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Cytotoxicity and genotoxicity assessment of the extract and lectins from Moringa oleifera Lam. Seeds

Avaliação da citotoxicidade e genotoxicidade do extrato e lectinas das sementes de Moringa oleifera Lam

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ABSTRACT
Moringa oleifera seeds are used globally as a treatment for water and contain the lectins cMoL and WSMoL, which display coagulant activity. In this study, we sought to determine the cytotoxicity and genotoxicity of the M. oleifera seed extract (SE), prepared with the same procedure that people use for treating water, as well as cMoL and WSMoL, in human peripheral blood mononuclear cells (PBMCs). Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay, while genotoxicity was evaluated using the comet assay, with cell nucleoids categorized in classes ranging from 0 (without damage) to 4 (maximum damage). The PBMCs treated with SE, cMoL, and WSMoL displayed viability higher than 60% in treatments with concentrations up to 100 µg/mL. In addition, SE and cMoL displayed low genotoxicity owing to the detection of nucleoids in class 1. However, the number of nucleoids in all classes increased when 50 and 100 µg/mL of WSMoL was administered, reaching a damage frequency of 50.0%. Although M. oleifera SE, cMoL, and WSMoL were not cytotoxic to PBMCs after 24 h of exposure, dose-dependent genotoxic effects were observed, especially with WSMoL. These findings indicate that caution must be exercised when selecting a lectin/extract concentration for water treatment.

Key-words: comet assay, water treatment, horseradish tree, carbohydrate-binding proteins, safety assessment.

RESUMO
As sementes de Moringa oleifera são utilizadas globalmente para o tratamento de água e contêm as lectinas cMoL e WSMoL, as quais apresentam atividade coagulante. Neste estudo, buscou-se determinar a citotoxicidade e genotoxicidade do extrato das sementes de M. oleifera (SE), preparado com o mesmo procedimento que a população usa para tratar água, bem como cMoL e WSMoL, em células mononucleares do sangue periférico humano. A viabilidade celular foi avaliada usando o ensaio do brometo de 3- (4,5-dimetiltiazol-2-il) 2,5-difeniltetrazólio (MTT), enquanto a genotoxicidade foi avaliada através do ensaio do cometa, com nucleoides celulares categorizados em classes que variam de 0 (sem danos) a 4 (dano máximo). As PBMCs tratadas com o extrato aquoso, cMoL e WSMoL exibiram uma viabilidade maior que 60% após o tratamento com concentrações de até 100 µg/mL. Além disso, SE e cMoL apresentaram baixa genotoxicidade devido à detecção de alguns nucleoides na classe 1. No entanto, o número de nucleoides em todas as classes aumentou nas concentrações de 50 e 100 µg / mL de

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WSMoL, atingindo uma frequência de dano de 50,0%. Embora o extrato aquoso de M. oleifera, cMoL e WSMoL não tenham sido citotóxicos para PBMCs após 24 h de exposição, efeitos genotóxicos dependentes da dose foram observados, especialmente com WSMoL. Esses achados indicam a necessidade de cuidado ao selecionar uma concentração de lectina ou extrato para o tratamento de água.

Palavras-chave: ensaio cometa, tratamento de água, proteínas de ligação a carboidratos, avaliação de segurança.

1 INTRODUCTION

Moringa oleifera Lam. is widely cultivated worldwide owing to its biotechnological and pharmacological applications (Gopalakrishnan et al., 2016). Seeds of this plant are used in the manufacture of cosmetics and biodiesel, traditional medicine, as well as human and animal feeding (Dhakad et al., 2019; Napoleão et al., 2019). In addition, they are commonly used as coagulant agents in several countries to treat water for human consumption (Hassan and Ibrahim, 2013).

The coagulant property of M. oleifera seeds is attributed to the presence of proteins, including lectins, which can form specific and reversible bonds with carbohydrates (Saini et al., 2016; Coelho et al., 2017). Two of the lectins isolated from moringa seeds, coagulant M. oleifera lectin (cMoL) and water-soluble M. oleifera lectin (WSMoL), display coagulant activity (Santos et al., 2009; Ferreira et al., 2011). WSMoL can also remove metals and residual aluminum sulfate from water (Freitas et al., 2016). Both lectins have been found to display in vitro anti-inflammatory activity in murine macrophages (Araújo et al., 2013), while WSMoL was demonstrated to be an immunomodulatory agent in human peripheral blood mononuclear cells (PBMCs) (Coriolano et al., 2018). The anticancer activity of cMoL against B16-F10 (murine melanoma) cells was reported by Luz et al. (2017). Further, the strong antibacterial activity of WSMoL against human pathogens has been reported (Ferreira et al., 2011; Moura et al., 2015, 2017; Coriolano et al., 2020). Both cMoL (Oliveira et al., 2011) and WSMoL (Coelho et al., 2009; Santos et al., 2012, 2014; Oliveira et al., 2017, 2020) are also reported as insecticidal agents.

Previous studies have reported the toxicity potential of some plant lectins, such as those from Jatropha curcas seeds (curcin), which strongly inhibit cell-free protein synthesis, as determined using a lysate of rabbit reticulocytes (half-maximal inhibitory

concentration (IC_{50}) of 0.42 nM); these lectins were found to be acutely toxic to mice with median lethal dose (LD_{50}) of 104.74 ± 29.45 mg/kg body weight (Lin et al., 2010).

The Viscum album lectin was found to be cytotoxic to RAW 264.7 macrophage cells at a concentration of 2.0 μg/mL (38±7.97 of cell viability). Further, its LD_{50} was found to be between 50 and 100 μg/kg body weight when intravenously injected into the mice (Sung et al., 2013). WSMoL was previously evaluated to determine its genotoxicity using cell-free plasmid DNA as well as Salmonella typhimurium (Ames and Kado) assays. Based on the results of these assays, this lectin was deemed non-mutagenic (Rolim et al., 2011).

Toxicity assessments are essential for ensuring the safety of plant extracts and compounds that display biotechnological potential. In the present study, we evaluated the cytotoxicity and genotoxicity of an M. oleifera seed extract (SE), prepared with the same procedure that Brazilian people use for the extract for treating water, as well as cMoL and WSMoL, in human PBMCs.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

Moringa oleifera seeds were collected in the city of Recife, Pernambuco, Brazil, under authorization (no. 38690) of the Instituto Chico Mendes de Conservação da Biodiversidade from the Brazilian Ministry of Environment. A voucher specimen was deposited (no. 73345), in the herbarium Dárdano de Andrade Lima at the Instituto Agronômico de Pernambuco (Recife, Brazil). The studies have been recorded (AA269CF and A3A079B) in the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen).

2.2 SE PREPARATION

Moringa oleifera SE was prepared in the same manner Brazilian people prepare it for the treatment of turbid water (Rolim et al., 2011). Briefly, seed powder (2 g) was added to distilled water (100 mL) and manually agitated for 5 min at 27 °C. Thereafter, the mixture was passed through a filter paper to obtain the SE. The SE was then passed through a sterile Millipore filter (pore size of 0.22 μm) and serially diluted in culture

medium to obtain required concentrations (6.25–200 µg/mL) for the cytotoxicity and genotoxicity assays.

2.3 PURIFICATION OF LECTINS

The lectins cMoL and WSMoL were isolated according to the methods of Santos et al. (2009) and Coelho et al. (2009), respectively. Briefly, seed powder (10 g) was subjected to extraction in 100 mL of 0.15 M NaCl (cMoL) or distilled water (WSMoL) at 28 °C. After filtration and filtrate centrifugation (3000 ×g for 15 min at 28 °C), the supernatants were collected. Proteins from the supernatants were precipitated with ammonium sulfate at 60% saturation (Green and Hughes, 1955) and the protein fractions were chromatographed on guar gel (cMoL) or chitin (WSMoL) columns equilibrated with 0.15 M NaCl. Adsorbed cMoL and WSMoL were eluted with 1.0 M NaCl and 1.0 M acetic acid, respectively. The carbohydrate-binding ability of the lectins was monitored using the hemagglutinating activity (HA) assay according to the method of Paiva and Coelho (1992), in microtiter plates using rabbit erythrocytes. The study was approved by the Ethics Committee on the Use of Animals of the *Universidade Federal de Pernambuco*, UFPE, process no. 23076.033782/2015-70. Lectin concentration was determined according to the method of Lowry et al. (1951), using bovine serum albumin as the standard. cMoL and WSMoL were passed through a sterile Millipore filter (pore size of 0.22 µm) and serially diluted in culture medium to obtain required concentrations (0.78–100 µg/mL) for the cytotoxicity and genotoxicity assays.

2.4 PBMCS

PBMCs were obtained from heparinized blood samples collected from non-smoking healthy volunteers (18–30 years old) who had not taken any drugs for at least 15 days before sample collection. All volunteers signed an informed consent form approved by the Ethics Committee on Research involving humans of the UFPE (process no. 60435316.0.0000.5208). To obtain PBMCs, blood (6 mL) was mixed with 3 mL phosphate-buffered saline (PBS) and 3 mL Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation (450 × *g*, 30 min, 25 ºC), the lymphocyte layer was carefully aspirated and transferred to another tube containing PBS. After centrifugation (350 × *g*, 20 min, 25 ºC), the supernatant was discarded and the pellet was resuspended

in RPMI 1640 culture medium (Sigma-Aldrich) supplemented with 20% fetal bovine serum, 3% phytohemagglutinin, 100 IU/mL penicillin, and 100 µg/mL streptomycin.

2.5 CELL VIABILITY ASSAY

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay according to the method of Mosmann (1983), with some modifications. Briefly, cells (1×10^6 cells/mL) were incubated in 96-well plates for 24 h for stabilization in RPMI medium (37 °C, 5% CO_2). Thereafter, they were treated with different concentrations of SE (1.56–200 µg/mL) or lectins (0.78–100 µg/mL) in RPMI medium, and incubated for 24 h. Three hours before the end of the incubation period, MTT (0.5 mg/mL) was added (25 µL). After the 24-h incubation period, the microplate was centrifuged (450 × *g*, 10 min, 25 ºC) and the medium was aspirated. Dimethyl sulfoxide (100 µL) was added to each well to solubilize the formazan crystals, and the optical density was measured in a microplate reader at 540 nm. Two independent experiments were performed in triplicate.

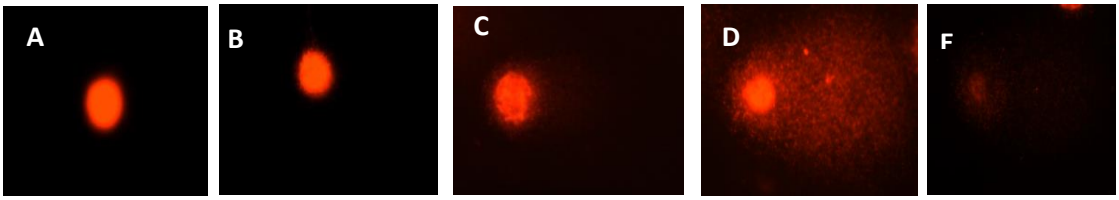
2.6 COMET ASSAY

Genotoxicity of the SE and lectins was evaluated using the alkaline version of the comet assay according to the protocol described by Singh et al. (1988), with some modifications. The tests were conducted in triplicate in two independent experiments. Of note, only those concentrations of SE and lectin were selected that did not inhibit more than 40% of the viability of PBMCs. Cells were seeded (3×10^6 PBMCs/mL) and incubated with SE (6.25–100 µg/mL) or lectin (25, 50, and 100 µg/mL) for 24 h, or with the positive control (4×10^{-2} M methyl methanesulfonate, MMS) for 4 h. Thereafter, the control and treated cells were harvested. Following centrifugation for 20 min at 450 × *g*, and 25 ºC, the pellet obtained was suspended in 0.5% low-melting point agarose at 37 °C, placed on fully frosted slides covered with a thin layer of normal-melting point agarose, and coated with a coverslip. The slides were incubated at 4 °C for 10 min to allow the agarose to solidify and were then immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, adjusted to pH 10.0, using NaOH) at 4 °C for 1 h. After lysis, the slides were placed in a horizontal electrophoresis chamber containing cold electrophoresis alkaline buffer (300 mM NaOH and 1 mM EDTA solution, pH 13.0) for

20 min for DNA denaturation. Electrophoresis was carried out at 40 V and 300 mA for 20 min in the dark to avoid DNA damage that was unrelated to the treatments. The slides were immersed in a neutralization buffer (0.4 M Tris, pH 7.5) for 15 min and fixed with cold absolute ethanol for 5 min.

DNA was stained with propidium iodide (20 µg/mL, 50 µL) and evaluated using a fluorescent microscope (Carl Zeiss, Göttingen, Germany). Two slides were examined for each well (six slides for treatment), and 100 nucleoids per slide were analyzed at a magnification of 40×. Nucleoids were classified as follows (Figure 1): absence of tail or without damage (class 0), tail smaller than the diameter of the head (class 1), tail with up to twice the diameter of the head (class 2), tail larger than twice the diameter of the head (class 3), and comet without head (class 4). The damage score of each treatment was verified by multiplying the number of nucleoids observed in each class by the value corresponding to each class (0, 1, 2, 3, or 4). The damage frequency (%) was calculated based on the number of cells with tails relative to cells with 0 damage owing to each treatment.

Figure 1. Representative images of nucleoids from human peripheral blood mononuclear cells (PBMCs) belonging to class 0 (A), class 1 (B), class 2 (C), class 3 (D), or class 4 (E)



2.7 STATISTICAL ANALYSIS

Results are presented as mean ± standard deviation. Cell viability percentage (%) was evaluated relative to that of the non-treated control, using the GraphPad Prism software (version 5.0; GraphPad Software, Inc., USA). One-way ANOVA, followed by the Newman-Keuls test, was used to evaluate the differences among the treatments administered. The level of statistical significance was set at $p < 0.05$.

3 RESULTS AND DISCUSSION

Moringa oleifera seeds have been widely used to treat drinking water in several countries (Sulaiman et al., 2017). Previous studies have demonstrated that WSMoL is

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present in the water treated with seeds and is one of the proteins responsible for their water coagulant activity (Ferreira et al., 2011). Additionally, cMoL can be present in the drinking water treated with moringa seeds and is an important natural coagulant. In fact, cMoL displays coagulant activity similar to aluminum sulfate, the most common synthetic coagulant used in water treatment (Santos et al., 2009). In the present study, we evaluated the cytotoxic and genotoxic effects of the SE and lectins extracted from moringa seeds, on human PBMCs. PBMCs include monocytes and lymphoblastoids, which are involved in cell-mediated cytotoxic innate and adaptive immunity; these cells circulate throughout the body and reflect the general state of an individual's health (He et al., 2018; Collins et al., 2008).

As shown in Table 1, the cells treated with 200 µg/mL SE had a viability of 49.36 ± 1.39; however, when other concentrations were employed, the viability was found to be greater than 60%. A cell viability >70% was detected when cMoL and WSMoL were administered. Previous studies have shown that 100 µg/mL of WSMoL is not cytotoxic to PBMCs after 24–72 h of incubation (Araújo et al., 2013; Coriolano et al., 2018, 2020). However, Araújo et al. (2013) showed that cMoL is toxic (IC_{50} = 11.72±1.51 µg/mL) to these cells after 72 h of exposure. The present study confirmed the low cytotoxicity of WSMoL to PBMCs and the non-cytotoxicity of cMoL after 24 h of incubation. These findings indicate that exposure time is a crucial factor that can influence the toxicity of these lectins. cMoL (1.5–16.0 µM) did not induce death of normal fibroblasts (GN) after 48 h of treatment (Luz et al., 2017), indicating that this lectin did not have an indiscriminate cytotoxic effect. Ampasavate et al. (2010) classified the toxicity degree of compounds as follows: IC_{50} < 10 µg/mL, very toxic; IC_{50} between 10 and 100 µg/mL, potentially toxic; and IC_{50} > 100 µg/mL, non-cytotoxic. If the IC_{50} of SE, WSMoL, and cMoL was found to be >100 µg/mL, these compounds would be considered non-cytotoxic to human PBMCs after 24 h of treatment.

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Table 1: Viability (%) of peripheral blood mononuclear cells after 24h of exposure to M. oleifera seed extract and lectins.

Concentration (µg/mL)	WSMoL	cMoL	Seed extract (SE)
0.78	100[a]	96.3 ± 1.33	-
1.56	98.33 ± 2.16[a]	83.63 ± 2.25 [a]	91.2 ± 2.71
3.12	98.33±1.77[ab]	85.78 ± 2.27 [a]	80.12 ± 1.76 [a]
6.25	90.3 ± 3.93[c]	84.42 ± 1.85 [a]	76.5 ± 1.92 [a b]
12.5	93.35 ± 1.6[abc]	82.5 ± 0.70 [a]	76.5 ± 2.32 [ac]
25	88.37 ± 1.77[c]	78.99 ± 2.25 [a]	79.44 ± 4.58 [ac]
50	85.1 ± 1.08[cd]	79.55 ± 2.33 [a]	71.52 ± 3.42[bc]
100	79.9 ± 1.3[d]	79.44 ± 0.85 [a]	60.21 ± 2.61
200	NT	NT	49.36 ± 1.39

Means followed by the same letter in the same column do not differ significantly. NT: no tested

As non-cytotoxic concentrations can also promote DNA damage and cause mutations that can lead to genomic instability, cancer, malformations, metabolic disorders, and neurological diseases (Poduri et al., 2013; Miyamae et al., 1998), the effects of SE and lectins on the DNA of PBMCs (Table 2) were evaluated. Accordingly, the comet assay was carried out using the extract and lectin concentrations that did not inhibit the viability of PBMCs by more than 40% in the MTT assay. Compared to the negative control, SE and cMoL displayed low but significant ($p < 0.05$) genotoxic effects, as the nucleoids in class 1 appeared at a slightly higher number. Further, significantly ($p < 0.05$) increased damage indices were observed for SE and cMoL at 50 and 100 µg/mL, respectively (Table 2). With regard to WSMoL, there was a significant ($p < 0.05$) increase in the number of nucleoids in all classes when treatments of 50 and 100 µg/mL were compared to the negative control. The damage indices also increased following all treatments, reaching 76.75% at the highest concentration (Table 2). The effects of SE, cMoL, and WSMoL were significantly ($p < 0.05$) higher than those of the positive control, MMS.

Table 2: Damage index for each class after 24 h exposed PBMCs to PBMCs treated with M. oleifera seed extract and lectins.

Treatment	Class					Damage index
	0	1	2	3	4	
NC	74.85±3.1#	20.28±2.12#	3.14±1.61#	1.57±0.75#	0.14±0.36#	31.85±2.9#
Seed Extract (SE)						
6.25 µg/mL	77.0 ± 1.53#	19.54 ± 1.80#	2.33 ± 0.5#	1.09 ± 0.3#	0	28.75 ± 2.34#
12.5 µg/mL	74.75 ± 1.73#	21.25 ± 2.16#	2.5 ± 0.57#	1.5± 0.5#	0	31.25 ± 3.07#
25 µg/mL	72.25 ± 1.70#	26.25± 1.89*#	1.25 ± 0.5#	0.75 ± 0.5#	0	31.0 ± 0.81#
50 µg/mL	69.25 ± 1.70*#	26.25 ± 1.5*#	3.75 ± 0.95#	0.75± 0.5#	0	36.0± 1.63*#
100 µg/mL	68.0 ± 1.41*#	27.0 ± 1.63*#	3.25 ± 0.95#	1.75 ± 0.5*#	0	38.75 ± 1.70*#
cMoL						
25 µg/mL	70.5±1.29#	25.5± 1.91*#	2.25±0.5#	1.75±0.5#	0#	35.25±0.95#
50 µg/mL	72.5±1.29#	20.0±0.81	3.75±0.5 #	3.0±0.81#	0.75±0.5#	39.5± 3.51*#
100 µg/mL	68.0±1.82*#	25.75±1.70*#	3.5±0.57	2.0±0.81#	0.75±0.5#	41.75 ± 2.62*#
WSMoL						
25 µg/mL	68.5±2.38#	23.75±2.95#	5.5±2.38*#	1.5±0.57#	0.75±0.5#	42.25±5.25*#
50 µg/mL	66.75±2.5*#	19.0±1.63#	6.0±0.81* #	6.25±0.95*#	2.0±0.81*#	57.75±5.67*#
100 µg/mL	50.0±3.55*#	32.25±5.90*#	8.75±0.5* #	7.0±2.44*#	1.5±0.57*#	76.75±5.61*#
MMS	2.50±0.57*	16.41±1.56*	28.5±1.73*	44.08±1.67*	7.66±0.86*	239.0±5.15*

NC = Negative control, Positive control = MMS. 0 = no damage; 400 = maximum damage. * p <0.05 in relation to the CN; # in relation to MMS; (ANOVA / Dunnet, p <0.05).

These findings were also evident when the frequency of damage (Table 3), which is essentially the percentage of cells displaying a tail, was assessed. The damage frequencies obtained with 50 and 100 µg/mL of SE were significantly ($p < 0.05$) higher than those of the negative control. This increase could be related to the increased concentration of cytotoxic and genotoxic compounds that have already been identified and reported in M. oleifera seeds, including some isothiocyanates (Ragasa et al., 2013; Villasenor et al., 1989). The genotoxicity of M. oleifera was also revealed using the Acinetobacter bioreporter model, which demonstrated that its genotoxicity was equivalent to that of 8.3 mg mitomycin C per 1.0 g of dry M. oleifera seed (Al-Anizi et al., 2014). Using cell-free plasmid DNA and the Salmonella typhimurium (Ames and Kado) assays, Rolim et al. (2011) demonstrated that the SE obtained from 800, 1000, and 1500 µg/mL concentrations of M. oleifera was mutagenic. Our results confirm that the

SE can be genotoxic. Therefore, caution should be exercised when selecting lectin concentrations for use in water treatment.

Table 3: Damage Frequency (%) observed in PBMCs exposed for 24 hours to *M. oleifera* seed extract and lectins.

Treatment	Damage Frequency
NC	25.14 ± 3.10#
MMS	97.5 ± 0.57*
Seed extract (SE)	
6.25 µg/mL	23.0 ± 1.77#
12.5 µg/mL	25.25 ± 2.41#
25 µg/mL	27.75 ± 1.54#
50 µg/mL	30.75 ± 1.17*#
100 µg/mL	32.00 ± 1.96*#
cMoL	
25 µg/mL	29.5 ± 1.29#
50 µg/mL	27.5 ± 1.29#
100 µg/mL	32.0 ± 1.82*#
WsMoL	
25 µg/mL	31.5 ± 2.38*#
50 µg/mL	33.25 ± 2.5*#
100 µg/mL	50.0 ± 3.55*#

NC = Negative control, Positive control = MMS. 0 = no damage; 400 = maximum damage. * p <0.05 in relation to the CN; # in relation to MMS; (ANOVA / Dunnet, p <0.05).

Regarding the lectins (Table 3), the frequency of damage to the cells treated with cMoL was significantly ($p < 0.05$) higher than that to the cells treated with the negative control, only when 100 µg/mL of cMoL was administered. However, the frequency of damage reached 50.0% when 100 µg/mL of WSMoL was used (i.e., half of the nucleoids displayed signs of damage). Currently, the genotoxicity mechanism of lectins is not completely known; however, some studies have indicated that some lectins can generate reactive oxygen species (ROS), which can promote DNA strand breaks (Bertholdo-Vargas et al., 2009). In addition, lectins can bind to specific carbohydrate structures on the cells, thereby causing dramatic changes in cellular morphology and metabolism, and can activate a cascade of signals that alter intermediary metabolism (Dang and van Damme, 2015; Yamamoto et al., 2013). Previous mutagenic and genotoxic assessments using Ames and Kado assays and a prokaryotic model, revealed that WSMoL is non-mutagenic or genotoxic at concentrations between 12.5 and 800 µg/mL (Rolim et al.,

2011). However, the genotoxic evaluation performed herein, with eukaryotic cells, revealed a dose-dependent damage. Thus, further studies must be carried out to elucidate the genotoxic effects of 100 µg/mL concentration of WSMoL on PBMCs.

4 CONCLUSION

According to the parameters employed in the present study, M. oleifera SE, which is often used to reduce water turbidity, and cMoL and WSMoL are non-cytotoxic to PBMCs after 24 h of exposure. However, the genotoxicity assessment using the comet assay revealed that all treatments, especially WSMoL, exerted a dose-dependent toxic effect. Thus, caution should be exercised when selecting a lectin/SE concentration for water treatment. Further research is needed to elucidate WSMoL genotoxic mechanisms.

DECLARATION OF INTEREST

The authors declare no conflicts of interest.

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