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Evaluation of the antioxidant, cytotoxic and radioprotective potencial of the lectin WSMoL from *Moringa oleífera* Lam. seeds

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Abstract: WSMoL (water-soluble lectin from the seeds of *Moringa oleifera* Lam.) is widely known for its biological properties, especially for its inflammatory, immunomodulatory and antitumor activity and is a promising candidate for a radioprotector of natural origin. **Aim of the study:** This study aimed to evaluate the antioxidant and cytotoxic potential and the radioprotective effect of WSMoL in human lymphocyte culture (*PBMC*). The concentrations analyzed were 850, 425, 212.5, 106.25 and 53.12µg/mL. **Materials and methods:** Antioxidant activity was tested using the DPPH and ABTS assays. Cell viability and cytotoxicity were assessed using the MTT assay on *PBMC* lymphocytes. Radioprotection was verified by the Alkaline Comet and Micronucleus with Blockage of Cell Cytokinesis assays after exposure to Cobalt-60 gamma radiation. **Results:** In the DPPH test, WSMoL was unable to capture the radical. In the ABTS test, the inhibition index (I%) was ≤10% and the IC_{50} was 71.42 g/L. The MTT test showed that the lectin was not cytotoxic and cell viability was over 79%, with a maximum (≤125%) at a concentration of 53.12 µg/mL. In the Alkaline Comet test, the Damage Index (DI) observed was high at concentrations of 850 and 53.12 µg/mL (≥170 ± 30). The Damage Frequency (*y*) observed through the Micronucleus assay was 0.260 for the two concentrations analyzed, similar to the *y* of the irradiated control group. **Conclusion:** WSMoL did not show significant antioxidant activity in the DPPH and ABTS tests. The lectin did not show a cytotoxic profile, and its use at a concentration of 53.12µg/mL is recommended. WSMoL showed no radioprotective capacity after exposure to gamma radiation at a dose of 2.5Gy.

Keywords: Lectin, WSMoL, Radioprotector, Radiation.

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Avaliação do potencial antioxidante, citotóxico e radioprotetor da lectina WSMoL das sementes da *Moringa oleífera* Lam.

Resumo: A WSMoL (lectina solúvel em água das sementes da *Moringa oleífera* Lam.) é amplamente conhecida por suas propriedades biológicas, principalmente por atividade inflamatória, imunomodulatória e antitumoral se apresentando como uma candidata promissora a radioprotetor de origem natural. **Objetivos:** Este estudo buscou avaliar o potencial antioxidante, citotóxico e o efeito radioprotetor da WSMoL em cultura de linfócitos humanos (*PBMC*). As concentrações analisadas foram de 850, 425, 212,5, 106,25 e 53,12µg/mL. **Materiais e métodos:** A atividade antioxidante foi testada através dos ensaios DPPH e ABTS. A viabilidade celular e citotoxicidade foram avaliadas pelo ensaio MTT em linfócitos *PBMC*. A radioproteção foi verificada pelos ensaios Cometa Alcalino e Micronúcleo com Bloqueio da Citocinese celular após exposição à radiação gama por Cobalto-60. **Resultados:** Pelo teste DPPH a WSMoL não foi capaz de capturar o radical. No ensaio ABTS o índice de inibição (I%) foi ≤10% e a IC_{50} igual a 71,42 g/L. A testagem MTT demonstrou que a lectina não foi citotóxica e a viabilidade celular encontrada foi superior a 79%, sendo máxima (≤125%) na concentração de 53,12 µg/mL. No ensaio Cometa Alcalino o Índice de Dano (DI) observado foi elevado nas concentrações de 850 e 53,12 µg/mL (≥170 ± 30). A Frequência de Dano (*y*) observada através do ensaio Micronúcleo foi de 0,260 para as duas concentrações analisadas, assemelhando-se à *y* do grupo controle irradiado. **Conclusão:** A WSMoL não apresentou atividade antioxidante significativa na testagem DPPH e ABTS. A lectina não exibiu perfil citotóxico, sendo recomendado seu uso concentração de 53,12µg/mL. A WSMoL não apresentou capacidade radioprotetora após exposição à radiação gama na dose de 2,5Gy.

Palavras-chave: Lectina, WSMoL, Radioprotetor, Radiação.

1. INTRODUCTION

One of the advances in science considered most important for screening, prevention and treatment of cancer patients has been ionizing radiation [1]. Although its use is fundamental, when it interacts with biological matter, ionizing radiation can cause alterations in the DNA molecule and produce free radicals that can cause cellular oxidative stress [2].

To date the FDA (*Food and Drug Administration*) has only authorized Amifostine (WR-2721) as a radioprotective agent, where it has been found that its molecule has the capacity to minimize adverse effects when applied before exposure to radiation. However, as it is a synthetic prodrug, it is highly toxic and can cause adverse effects such as nausea and vomiting [3].

Moringa oleifera Lam. popularly known as white acacia, is a plant native to southern Africa with a distribution in tropical and arid climate countries [4]. Its pharmacological potential, due to the presence of bioactive and biofunctional compounds, guarantees the effectiveness of its biological applications [5,6].

Isolated from the seeds of *Moringa oleifera* Lam. and purified primarily by Santos *et al.* (2005), WSMoL is a water-soluble acid lectin with specificity for cell surface carbohydrates, a molecular weight of 60kDa and the ability to bind to chitin [7,8].

Among the pharmacological properties already scientifically proven are in vivo antitumor activity against sarcoma-180, immunoregulatory effect with increased expression of pro-inflammatory cytokines, hypoglycemic potential, nematicide and antidepressant [9-12].

Thus, in view of the growing demand for new, non-genotoxic radioprotectants with proven clinical efficacy, WSMoL is a promising candidate of plant origin. In addition, studies with WSMoL suggest that the lectin possesses antioxidant capacity, which is strategically fundamental as a countermeasure to the deleterious effects of ionizing radiation.

2. MATERIALS AND METHODS

The DPPH (2,2-diphenyl 1-picrylhydrazyl radical) and ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) tests were used to assess antioxidant activity. The WSMoL was isolated according to the procedure described by Coelho *et al.* [8] and donated by the Protein Biochemistry Laboratory (BioProt) of the Biochemistry Department of the Federal University of Pernambuco. The isolated lectin was dissolved in 0.15M saline and used throughout the experiment. The DPPH test followed the methodology adapted from Blois (1958) [13]. Ascorbic acid 500µM with the DPPH radical and saline solution with DPPH were used as positive and negative controls, respectively.

For the ABTS method, the methodology according to Roberta *et al.* (1999) [14] was followed, with the positive control being gallic acid 500Mm / ABTS and the negative control, ethanol PA and the radical. When reading the absorbance, the ELISA was adjusted to a wavelength of 517nm (DPPH) and 734nm (ABTS). For both tests, 5 concentrations of lectin were analyzed, starting at 850µg/mL. At the end, the Inhibition Index (I%), which measures in percentage how much of the radical was reduced, and the IC_{50}, which measures the concentration needed to inhibit 50% of the radical under study, were calculated.

Using the MTT test (3-(4,5-Dimethylthiazol -2YL) -2,5-Diphenyl Tetrazoline Bromide), the cytotoxic action of the lectin was assessed in a *PBMC* cell culture at a concentration of 10^6 cells/mL. The five concentrations of WSMoL were tested using the Cell Proliferation kit (ROCHE©), according to the manufacturer's recommendations. Phytohemagglutinin (5mg/mL) was used as a positive control (process approved by the CCS-UFPE humans Use Ethics Committee, process 50965221.5.0000.5208).

The lymphocyte isolate was obtained by concentration gradient, from the dilution and consecutive centrifugations of whole peripheral blood from healthy donors with Ficoll-Paque Plus© (1:3 v/v). Cell viability was checked using 0.4% Trypan blue and cell counting in a Neubauer chamber; samples with viability ≥ 90% were accepted [15].

The genotoxicity and detection of the radioprotective effect of WSMoL was analyzed by the Alkaline Comet Assay, as protocoled by Singh *et al.* (1988) and Arivalagam *et al.* (2015) [16-17]. The samples were separated into groups according to radiation exposure: Group 1 (non-irradiated cells in RPMI medium), Group 2 (irradiated cells in RPMI medium), Group 3 (Amifostine, cells and RPMI medium), Group 4 and 5 (WSMoL 850µg/ml, cells, RPMI medium / Irradiated and non-irradiated).

They were incubated for 3 hours and 30 minutes in a dark environment at 37°C and after this period, the groups to be irradiated were exposed to radiation from a gamma source (Gammacell 220 60Co) at a dose of 2.5Gy (dose rate: 0.975 kGy/h and irradiation time: 4.716 sec), at the Department of Nuclear Energy (DEN-UFPE).

After irradiation, centrifugation, lysis, neutralization, electrophoresis and slide fixation steps were carried out. At the end of the experiment, the slides were stained with SYBR safe (Invitrogen®) and 500 cells per sample group were counted using fluorescence microscopy. The Damage Index and Damage Frequency were calculated by counting the degrees of damage of the comets counted after reading the slides [18-19].

The assessment of genotoxicity and radioprotection using the Micronucleus with Cytokinesis Blocking technique was carried out as recommended by the IAEA (International Atomic Energy Agency, 2011) [20]. Samples of 0.5mL of whole blood were incubated with the lectin WSMoL at concentrations of 850 and 53.12µg/mL for 3h, 37°C.

As a positive and negative control group, 0.5mL of blood was used without prior treatment with the lectin. After 3 hours, the samples were irradiated with a gamma source (Gammacell 220 60Co) at a dose of 2.5Gy. For comparison purposes, the negative control, a group treated with lectin at a concentration of 850 and a group treated with a concentration of 53, 12µg/mL were not exposed to radiation.

Once the cell pellet had been obtained, the slides with the samples were stained with 5% Giemsa and 500 binucleated cells were counted using light microscopy, as well as binucleated cells with apparent micronuclei.

All the tests carried out in this study were performed in triplicate. A statistical difference was defined as $p<0.05$. Using GraphPad Prism 7.0 software, it was possible to compare the results between groups using analysis of variance (ANOVA) and Bonferroni post-hoc.

3. RESULTS AND DISCUSSIONS

Table 1 shows the percentage inhibition of the DPPH and ABTS tests by the WSMoL lectin. It can be seen that there was no antioxidant action by the molecule, which maintained its I% with negative values at all concentrations, as well as the IC_{50}. On the other hand, in the ABTS test, the I% was positive, but did not exceed 8% and the IC_{50} was significantly high.

Table 1: Percentage inhibition of DPPH and ABTS tests by WSMoL.

Concentration (µg/mL)	I% DPPH	I% ABTS
850	-5.82	6.95
425	-6.73	7.56
212,5	-5.08	7.09
106,25	-5.68	7.75
53,12	-1.94	7.56
IC_{50}	**-16124.97**	**71420.94**

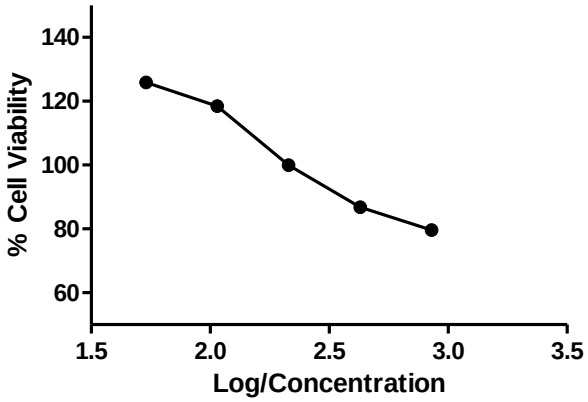
Source : Ferreira *et al.*

From the equation of the line in both tests, the R^2 value obtained was far from 1 ($R^2=0.2897$ for DPPH and $R^2= 0.4716$ for ABTS), indicating a weak correlation between concentration and absorbance [21]. Although WSMoL is not known at the molecular level, the structure of the phenolic compounds in a molecule, as well as the position and number of the hydroxyl group, are fundamental in terms of their ability to donate electrons and stabilize free radicals which, according to Aliboudhar (2014) [22], is decisive in eliminating these radicals, enabling the radioprotection of a given system.

Furthermore, according to Gulcin (2020), the efficiency of a studied substance in capturing free radicals is directly linked to the IC_{50} concentration, so the lower the IC_{50} value, the greater its ability to eliminate free radicals from the medium [23]. Therefore, the low antioxidant activity of the WSMoL lectin may be related to its chemical characteristics.

To obtain the IC_{50} using GraphPad Prism 7.0, the concentration values were plotted in terms of Log base 10, where the highest concentration corresponded to 2.93 and the lowest to 1.73 (Figure 1). The IC_{50} value found by the method was 1597µg/mL. When comparing groups, there was a statistical difference ($p \leq 0.0161$). The cell viability found ranged from 79.64 to 125.84%, with the maximum value referring to the concentration of 53.12µg/mL.

Figure 1: Cell viability by MTT.



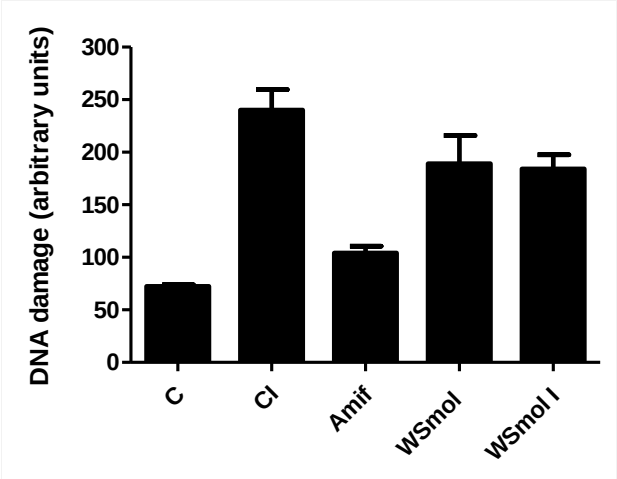
Source: Ferreira *et al.*

Peripheral blood mononuclear cells are the target of studies evaluating the cytotoxicity of natural or synthetic substances, as well as indicating crucial parameters on the immunomodulatory response in healthy cells. In genotoxicity tests resulting from exposure to ionizing radiation, *PBMC* cells are crucial for establishing the responsive use of innovative products because they are highly sensitive to low doses of radiation [24-26].

As for the use of *PBMC*, in studies with extracts and bioactives isolated from *Moringa oleifera* Lam. seeds, it was observed that the seeds contain glucomoringin isothiocyanate (GMG-ITC), a compound with promising therapeutic potential in the treatment of cancer, but highly

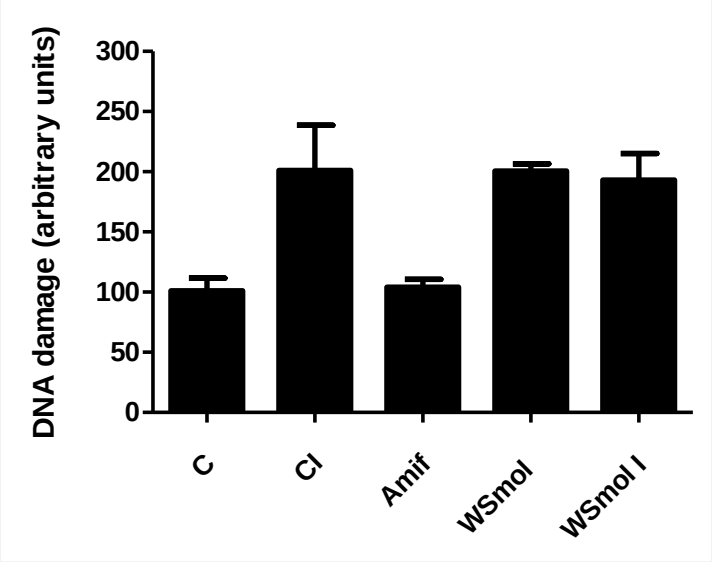
cytotoxic to mononuclear cell lines [27-29]. However, unlike the extracts, the lectin WSMoL does not show cytotoxicity for the lymphocyte isolate, as found in this study by Araújo *et al.*(2013) [30]. The measurement of the damage index (DI) using the alkaline comet assay can be seen in figures 2 and 3, for the concentrations of 53.12 and 850 µg/mL, respectively.

Figure 2: DNA damage assessment: Comet assay with lectin WSMoL 53.12µg/mL. C* = Non-irradiated control / CI = Irradiated control / Amif = Amifostine (Control +) / WSMoL = non-irradiated lectin group / WSMoL I = irradiated lectin group.



Source : Ferreira *et al.*

Figure 3 - DNA damage assessment: Comet assay with lectin WSMoL 850µg/mL. C* = Non-irradiated control / CI = Irradiated control / Amif = Amifostine (Control +) / WSMoL = non-irradiated lectin group / WSMoL I = irradiated lectin group.

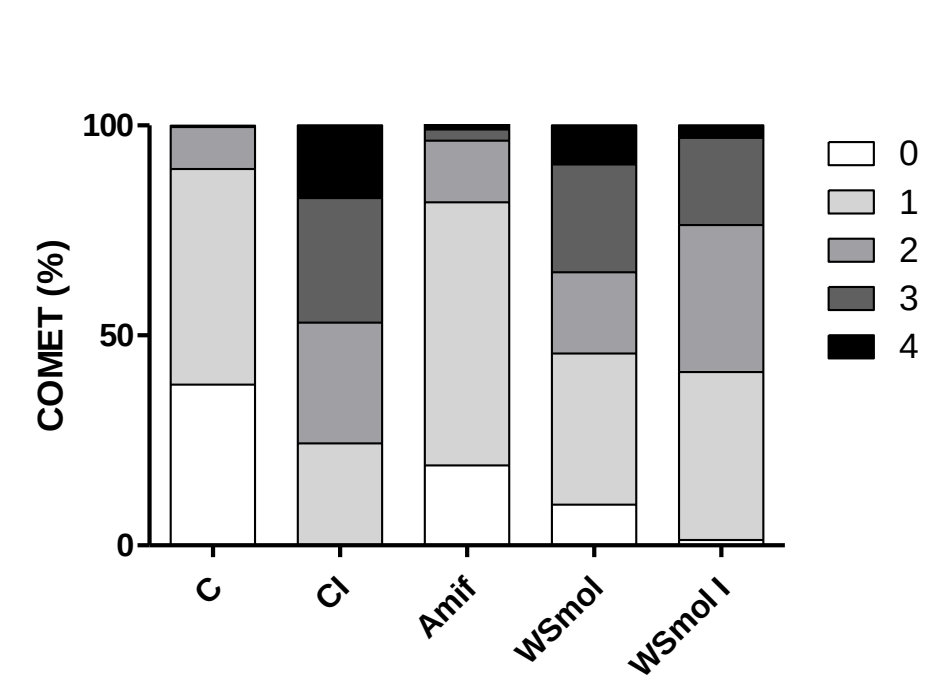


Source : Ferreira *et al.*

Bonferroni's analysis showed no statistically significant difference between the sample groups pretreated with the lectin and the DI of both groups was ≥ 170 ± 30. Comparing the non-irradiated control group (DI = 72.33 ± 33) and amifostine (DI = 104 ± 11) with the samples incubated with the lectin, it can be seen that WSMoL was unable to radioprotect the cells at the established dose of 2,5Gy. According to Siqueira *et al.*(2014), the absence of a substance's radioprotective property may be directly related to its limited antioxidant activity [31].

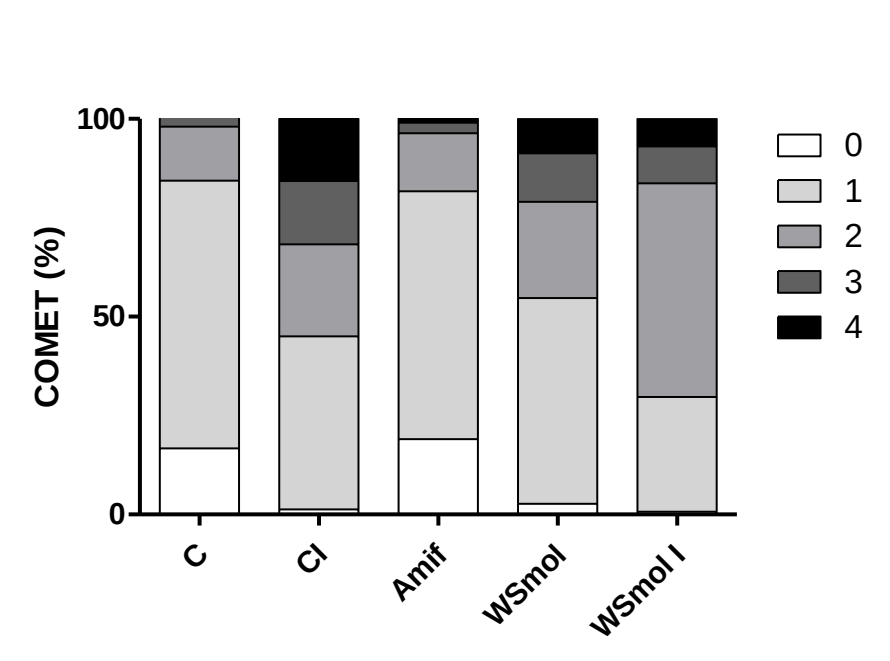
With regard to determining the percentage of comets by degree of damage after incubation and exposure to radiation, it was found that the lectin-treated groups - although not irradiated - showed a similar distribution of comets (DNA fragmentation) to the irradiated control group (Figures 4 and 5), with no statistically significant difference ($p \geq 0.05$).

Figure 4 - Comet percentage in *PBMC* cells treated with WSMoL 53.12µg/mL at an absorbed dose of 2.5 Gy. C* = Non-irradiated control / CI = Irradiated control / Amif = Amifostine (Control +) / WSMoL = non-irradiated lectin group / WSMoL I = irradiated lectin group.



Source : Ferreira *et al.*

Figure 5: Comet percentage in *PBMC* cells treated with WSMoL 850µg/mL at an absorbed dose of 2.5 Gy. C* = Non-irradiated control / CI = Irradiated control / Amif = Amifostine (Control +) / WSMoL = non-irradiated lectin group / WSMoL I = irradiated lectin group.



Source : Ferreira *et al.*

As for genotoxicity by cleavage with plasmid DNA, Rolin *et al.* observed no toxicity in samples tested with extracts, however, with WSMoL lectin the results were inconclusive, making it impossible to compare with the findings of this study [32].

A total of 3.000 BN cells were analyzed in the micronucleus test. The non-irradiated group incubated with WSMoL 53.12µg/mL showed a similar frequency of MN to the non-irradiated control, allowing us to infer that the lectin at this concentration has no genotoxic properties in *PBMC* culture. By Bonferroni pairwise comparison, there was no statistical difference between the samples treated with the lectin at a concentration of 850µg/mL and the irradiated control (Table 2), confirming that the lectin was not radioprotective.

Table 2: Micronucleus frequency (y) after treatment with WSMoL and 2.5Gy irradiation

Groups	*y*
Non-Irradiates Control	**0.042 ± 0.01**
Irradiated Control	**0.272 ± 0.02**
WSMoL 53,12µg/mL (Non-Irradiates)	**0.034 ± 0.01**
WSMoL 53,12µg/mL (Irradiated)	**0.260 ± 0.02**
WSMoL 850µg/mL (Non-Irradiates)	**0.132 ± 0.02**
WSMoL 850µg/mL (Irradiates)	**0.260 ± 0.02**

Source : Ferreira *et al.*

Due to the DNA damage observed in the alkaline comet assay and the high micronucleus frequency found in the CBMN test, especially at a concentration of 850µg/mL, it is recommended that an alternative evaluation with the modified enzymatic comet assay, be carried out with the enzymes Exonuclease III and/or FPG (Formamidopyrimidine-DNA Glycolysase) to elucidate the genotoxic profile of the lectins, i.e. whether the damage to the DNA molecule is due to ionizing radiation or the WSMoL itself [33].

4. CONCLUSIONS

WSMoL showed no antioxidant activity in the DPPH and ABTS radical scavenging tests. At the concentrations analyzed, the lectin was not cytotoxic. WSMoL was unable to radioprotect *PBMC* cells when exposed to 2.5 Gy gamma radiation.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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