.45	0.09 ± 0.02	0.74 ± 0.09	32.20* ± 1.13	33.45* ± 0.36
		1,50	8.61 ± 0.57	3.82 ± 0.65	0.17 ± 0.06	0.30 ± 0.04	56.19* ± 5.34	25.25* ± 0.54
	B	0,075	2.92 ± 0.58	0.42 ± 0.14	2.55 ± 0.49	0.45 ± 0.09	11.44 ± 0.30	5.34 ± 0.04
		0,15	3.85 ± 0.82	0.40 ± 0.09	1.28 ± 0.16	1.55 ± 0.16	14.21* ± 0.49	9.21 ± 0.32
		0,375	5.31 ± 0.57	0.59 ± 0.16	0.36 ± 0.12	0.97 ± 0.08	21.37* ± 4.15	24.12* ± 0.53
		0,75	3.43 ± 0.58	4.42 ± 0.60	0.51 ± 0.03	1.01 ± 0.18	36.35* ± 1.13	27.44* ± 0.96
		1,50	3.11 ± 0.33	4.15 ± 0.43	0.10 ± 0.02	0.32 ± 0.18	54.46* ± 2.08	41.34* ± 1.94
	C	0,075	4.82 ± 0.52	1.16 ± 0.23	0.28 ± 0.06	0.86 ± 0.17	27.78* ± 0.83	34.50* ± 0.29
		0,15	19.50* ± 1.29	0.70 ± 0.12	0.19 ± 0.03	0.59 ± 0.16	35.60* ± 1.53	35.46* ± 0.68
		0,375	14.42 ± 0.77	3.89 ± 0.37	0.03 ± 0.03	0.46 ± 0.10	35.63* ± 1.66	20.64* ± 0.39
		0,75	25.34* ± 0.70	25.46* ± 0.49	0.00 ± 0.00	0.11 ± 0.00	45.88* ± 3.15	18.85 ± 0.29
		1,50	37.64* ± 1.07	54.20* ± 0.42	0.27 ± 0.27	0.14 ± 0.14	77.09* ± 5.86	69.06* ± 2.23

Table 3: Results of the mitochondrial potential assay and DNA fragmentation assay on K562 cells (percentage ± SE) treated with different concentrations of the three extracts of *Gymnema sylvestre*at 4h and 24 hours. *=p<0.05

K562 cell line			**% of hyperpolarized cells (mean ± SE)**		**% of depolarized cells (mean ± SE)**		**% of DNA fragmentation (mean ± SE)**	
			4h	**24h**	**4h**	**24h**	**4h**	**24h**
CTRL			1.03 ± 0.22	1.37 ± 0.29	5.12 ± 0.23	5.69 ± 1.37	4.17 ± 0.87	6.09 ± 2.61
Samples (mg/ml)	A	0,075	0.45 ± 0.36	0.25 ± 0.12	1.30 ± 0.59	2.16 ± 0.03	3.88 ± 0.81	4.22 ± 1.33
		0,15	0.33 ± 0.19	0.15 ± 0.07	1.11 ± 0.39	2.19 ± 1.23	3.61 ± 0.77	3.78 ± 1.23
		0,375	4.13 ± 2.30	2.55 ± 0.85	1.96 ± 1.05	1.44 ± 0.51	4.36 ± 1.05	4.77 ± 1.32
		0,75	3.70 ± 1.60	22.41* ± 2.14	2.23 ± 0.31	0.84 ± 0.63	4.29 ± 0.96	4.51 ± 1.28
		1,50	38.60* ± 6.20	44.81* ± 0.29	2.35 ± 0.67	3.03 ± 0.75	4.09 ± 0.83	6.28 ± 1.15
	B	0,075	1.17 ± 0.48	1.08 ± 0.12	1.92 ± 0.79	1.77 ± 0.28	4.34 ± 1.27	5.30 ± 1.56
		0,15	0.96 ± 0.07	0.79 ± 0.49	4.04 ± 1.71	2.52 ± 0.21	3.94 ± 0.97	5.18 ± 1.16
		0,375	0.68 ± 0.21	0.34 ± 0.22	1.82 ± 0.61	2.48 ± 0.78	5.17 ± 0.96	3.66 ± 0.86
		0,75	2.25 ± 1.59	17.45* ± 1.82	1.44 ± 0.03	2.91 ± 2.09	4.16 ± 0.84	4.28 ± 1.33
		1,50	39.20* ± 8.93	40.13* ± 7.19	0.62 ± 0.39	3.11 ± 1.64	4.11 ± 0.90	4.44 ± 0.76
	C	0,075	1.45 ± 0.55	8.73 ± 5.75	2.30 ± 2.09	1.18 ± 0.26	4.67 ± 1.07	4.50 ± 1.05
		0,15	4.23 ± 2.95	23.03* ± 2.20	3.03 ± 2.26	1.40 ± 0.15	5.54 ± 0.92	4.48 ± 1.19
		0,375	49.30* ± 17.59	57.51* ± 8.25	2.17 ± 1.86	2.99 ±.185	5.05 ± 0.86	3.52 ± 0.85
		0,75	84.29* ± 7.19	56.50* ± 0.68	0.85 ± 0.65	12.39 ±.631	3.42 ± 0.68	6.31 ± 1.10
		1,50	67.68* ± 7.56	5.65 ± 5.65	27.16* ± 1.80	89.92* ± 10.08	8.11 ± 1.47	44.07* ± 8.28

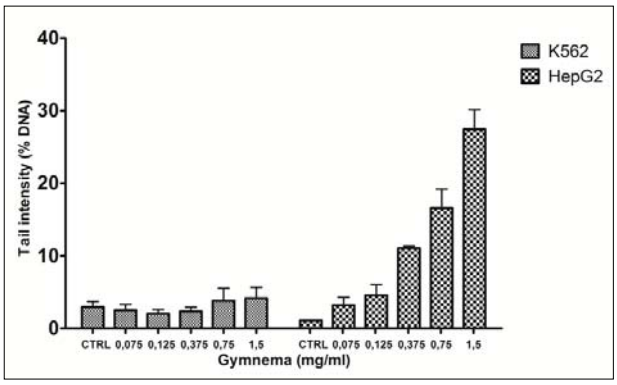

Fig 1: Trend of genotoxicity of *Gymnema sylvestre* (Sample A) on HepgG2 and K562 cells after 4 hours of treatment, express ad percentage of Tail Intensity ± SE.

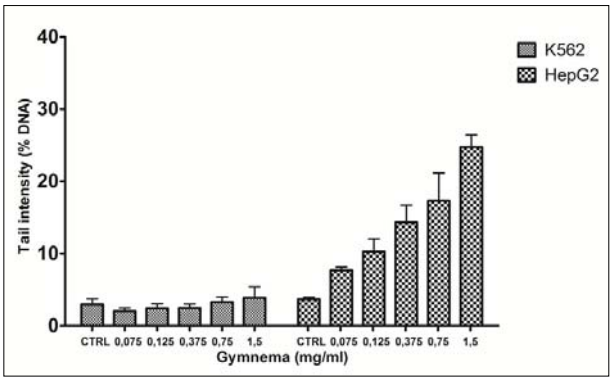

Fig 2: Trend of genotoxicity of *Gymnema sylvestre* (Sample B) on HepgG2 and K562 cells after 4 hours of treatment, express ad percentage of Tail Intensity ± SE.

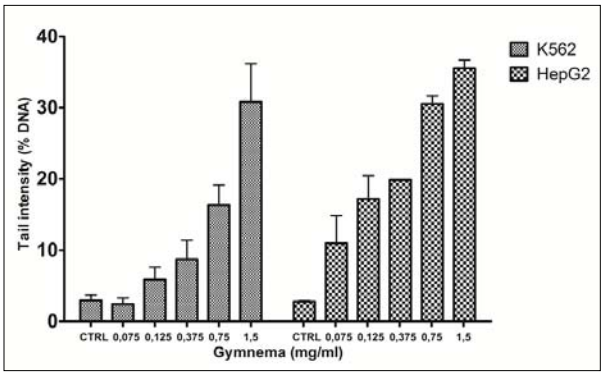

Fig 3: Trend of genotoxicity of *Gymnema sylvestre* (Sample C) on HepgG2 and K562 cells after 4 hours of treatment, express ad percentage of Tail Intensity ± SE.

4. Conclusions

The different behavior of *Gymnema sylvestre* extracts observed in the two cell lines used for *in vitro* testing could be explained by considering the inherent characteristics of cells. In fact, HepG2 cells express many of the phase 1 and phase 2 detoxification enzymes, while K562 cells do not. It could be therefore assumed that some of the bioactive compounds in *Gymnema sylvestre* cause a hyperpolarization of the mitochondrial membrane, which may lead to an induction of apoptosis. These molecules are likely metabolized by enzymes expressed in HepG2 cells, reducing the effect of hyperpolarization of mitochondria. At the same time, these metabolites might activate pathways that do not involve the alteration of mitochondrial potential, carrying instead to the fragmentation of DNA, being the latter one of the final stages of apoptosis. However, these mechanisms should be explored through specific biochemical studies, in order to understand if these assumptions are correct. The fact that *Gymnema sylvestre* was found to be capable of inducing DNA damage and apoptosis in 2 cell lines, both derived from a human tumor, could led to the assumption that this plant could have anticancer properties. On the other hand, the observed genotoxic activity should not be underestimated. These observations need further investigation to confirm potential useful biological activities as well as safety for humans. In particular, it would be necessary to repeat the same experimental protocol *in vitro* using primary human cell lines (non-tumor derived) and then assessing the anti-tumor properties on specific animal models, such as nude mice which have been implanted with human cancer cells. Positives results of such tests could open the way for conducting clinical trials.

5. Conflict interests

The authors declare that they have no conflict of interests.

6. References

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