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Euphorbia tirucalli Aqueous Extract Induces Cytotoxicity, Genotoxicity and Changes in Antioxidant Gene Expression in Human Leukocytes

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Abstract

Euphorbia tirucalli, popularly known as "*avelós"*, is a toxic plant used as a tea in the Brazilian folk medicine as antibacterial, antiviral and anticarcinogenic agent. However, there is no scientific report about its potential toxicity in human cells. Therefore, the objective of the present study was to evaluate the *in vitro* genotoxicity and cytotoxicity of aqueous extract of *E. tirucalli* in human leukocytes using comet assay and trypan blue exclusion assay, respectively. In addition, the effect of *E. tirucalli* in the osmotic fragility was investigated in human erythrocytes. The expressions of selected antioxidant mRNA genes (SOD2, CAT and GPx4) as well as tumor protein p53 (TP53) was evaluated by qRT-PCR. Exposure of human leukocytes to high concentrations of aqueous extract of *E. tirucalli* (100-150 µg/mL) caused significant increase in DNA damage. Leukocytes viability was decreased in the presence of 50 to 150 µg/mL *E. tirucalli* extract. *E. tirucalli* did not change the osmotic fragility of human erythrocytes. High concentrations *E. tirucalli* (10-50 µg/mL) significantly up-regulated the mRNA of SOD2 and CAT and decreased the mRNA of GPx4 expresssion in human leukocytes. In addition, the mRNA gene expression of SOD2 was down-regulated at the highest concentration tested (150 µg/mL). In summary, based on the results of genotoxicity observed in our study, we recommend caution regarding the acute or chronic use of this homemade preparation. Taken together, our results suggest that the aqueous extract of *E. tirucalli* induces genotoxicity and cytotoxicity to human leukocytes, possibly by interacting with the antioxidant enzyme system, thereby, increasing the formation of ROS and decreasing the cellular tolerance level to chemical constituents of this plant.

Key-words: *Euphorbia tirucalli*, avelos, genotoxicity, cytotoxicity and gene expression.

1.Introduction

Exposure to a variety of xenobiotics generates reactive oxygen species (ROS), which may be cytotoxic and/or genotoxic [1-4]. At low levels, ROS can physiologically modulate cell signaling [5-7], but elevated levels of ROS can induce oxidation of proteins, lipids and DNA, which can result in cell damage, apoptosis or other types of cell death [7-9].

The cell redox signaling system is controlled by a fine balance between ROS generation and antioxidant mechanisms, which can involve the activation of multiple genes encoding antioxidant enzymes [10-14]. In addition to antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX); the redox balance of mammalian cell can be modified by vitamins (for instance, vitamin C and E) and by flavonoids from exogenous sources [15-19].

For several millennia, plants have been used for pharmaceutical and dietary therapy [20-22]. The consumption of natural antioxidants has been reported to be associated with reduced risk of pathological conditions such as cancer and cardiovascular diseases [10, 23, 24]. Thus, there is a growing interest in natural compounds isolated from plants as potential source of novel anticancer drugs [10]. Epidemiological points of evidence suggest that phytochemicals may in part exert beneficial effects via modulation of cell redox status [25]. Accordingly, *in vitro* and *in vivo* studies have shown that cellular metabolism and antioxidant enzymes expression can be modulated by phytochemicals [26-31]. However, despite the "apparent" health beneficial effects of plants extracts, they can also be toxic [32-34]. The toxicity may be due, in part, to the pro-oxidant activity of their chemical constituents [35-36]. Therefore, it is important to evaluate the toxicology of plant extracts to determine their safety as phytomedicinal preparations.

Euphorbia tirucalli L. (Euphorbiaceae family), known as "Aveloz" in English, is a shrub endemic to the Northeastern, Central and Southern Africa, which grows in dry areas[37-39]. Although *E. tirucalli* is known as a toxic plant [40], it has been used in traditional medicine in many cultures. It is used in African folk medicine against warts, snake bites, cough, sexual impotence, hemorrhoids, epilepsy and cancer [37-39]. In India, it is used as ornamental plant and for the treatment of asthma, leprosy, leucorrhoea and tumors [37]. In Brazilian folk medicine, *E. tirucalli* is used to treat rheumatism, cancer, tumors and as laxative agent [41]. Phytochemical analyses of the branches and latex of *E. tirucalli* have indicated the presence of a wide range of bioactive compounds such as alkaloids, flavonoids, polyphenols [42], triterpenoids [43], and phorbol diterpene esters [44-46]. Extracts from *E. tirucalli* exhibit have been reported to exhibit a variety of pharmacological activities including antiviral [47], molluscicide [48], antibacterial [49] and myelomodulating activities [47].

In contrast, isolated components of *E. tirucalli* have been shown to induce inflammation [50], apoptosis in tumor cells [51], cytotoxicity [47] and hepatotoxicity [52-54]. Optimistically, the toxicity and the pro-apoptotic effects of *E. tirucalli* components against tumor cells may be beneficial in the treatment of cancer. Although the mechanisms by which *E. tirucalli* induces toxicity is still elusive, it has been suggested that it may modulate leukocyte functions and alter genes expression of the cells involved in the immune response [55]. In the present study, we investigated the *in vitro* mechanism of toxicity of *E. tirucalli* in human leukocytes. Specifically, its potential genotoxicity and cytotoxicity to human leukocytes were determined as well as its potential modulatory effects in the expression of mRNA of antioxidant (CAT, GPX4 and SOD2) and tumor suppressor protein p53 (TP53) genes. The chemical composition

of the crude extract of *E. tirucalli* and its effects on the osmotic fragility of human erythrocytes was also determined.

2. **Materials and Methods**

2.1. Chemicals

All chemical used were of analytical grade. DMSO (Dimethyl sulfoxide), trypan blue, dextran, tungstosilicic acid and Triton X-100 were obtained from Sigma, (St. Louis, MO., USA). NaCl, EDTA, and Tris were obtained from commercial sources. Other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Plant material

E. tirucalli was collected in Company of Agricultural Research and Rural Extension (EPAGRI) of Chapecó/SC, Brazil, (latitude (26º 57'N, longitude 51º 23.76' W) in January 2010. The plant was identified by Prof. Dr. Thais Scotti do Canto-Dorow, from the Herbarium Department of Biology of Universidade Federal de Santa Maria, where a specimen was deposited (number SMDB 14.451). The plant identification was based on both macro-morphological study of the vegetative and reproductive structures of *E. tirucalli* under a stereoscopic microscope.

2.3. Preparation of extract

The aqueous extract from the branches of *E. tirucalli* was obtained through decoction method, according to its use in folk medicine. This was prepared by soaking 8 g of dried pharmacogen (branches) in 150 mL of distilled water for 5 min. The extract was filtrated, lyophilized and stored at -80ºC.

128 *2.4. Identification and Quantification of phenolics and flavonoids compounds by HPLC-*
129 *DAD*

130 The High-Performance Liquid Chromatography (HPLC) profile of aqueous extract from
131 the branches of *E. tirucalli* was performed according to Laghari et al [56] with slight
132 modifications. Identification of phenolics and flavonoids compounds was performed by
133 comparing their retention time and UV absorption spectrum with those of commercial
134 standards analyzed under identical analytical conditions. Reverse phase
135 chromatographic analyses were carried out under gradient conditions using C_{18} column
136 (4.6 mm x 250 mm) packed with 5 μm diameter particles. The mobile phase was water
137 containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5%
138 of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10,
139 20, 30, 40, 50 and 80 min, respectively. All the samples and mobile phase were filtered
140 through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior
141 to use. Stock solutions of standards references were prepared in the HPLC mobile phase
142 at a concentration range of 0.031 – 0.250 mg/mL for kaempferol, quercetin and rutin;
143 and 0.006 – 0.250 mg/mL for gallic, caffeic and chlorogenic acids. The flow rate was
144 0.6 mL/min, injection volume 40 μL and the wavelength were 254 nm for gallic acid,
145 327 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and
146 kaempferol. The chromatography peaks were confirmed by comparing its retention time
147 with those of reference standards and by DAD spectra (200 to 500 nm).

148

149 *2.5. DPPH radical scavenging activity*

150 The free radical scavenging capacity of *E. tirucalli* extract was analyzed by DPPH [57],
151 with some modifications. Briefly, fifty μL of *E. tirucalli* extract or ascorbic acid (1 to

150 µg/ml) was mixed with 100 µL of DPPH (3 mM) in microplate wells. The microplate was kept in the dark at room temperature for 30 min and the absorbance was read at 517 nm. Vitamin C was used as a positive control. The radical scavenging activity was measured as a decreased in the absorbance of DPPH and was calculated as follows: % inhibition = 100-([$A_{sample} - A_{blank}$] / $A_{control}$) × 100, where, $A_{control}$ is the absorbance of the control group, $A_{sample,}$ is the absorbance of the tested sample and A_{blank} is the absorbance of the blank (i.e., extract without DPPH)[58].

2.6. Blood Sampling and preparation of human leukocytes and erythrocytes

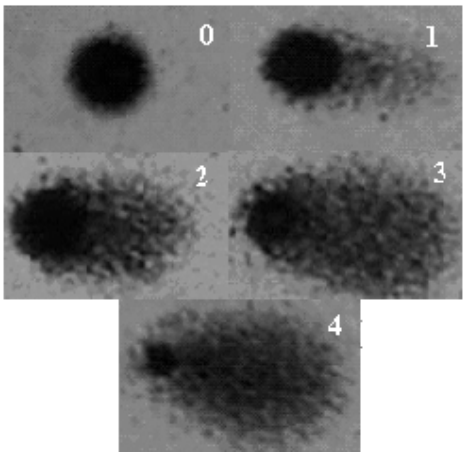
Heparinized venous blood was obtained from healthy volunteer donors from the Hospital of Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil (age 30 ± 15). The protocol used in this study has been approved by the Ethical Committee of the UFSM (number 089.0.243.000-07). The leukocytes were separated by differential erythrocyte sedimentation utilizing dextran 5%. Leukocytes were then adjusted to 2 x 10^6 / mL with Hank's buffer solution saline (HBSS) / heparin (KCl 5.4 mM, Na_2HPO4 0.3 mM, KH_2PO4 0.4 mM, $NaHCO_3$ 4.2 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ 0.5 mM, MgSO4 0.6 mM, NaCl 137 mM and D-glucose 10 mM, Tris–HCl 10 mM, heparin 15 UI / mL, pH 7.4). For erythrocyte preparation, the heparinized blood was centrifuged at 2000 rpm for 10 min at room temperature and the plasma was aspirated. The cell pellet was washed three times with phosphate buffer saline (6.1 mM, containing NaCl 150 mM in pH 7.4).

2.7. Comet assay

The comet assay was carried out according the method described by Collins [59]. Peripheral blood leukocytes were incubated for 3 h in the absence or presence of

different concentrations (10-150µg/mL) of the *E. trucalli* extract. Then, fifteen µL leukocytes suspension (2 x 10^6 leukocytes/mL) were mixed with low-melting point agarose and subsequently added to 90 µL of LMP agarose 0.75 % (w/v), mixed, and placed on a microscope slide pre-coated with normal melting point agarose 1 % (w/v). A coverslip was added and the samples were left to solidify at 4ºC. The coverslips were removed and the slides were placed on a lysis solution (NaCl 2.5 M; EDTA 100mM; Tris-HCl 8mM; TritonX-100 1%; pH 10-10.5) during 24 h at 4ºC. Afterwards, the slides were incubated in an electrophoresis solution (NaOH 300 mM; EDTA 1 mM; pH 13.5) for 20 min at 4ºC, under specific conditions (25V; 300mA; 7W). All the steps were performed in the dark. After electrophoresis, the slides were neutralized in Tris-HCl 400 mM; pH 7.5, rinsed three times in distilled water, and left to dry at room temperature. The slides were re-hydrated for 3 min in distilled water and fixed for 10 min in trichloroacetic acid 15% (w/v), zinc sulfate 5 % (w/v) and glycerol 5% (v/v), rinsed three times in distilled water, and left to dry at room temperature. Then, the rehydrated slides were stained with sodium carbonate 5%, ammonium nitrate 0.1%, silver nitrate 0.1%, tungstosilicic acid 0.25% and formaldehyde 0.15% (w/v). The staining was stopped with 1% acetic acid and slides were air-dried. One hundred randomly selected leukocytes per sample were scored visually according to tail size and intensity in five classes (from undamaged, 0; to maximally damaged, 4) as depicted in Fig. 1. The image of Fig. 1 were captured using Molecular Microscope (Bioval) connected with CCD color camera 480 Lines Bivolt (Model-VI-6633). Damage index (DI) was defined as follows: DI = 1n1+2n2+3n3+4n4, where, n1 to n4 represent the number of cells with damage level 1, 2, 3 and 4 respectively. Methanesulfonate (MMS) (10 µM) was used as positive control, while distilled water was used as negative control.

Fig 1. Visualization of different levels of DNA damage in human leukocytes.



2.8. Cell viability analysis

The cell viability was determined by Trypan blue exclusion test following the method of Mischell and Shiingi [60]. The leukocytes were incubated for 3 h, as described in the comet assay. After, fifty µL of Trypan's Blue (0.4%) was mixed with 50 µL of leukocytes and the mixture was allowed to stand at room temperature for 5 min. Cell viability was determined microscopically and calculated as the number of living cells divided by the total number of cells multiplied by 100. The comet assay was performed only when cell viability was > 90%, as defined by the Singh et al [61]. The combination of hydrogen peroxide (H_2O_2) (2mM) and sodium azide (1mM) was used as positive control.

2.9. Osmotic fragility assay

The osmotic fragility of erythrocytes was estimated by measuring their resistance to lysis by decreasing concentration of saline solutions as described by Oyewale [62] with minor modifications. Five hundred microliters of erythrocytes suspension treated with aqueous extract was mixed with 900 µL buffer solution (TFK 6.1 mM, NaCl 150mM,

223 pH 7.38) and incubated for 3h at 37°C. After incubation, the erythrocytes were
224 centrifuged at 2500 rpm for 10 min and then suspended in varying concentrations of
225 NaCl (0 to 0.9%). Subsequently, samples were centrifuged and the hemoglobin content
226 of the supernatant was measured at 540 nm using microplate reader (SpectraMax,
227 USA). Results are expressed as the percentage of positive control - Triton-total
228 hemolysis.

229 *2.10. Analysis of gene expression using RT-PCR*

230 Human leukocytes were exposed to different concentrations of the *E. tirucalli* aqueous
231 extract (10-150 µg/mL) for 3 hours. Then the mixture was centrifuged at 700 g for 5
232 min, the supernatant was discarded and the pellet suspended in 1 mL Trizol ™
233 (Invitrogen, USA). Purification of total RNA was made according to the manufacturer's
234 protocol (Invitrogen, USA). RNA samples were treated with DNAse (DNAse
235 amplication grade, Invitrogen, USA) and reverse-transcribed to cDNA using the enzyme
236 reverse transcriptase MLVRT-M- (Moloney Murine Leukemia Virus Reverse
237 Transcriptase) according to the instructions of the manufacturer (Invitrogen). The
238 sequences of qPCR primers used are presented in Table 1.

239

240 Table 1 Sequences of qPCR primers

Gene name	Symbol	Primer Sequence (5'-3')	Amplicon
Catalase	CAT	TGGAAAGAAGACTCCCATCG CCAACGAGATCCCAGTTACC	132pb
Glutathione Peroxidase 4	GPX4	AGATCCAACCCAAGGGCAAG GACGGTGTCCAAACTTGGTG	72 pb
Superoxide Dismutase 2	SOD2	TCACATCAACGCGCAGATCA CTGGGCTGTAACATCTCCCTT	124pb
Tumor Protein p53	TP53	AAAGTCTAGAGCCACCGTCCA CAGTCTGGCTGCCAATCCA	121pb
Nuclear Factor (erythroid-derived 2)-like 2 Avtina B	NFE2L2	AACCAGTGGATCTGCCAACT ACGTAGCCGAAGAAACCTCATT	134 bp
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	TTCGTCATGGGTGTGAACC AGTTGTCATGGATGACCTTGG	112pb

241

242 All the samples were run in triplicate.

243 *2.11. Statistical analysis*

244 Statistical analyses were performed using analysis of variance (ANOVA) followed by
245 Newman-Keuls multiple test when appropriate. The results are expressed as mean ±
246 SEM for three to four independent replicates. The differences were considered
247 statistically significant when the p values were less than 0.01.

248

3. Results

3.1. HPLC analysis

The HPLC profile of the branches of aqueous extract of *E. tirucalli* revealed the presence of the gallic acid derivative (tR = 10.54 min; peak 1), gallic acid derivative (tR = 10.82 min; peak 2), gallic acid (tR = 15.17 min; peak 3), chlorogenic acid (tR = 27.08 min; peak 4), caffeic acid (tR = 31.17 min; peak 5), rutin (tR = 39.19 min; peak 6), flavonoid derivative (tR = 44.59 min; peak 7), quercetin (tR = 48.83 min; peak 8) and kaempferol (tR = 57.66 min; peak 9) (Fig. 2 and Table 2).

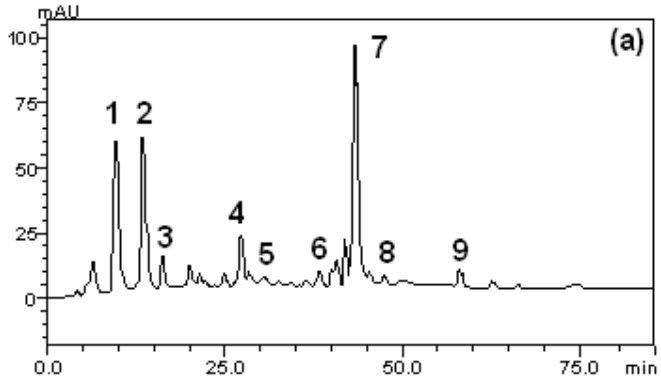


Fig 2. Representative HPLC profile of aqueus extract of the branches of *E. tirucalli* L. Gallic acid derivatives (peak 1 and 2), Gallic acid (peak 3), chlorogenic acid (peak 4), caffeic acid (peak 5), rutin (peak 6), flavonoid derivative (peak 7), quercetin (peak 8) and kaempferol (peak 9). Calibration curve for gallic acid: Y = 11611x + 1468.8 (r = 0.9999); chlorogenic acid: Y = 14762x + 1257.5 (r = 0.9997); caffeic acid: Y = 11526x + 1293.1 (r = 0.9995); rutin: Y = 13035x – 1045.9 (r = 0.9998); quercetin: Y = 15105x – 1192.3 (r =0.9998) and kaempferol: Y = 15223x – 1303.9 (r = 0.9999). All chromatography operations were carried out at ambient temperature and in triplicate.

Table 2 Quantification of some Phenolic acids and Flavonoids from the aqueous extract of *E. tirucalli*

		E. tirucalli	
Peak	**Compounds**	**Quantity (mg/g)**	**Percent**
1	Gallic acid derivative (peak 1)	11.94 ± 0.05 a	1.19
2	Gallic acid derivative (peak 2)	12.65 ± 0.02 a	1.26
3	Gallic acid	2.73 ± 0.11 b	0.27
4	Chlorogenic acid	5.09 ± 0.08 c	0.51
5	Caffeic acid	0.92 ± 0.06 d	0.09
6	Rutin	1.65 ± 0.13 e	0.16
7	Flavonoid derivative #	20.73 ± 0.04 f	2.07
8	Quercetin	1.49 ± 0.05 e	0.14
9	Kaempferol	11.94 ± 0.05 a	0.20

Results are expressed as mean ± standard deviations (SD) of three determinations.

3.2. DPPH radical scavenging activity

E. tirucalli extract (1-150 µg/mL) exhibited weak and concentration dependent DPPH radical scavenging activity (maximal inhibitory effect of 20%).

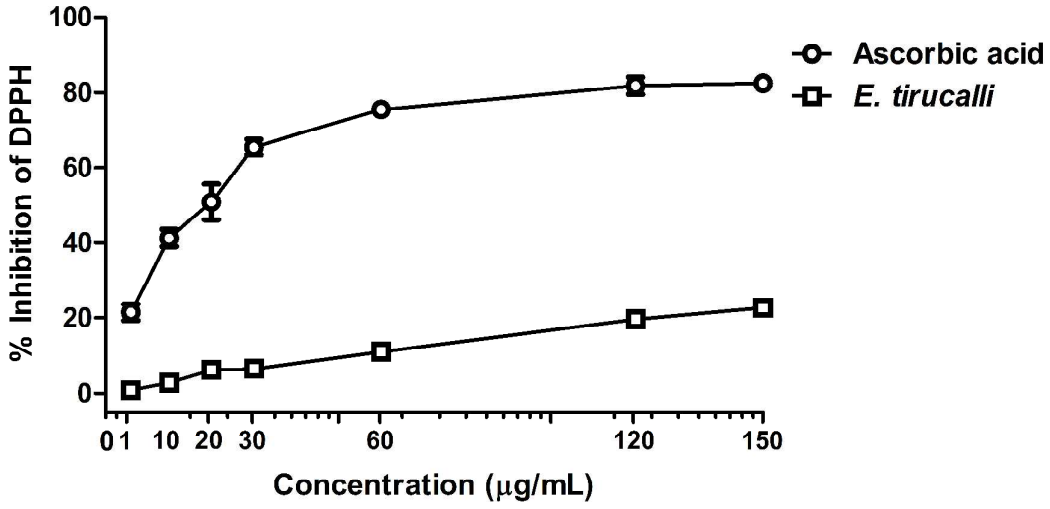


Fig. 3 DPPH Radical Scavenging Activity of *E. tirucalli* aqueous extract. Ascorbic Acid was used as positive control and results are the mean of n = 3 independent experiments performed in triplicate.

3.3. *E. tirucalli aqueous extract causes cytotoxicity to human leukocytes*

The cytotoxicity of *E. truncalli* extract in human leukocytes was evaluated after 3 h of exposure. Relatively high concentrations of *E.tirucalli* extract (50-150 µg/mL) caused significant decrease in cellular viability (varying from 15 to about 30%; $p < 0.01$; Fig. 4). In contrast, lower concentrations of the plant extract (1-25 µg/mL) did not cause any significant toxic effects on leukocytes when compared to the control ($p > 0.05$; (Fig. 4) Hydrogen peroxide + Azide (positive control) caused a decrease in cellular viability of about 35% (Fig. 4).

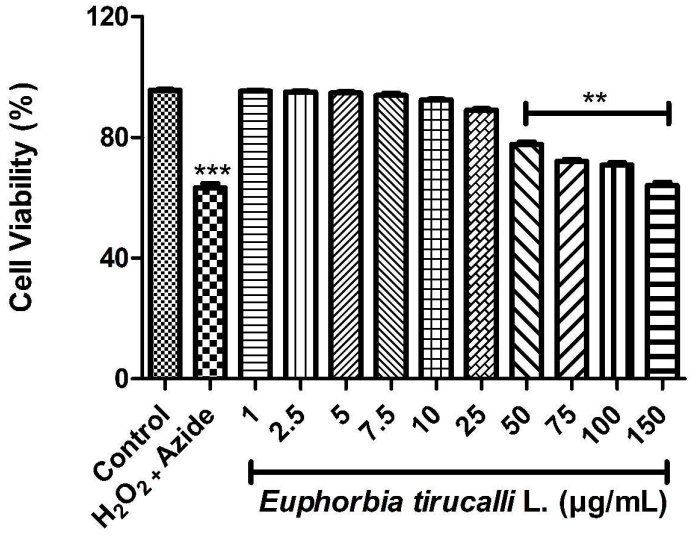


Fig 4 Effect of aqueous extract of *E. tirucalli* on the cell viability of human leukocytes *in vitro*. Hydrogen peroxide+Azide were used as positive control. $^{**}p < 0.01$ and $^{***}p$

307 < 0.001 versus control. Data are expressed as means and ± SEM of four independent
308 experiments.

309 *3.4. Genotoxicity effect of E. tirucalli aqueous extract (Comet Assay)*

310 Incubation of human leukocytes with the plant extract at concentrations of 100 and 150
311 µg/mL resulted in a significant increase DNA damage, when compared with the control
312 ($p < 0.05$ and $p < 0.01$, respectively; Table 3), suggesting a possible induction of DNA
313 single-strand breaks. However, at concentrations lower than 100 µg/mL, *E. tirucalli* did
314 not cause DNA damage when compare with the control group ($p > 0.10$; Table 3). The
315 positive control, methyl methanesulphonate (MMS) (10 µM) caused a marked increase
316 in the DNA damage index when compared with the control ($p < 0.001$; Table 3).

317 **Table 3** DNA Damage Index in Human Leukocytes (the Comet Assay) after Exposure
318 to Aqueous Extract of *E. tirucalli*.

Treatment	Concentration	Level of DNA damage a (percentage of cells)					D.I.
		0	1	2	3	4	
Control (H_2O)	0	95.87 ± 0.62	3.00 ± 0.84	1.12 ± 0.23	0 ± 0	0 ± 0	5.25 ± 0.43
MMS (PC)	10 µM	20.62 ± 2.26	20.37 ± 3.18	29.62 ± 2.65	11.12 ± 3.39	18.25 ± 2.78	186.0 ± 4.97***
E. tirucalli	10 µg/mL	95.12 ± 0.23	4.12 ± 0.37	0.75 ± 0.32	0 ± 0	0 ± 0	5.62 ± 0.42
	25 µg/mL	94.87 ± 0.23	4.25 ± 0.43	0.75 ± 0.14	0 ± 0	0 ± 0	5.75 ± 0.32
	50 µg/mL	94.50 ± 0.35	4.25 ± 0.32	1.25 ± 0.32	0 ± 0	0 ± 0	6.75 ± 0.59
	75 µg/mL	94.25 ± 0.59	4.75 ± 0.82	1.00 ± 0.45	0 ± 0	0 ± 0	6.75 ± 0.66
	100 µg/mL	92.62 ± 0.12	4.00 ± 0.20	2.50 ± 0.20	0.87 ± 0.23	0 ± 0	8.12 ± 0.51*
	150 µg/mL	91.87 ± 0.12	4.62 ± 0.12	2.5 ± 0.12	1.00 ± 0.20	0 ± 0	12.60 ± 0.52**

Results are expressed as mean ± SEM of four independent experiments. D.I., Damage index; PC, positive control. *p < 0.05,** p < 0.01 and ***p< 0.001 as compared to control values.

3.5. Osmotic fragility test

E. tirucalli aqueous extract (10-200 µg/mL) did not modify the the osmotic fragility of human erythrocytes to hypoospotic salt concentrations of NaCl (0-0.65%; p > 0.05; Fig.5).

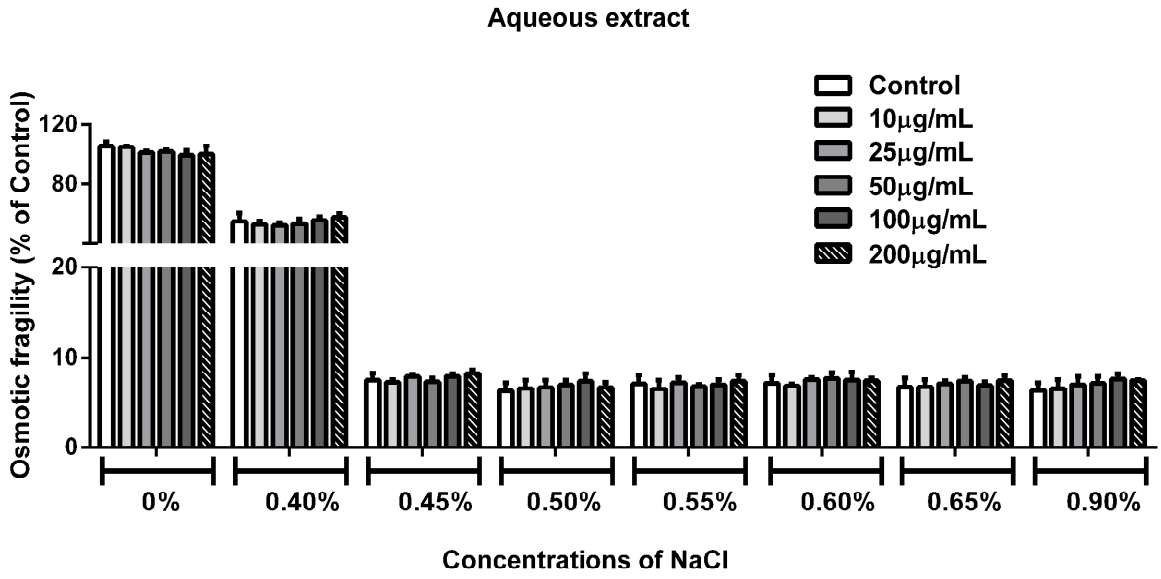


Fig 5. Osmotic fragility of erythrocytes treated with aqueous extract of *E. tirucalli*. Hemolysis was expressed in percentage of the positive control (Triton X-100). The bars represent the means of n = 3 independent experiments performed in duplicate ± SEM

3.6. CAT, GPx4, and SOD2 mRNA gene expression after exposure of human leukocytes to aqueous extract of E. tirucalli.

343 Exposure of human leukocytes to *E. tirucalli* (10-50 µg/mL) caused a significant
344 increase in expression of Catalase (Fig. 6A) and MnSOD mRNA (Fig. 6B), when
345 compared to the control ($p < 0.05$). In contrast, highest concentration of the extract
346 significantly decreased the expression of MnSOD (Fig. 6B). At 150 µg/mL, *E. tirucalli*
347 caused a significant decrease in SOD2 mRNA expression ($p<0.05$; Figure 6B).
348 Exposure to the extract at the lowest and highest concentrations tested caused a
349 significant decrease in the expression of GPx4 when compared to the control (Fig. 6C, p
350 < 0.05). The expression of TP53 and Nrf2 mRNA in leukocytes treated with *E. tirucalli*
351 was not changed when compared to the control (Fig. 6D and E).

352

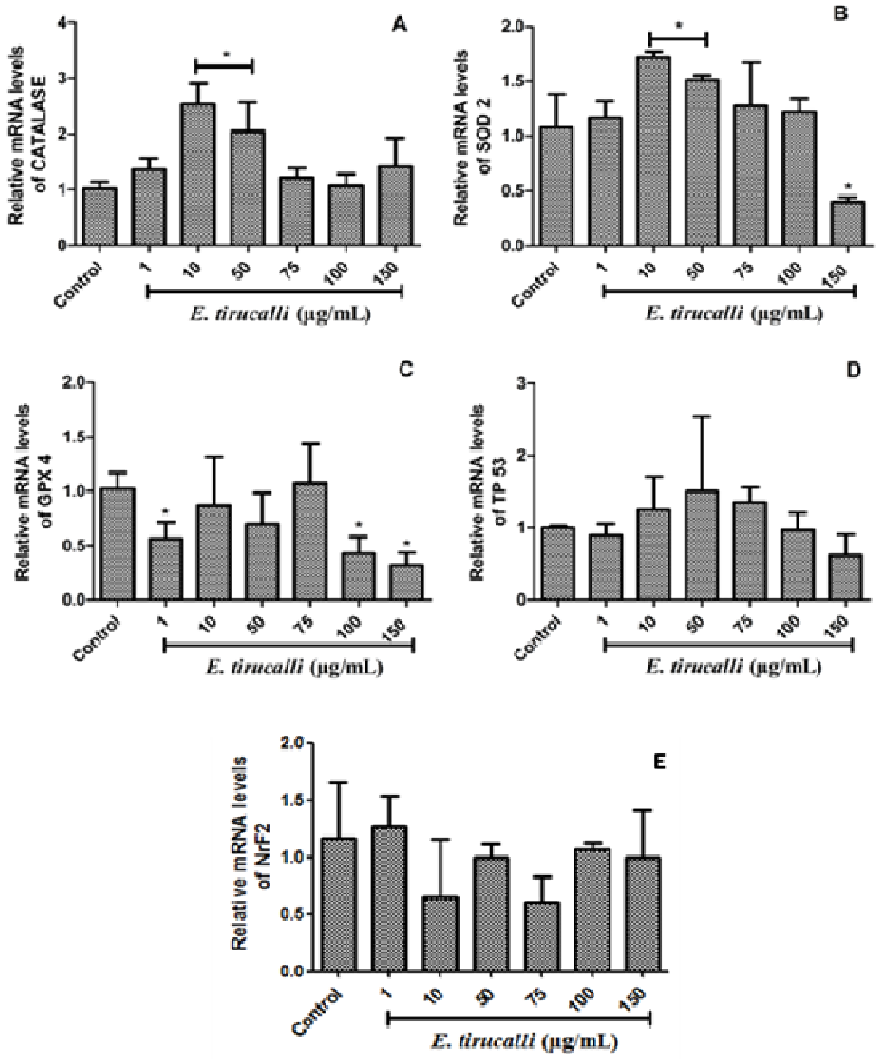


Fig. 6 Relative mRNA levels of (A) Catalase, (B) MnSOD, GPx4 (C), TP53 (D) and Nrf2 (E) following treatment of human leukocytes with different concentrations of aqueous extract of *E. tirucalli*. Values are the mean ± SEM of n = 3 independent experiments performed in triplicates. *$p < 0.05$ as compared to control values.

4. Discussion

Safety evaluation of plant extracts used in folk medicine has generated considerable interest to the scientific community in an attempt to identify those that can potentially cause toxicity to humans, including those with carcinogenic properties [63]. In this context, the objective of the present study was to investigate the genotoxicity and cytotoxicity of aqueous extract of branches from *E. tirucalli.* Exposure of human leukocytes to relatively high concentrations of *E. tirucalli* extracts (50-100 μg/ml) caused genotoxicity and cytotoxicity, which were associated with changes in the expression of some antioxidant genes. However, *E. tirucalli* did not modify the expression of the transcription factor Nrf2 and the tumor protein p53 (TP53).

DNA is an important target of ROS in the living cells. Here, the effect of *E. tirucalli* on DNA of leukocytes was evaluated by the comet assay, which gives an index of genotoxicity. [64]. Since we have used a crude extract of *E. tirucalli,* the increase in DNA damage might be the result of the synergistic interaction of some plant constituents [65]. However, we must emphasize that the increase in DNA damage caused by *E. tirucalli* was very small in comparison to the positive control methyl methanesulfonate (MMS). Although the phytoconstituent(s) involved in the genotoxic effect of *E. truncalli* is (are) unknown, some compounds identified here (Table 1, Fig. 2), for instance, kaempferol, quercetin, and rutin have been reported to possess toxic and genotoxic effects at high concentrations [66-68]. Our results are at variance with those of Oliveira and Nepomuceno [68] and Paiva et al. [69] who have not found genotoxic effects for *E. tirucalli* latex. Here high concentrations of the aqueous extract of the branches of *E. tirucalli* also induced cytotoxicity in leukocytes. Although it is difficult to extrapolate *in vitro* results to *in vivo* situations, particularly regarding the popular medicinal use of

E. tirucalli, the results obtained here suggest that caution should be taken regarding the dosage and frequency of ingestion of *E. tirucalli* teas in folk medicine.

Under physiological conditions, ROS are generated as by-products of the mitochondrial respiratory chain, but are neutralized by primary antioxidants defense system composed by antioxidant enzymes, for instance, SOD, CAT, GPx [70]. In the mitochondria, MnSOD and GPx4 play central role in protecting against oxidative damage [70-72], and their antioxidant properties depend on the level of their expression [72]. In the current study, the effect of aqueous extract of the branches of *E. tirucalli* on genes expression of mitochondrial antioxidant enzymes defense system (MnSOD, CAT, GPx4) was investigated in human leukocytes. Our results showed a significant increase in the expression of CAT and MnSOD genes even after a short time of exposure to *E. truncalli*, which can be a compensatory response to overcome the oxidative stress caused by *E. tirucalli.* The decrease in the expression of mRNAs coding for the antioxidant enzymes MnSOD and GPx4 observed after exposure to high concentrations may suggest that leukocytes were stressed. ROS may modify gene expression patterns, but the mechanisms of this modulation are still elusive and need to be better understood. Here, we can presume that the decrease in MnSOD and GPX4 genes expression have made leukocytes prone to oxidative stress. Consequently the increase in DNA damage index and cell death can be related to oxidative stress. The TP53 gene, known as "the guardian of genome", has been shown to be induced in response to DNA damage and its induction results from different processes including oxidative modifications [73]. However, here we did not observed significant increase in the expression of TP53 following treatment with *E. tirucalli*, which may be related to the short-term exposure used here.

Osmotic fragility is a good indicator for general screening of cytotoxic substances and has been found to be altered in various pathological conditions including anemia and cancer [74, 75]. Here, human erythrocytes were also used to investigate the toxic effect of the aqueous extract of *E. tirucalli.* But *E. tirucalli* did not affect the osmotic fragility of erythrocytes. The differences in the responses of leukocytes and erythrocytes to *E. tirucalli* may be related to differences in the metabolism and protein biosynthesis in red blood cells and leukocytes [76]. In fact, erythrocytes are equipped with more non-enzymatic and enzymatic antioxidants (including GPX, CAT and SOD) than leukocytes, which may have protected membranes of erythrocyte from the toxicity of *E. tirucalli.*.

5. Conclusion

In summary, the present study investigated for the first time the toxicity of the aqueous extract of branches of *E. tirucalli* in human cells. Treatment of human leukocytes with high concentration of aqueous extracts of *E. tirucalli* promoted genotoxicity and cytotoxicity, which was associated with changes in the expression of MnSOD, CAT, and GPx. These results indicate that *E. tirucalli* may interact with the antioxidant enzyme system in leukocytes. In contrast, the extract did not modify the fragility of erythrocytes to hipoosmotic solutions. Of particular toxicological significance, *E. tirucalli* up-regulated the expression of antioxidant enzymes genes; however, the exact mechanism of action of the extract on the examined genes needs to be further explored. It is difficult to extrapolate the *in vitro* results obtained here to the real world of the medicinal use of this plant. Nevertheless, the *in vitro* toxicity of *E. tirucalli* at relatively high concentrations of its aqueous extracts indicates that caution should be taken regarding the dosage and frequency of *E. tirucalli* use in the alternative medicine.

Furthermore, efforts have to be done to identify overt and hidden signs of toxicity in subjects taking *E. tirucalli* for medicinal purposes.

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