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# Superoxide Anion Release by Human Blood Phagocytes Can Increase the Microbicidal Activity Induced by a New Microemulsioned System Containing Cotton Oil

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## Abstract

The study aimed to develop and characterize a microemulsified system based on cotton oil and verify its effect on superoxide release anion and microbicidal activity by human peripheral blood cells. Microemulsions were formulated using distilled water, degummed cotton oil, Span 80 (SP), Tween 80 (TW), and 1-butanol (BT). The pseudo-ternary diagram delimited ME regions, and the points were pre-selected. The physical-chemical and rheological characterization of the microemulsions was carried out. The ME activity on the interactions between leukocytes and bacteria was analyzed by superoxide release, phagocytosis, and microbicidal activity. The developed formulation was classified as Oil/Water, with an average pH of 5.76, and the viscosity showed resistance to temperature changes. The rheological model of the Power Law classified the microemulsion as a non-Newtonian fluid with pseudoplastic characteristics. The cell viability of cotton oil microemulsion was greater than 90%. There was an increase in the superoxide release by MN phagocytes when treated with cotton oil microemulsion. The cotton oil microemulsion increased phagocytosis and microbicidal activity. The present study suggests that cotton oil is an alternative biomaterial for therapeutic applications, especially in treating infections.

## Keywords

Cotton Oil, Microemulsion, Phagocytosis, Infection, Human Phagocytes

## 1. Introduction

Vegetables represent one of the alternatives among the various sources of inputs

needed to maintain society and have its main advantage: it is a renewable source [1]. Vegetable oils and fats are defined as products consisting mainly of fatty acids of vegetable species; may contain small amounts of other lipids, such as phospholipids, unsaponifiable constituents, and free fatty acids naturally present in oil or fat [2].

Among vegetable oils, cotton oil stands out with increased consumption and use due to its characteristic stability provided by the content of linoleic, palmitic, oleic, and stearic acids [3]. Also, this oil has a high concentration of antioxidants, such as vitamin E, contributing to increasing its conservation and helping in the organism's defense. Cotton oil can also maintain its properties even after heating, making it different from other vegetable oils [4].

The use of plants and oils for therapeutic purposes is a widespread practice in popular medicine and has increased in recent decades. Several studies using oils from different vegetables have reported beneficial effects on infectious processes with increased defense cell microbicidal activity [5] [6], however, to cotton oil, the results remain to be elucidated. There is a lack of information about its constituents, as well as about the biological potential offered to human health [7].

On the other hand, the interest in natural products and their association with nanoscience and nanotechnology makes this area a new knowledge level, with immense scientific and economic impacts [8]. Among the proposed actions, those related to health stand out, including the development of raw materials and functional materials associated with intelligent delivery systems [9] [10].

The development of drug and product delivery systems with therapeutic potential has increased in recent years, aiming to improve the treatment of diseases, increase efficacy, reduce toxicity, and increase bioavailability [11].

Microemulsions (ME) are modified systems for delivering drugs natural or synthetic products with therapeutic potentials. They are characterized as spherical aggregates with diameters less than 1400 Å, typically in the order of 100 Å [12]. From a pharmaceutical point of view, ME can be defined as transparent emulsions, in which an oil or lipophilic drug is dispersed in an aqueous medium (or vice versa), containing a surfactant, associated or not with an appropriate co-surfactant, generating a thermodynamically stable system [13].

Considering that cotton oil components are rich in linoleic acid and vitamin E, they can act in immunological processes; incorporating cotton oil in a microemulsified system can modulate phagocytes' functional activity in human peripheral blood. Thus, this study aimed to develop and characterize a microemulsified system based on cotton oil and verify its effect on superoxide release anion and microbicidal activity by human peripheral blood cells.

## 2. Materials and Methods

### 2.1. Composition of System

The microemulsions were formulated using distilled water, degummed cotton oil, from industrial extraction, with Hydrophilic-Lipophilic Balance (HLB) be-

tween 6.0 and 12.0, Sorbitan Oleate - Span 80® (SP) with HLB of 4.3 (Emfal®, Betim, Brazil), Polysorbate 80 - Tween 80® (TW) with HLB of 15.0 (Vetec®, Rio de Janeiro, Brazil) and 1-butanol (BT) (Vetec®, Rio de Janeiro, Brazil). The system was abbreviated as SP/TW/BT [14].

## 2.2. Determination of the Mixing Ratio for the Surfactants

To act as a co-surfactant, BT was used at a ratio of 10% of the surfactant mixture. The HLB of the mixture of surfactants and the fraction of surfactants to achieve the HLB required by the oil phase was calculated taking into account only the HLB of the surfactants SP and TW, according to Equations (1) and (2) [14]:

$$HLB_r = \frac{\left(HLB_{T1} \times F_{T1}\right) + \left(HLB_{T2} \times F_{T2}\right)}{(10) - \left(F_c\right)}. \quad (1)$$

$$F_{T1} + F_{T2} + F_c = 10. \quad (2)$$

where: $HLB_r$ represents the HLB required by the oil phase; $HLB_{T1}$ represents the $HLB$ of surfactant 1; $HLB_{T2}$ represents the HLB of surfactant 2; $F_{T1}$ represents the fraction of surfactant 1; $F_{T2}$ represents the fraction of surfactant 2; $F_c$ represents the fraction of the co-surfactant, with $F_c = 1$.

## 2.3. Development of Microemulsion Systems

Pre-established quantities of the components were used for the development of the system. After 24 hours at a temperature of 25˚C, the samples were classified visually into regions of MEL (liquid microemulsion), EL (liquid emulsion), EG (gel emulsion), and SF (phase separation).

Titrations were performed using samples present predominantly in the MEL region. During the process, the mixtures were vortexed. After homogenizing each volume of the titrant, the formulations were visually classified after 24 hours [14].

## 2.4. Construction of Pseudoternary Diagrams

The pseudoternary diagrams were constructed using the SigmaPlot 8.0 program with the samples' data and titrations performed. The upper vertex represents 100% surfactant/co-surfactant, the lower right represents 100% oily phase, and the bottom left represents 100% aqueous phase [14].

## 2.5. Pre-Selection of Systems

From the determination of the domain regions in the pseudoternary diagram, the composition of the systems that fall within the MEL region was determined. In this region, points distributed on straight lines that cut the region's segment were pre-selected to obtain representative samples of the systems characterized in later tests [14].

## 2.6. Quality Control of the Microemulsions

A method to verify the physicochemical parameters was designed to test the

formulations' suitability to act as delivery vehicles and perform each sample's initial characterization. The physicochemical characterization of formulations was made after 24 hours of preparation and was repeated at the end of the stability cycle.

For the analysis of heterogeneity, aliquots of the samples that showed greater visual homogeneity were made, and these were subjected to centrifugation at 300 xg, for 30 minutes in a BABYI centrifuge (FANEM®, São Paulo, Brazil). After centrifuging, samples with visual heterogeneity were excluded from the study.

The formulations' pH was verified using a pH meter (DEL Lab®, Araraquara, Brazil) previously calibrated with a standard solution of pH = 7.0 and pH = 4.0, to verify stability in the face of the possibility of decomposition reactions.

Electrical conductivity was evaluated by inserting the electrode directly into each sample, using a conductivity meter (LIDA®, São Paulo, Brazil) calibrated with standard KCL 0.1 mol/L solution, to identify whether the type of system is water in oil (W/O) or oil in water (O/W), and the possibility of a tendency to phase inversion.

For the stability study, the selected samples were submitted to different temperatures, being: refrigeration at 5˚C ± 1˚C and heating 40˚C ± 1˚C every 24 hours, completing the cycle on the 14th day. At the end of the cycle, the system's points with the highest thermal resistance were identified. After checking the thermal stability test, the sample's pH stability was verified, which showed effective results.

## 2.7. Rheological Characterization

The rheological parameters were determined in Modular Compact Rheometer - MCR 102 (Anton Paar®GmbH, Ostfildern, Germany). In all experiments, 600 µL of the microemulsions were added to the reading plate's surface, removing excess samples when necessary. The readings were performed with constant control of the measurement gap with 0.099 mm TruGapTM support, ToolmasterTM CP 50 cone measuring cell (angle 1˚), and temperature control with T-ReadyTM feature, using Rheoplus V3.61 Software.

For the flow curves, the established parameters were based on the control of the shear stress ($\tau$) of 0 Pa·s to 5 Pa·s for the ascending curve and of 5 Pa·s to 0 Pa·s for the descending curve, and the curves viscosity of 0 Pa·s to 0.4 Pa·s for the rising curve and 0.4 Pa·s to 0 Pa·s for the falling curve. These tests were carried out at a temperature of 25˚C. The flow curve and viscosity curve data for each sample obtained were adjusted to the Ostwald de Waele rheological model (Power Law).

## 2.8. Modulation of Blood Phagocyte Functional Activity by Degummed Cotton Oil Microemulsion

### Blood sampling and blood cell separation

Blood samples of approximately 10 mL were collected from 12 volunteer donors in tubes with an anticoagulant. The institutional Research Ethics Commit-

tee approved this study of the Federal University of Mato Grosso (Protocol number: 1.415.375). All of the subjects gave written informed consent before entering the experimental protocol. The samples were centrifuged (160 ×g; 15 min) to separate plasma from the cells. Cells were separated over a Ficoll-Paque gradient [Pharmacia, Upsala, Sweden], producing preparations with 95% of pure mononuclear cells analyzed by light microscopy. Purified monocytes were resuspended independently in serum-free 199 medium at a final concentration of $2 \times 10^6$ cells/mL. The cells were used immediately for superoxide release, phagocytosis, and microbicidal activity assays.

**Phagocyte treatment with degummed cotton oil and microemulsion**

The MN phagocytes [$2 \times 10^6$ cells/mL] and cotton oil microemulsion [50 µL - final concentration 100 ng/mL] were incubated and immediately used in the assays. A control was performed with only Medium 199.

***Escherichia coli* strain**

The enteropathogenic *Escherichia coli* [EPEC] used was isolated from stools of an infant with acute diarrhea [serotype 0111: $H^-$ $AL^-$, $eae^+$, $eaf^+$, $bfp^+$]. This material was prepared and adjusted to $10^7$ bacteria/mL, as previously described [15].

**Superoxide anion release**

Superoxide release was determined by cytochrome C [Sigma, St Louis, USA] reduction. Briefly, mononuclear phagocytes and bacteria were mixed and incubated for 30 min. to assess phagocytosis. Cells were then resuspended in PBS containing 2.6 mM $CaCl_2$, 2 mM $MgCl_2$, and cytochrome C [Sigma, St Louis, USA; 2 mg/mL], and degummed cotton oil [50 µL] or degummed cotton oil microemulsion [50 µL] was added. The suspensions [150 µL] were incubated for one hour at 37˚C on culture plates. A control was performed using only the spontaneous release of cells. The reaction rates were measured by absorbance at 620 nm, and the results were expressed as nmol/$O_{2^-}$. All of the experiments were performed in duplicate.

**Cellular viability and bactericidal assay**

Cellular viability, phagocytosis, and microbicidal activity were evaluated using the acridine orange method. Equal volumes of bacteria and cell suspensions were mixed and incubated at 37˚C for 30 min under continuous shaking. Phagocytosis was stopped by incubation on ice to eliminate extracellular bacteria. The suspensions were centrifuged twice [160 ×g; 10 min, 4˚C]. Cells were resuspended in Medium 199 and centrifuged. The supernatant was discarded, and the sediment was dyed with 200 µL acridine orange [Sigma, St Louis, USA; 14.4 g/L] for 1 min. The sediment was resuspended in cold Medium 199, washed twice, and observed under immunofluorescence microscopy at 400× and 1000× magnification.

The viability index was calculated by counting the number of orange-stained [dead] and green-stained [alive] cells out of 100. The phagocytosis index was calculated by counting the number of cells that ingested at least 3 bacteria in a

pool of 100 cells. To determine the bactericidal index, we stained the slides with acridine orange and counted 100 cells with phagocytized bacteria. The bactericidal index was calculated as the ratio between orange-stained [dead] and green-stained [alive] bacteria × 100 [16]. All of the experiments were performed in duplicate.

**Statistical analysis**

Data were expressed as the mean ± standard deviation [SD]. Statistically significant differences were evaluated using analysis of variance [ANOVA] followed by the Tukey post-test. Statistical significance was considered for a p-value less than 0.05.

## 3. Results

### 3.1. Determination of the Surface Mixture

The concentration of the BT co-surfactant was 10%. The percentage of SP and TW surfactants were calculated according to Equation 1 to achieve the Hydrophilic-Lipophilic Balance (HLB) required by Degummed Cotton Oil, as shown in Table 1. The fraction of the SP/TW/BT surfactant mixture, determined according to Equation 2, resulted in the HLB required by the oil phase.

### 3.2. Development of Microemulsioned Systems

We obtained 36 microemulsion formulations (Figure 1) with different stability characteristics, according to the proportions pre-established by the diagram of distilled water and cotton oil associated with the mixture of surfactants SP/TW/BT (2.0:7.0:1.0).

As shown in Figure 1, among the 36 formulations were obtained by mixing SP/TW/BT surfactants associated with Degummed Cotton Oil and distilled water, it was possible to visualize the formulations with phase separation characteristics (1, 5, 6, 9, 12, 13 14, 16, 19, 20, 24, 25, 28, 29, 32, 33, 34, 35 and 36), emulsion gel (3, 4, 11 and 18), emulsion liquid (2, 10, 17, 22, 23, 27 and 31), microemulsion liquid (7, 8, 15, 21, 26 and 30).

### 3.3. Pseudoternary Diagram and Pre-Selection of Materials

The pseudoternary diagrams that classify the region's points and domains are shown in Figure 2. In Figure 2(a), it is possible to observe that the formulations

Table 1. Concentration, fraction and hydrophilic-lipophilic balance (HLB) resulting from the mixture of surfactants SP/TW/BT.

| Surfactants | SP | TW | BT |
|---|---|---|---|
| Concentration (%) | 20 | 70 | 10 |
| Fraction | 2.0 | 7.0 | 1.0 |
| HLB | | 12.62 | |

Where: HLB = resulting from the mixture of surfactants.

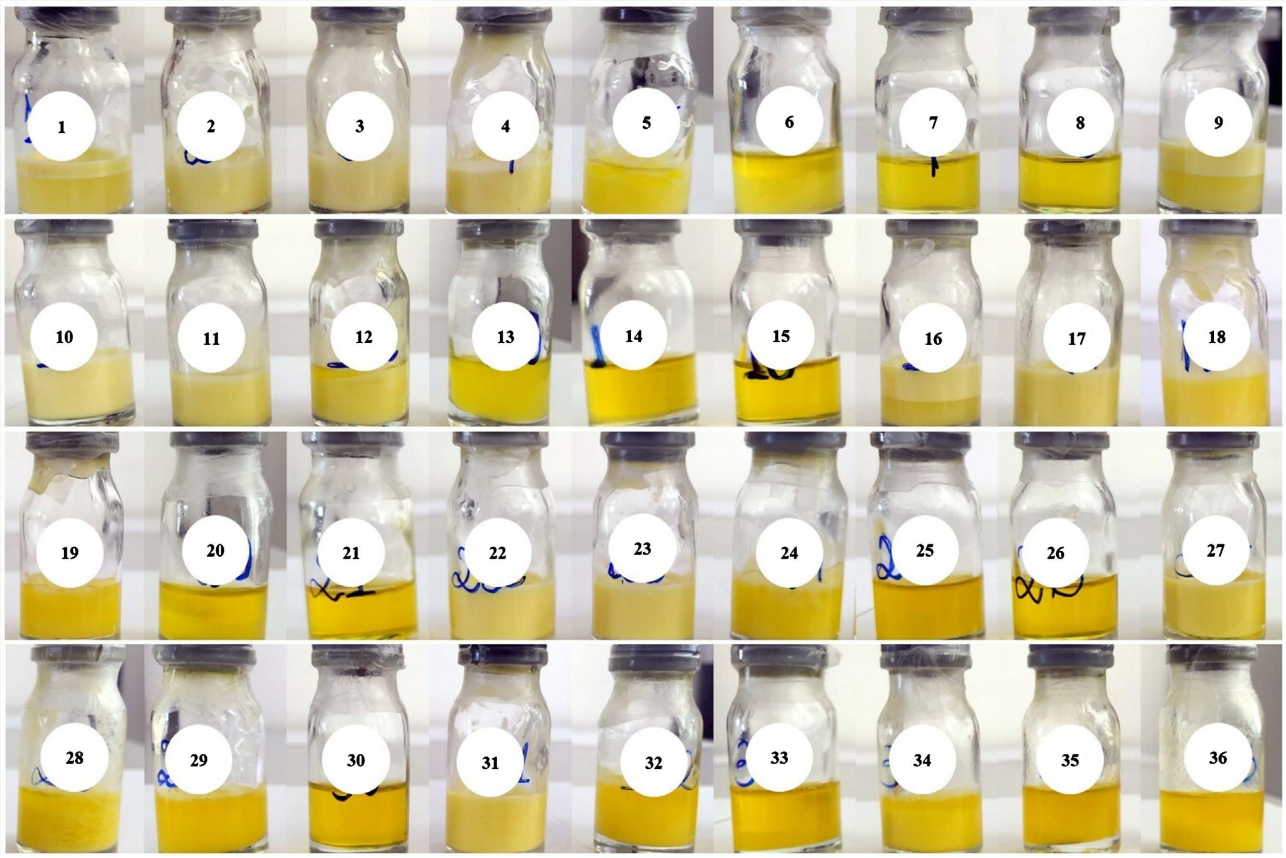


**Figure 1.** Formulations obtained from the SP/TW/BT system of 36 points with divergent equilibrium characteristics.

found above 40% of surfactants promoted stable materials (MEL). The samples found in this MEL region were used to study the SP/TW/BT system.

Figure 2(b) shows the region domains from the point diagram and the titrations. However, as in the point diagram, the materials located below the 40% mixture of surfactants showed instability and phase separation (EL, EG, and SF).

### 3.4. Physical-Chemical Characterization and Stability Study

The results of the physical-chemical analyzes (Analysis of Heterogeneity, Electrical Conductivity, and pH) of the points under alternating temperature cycles are shown in Table 2.

After the first analysis of heterogeneity (before the stability test), formulation 2M showed characteristics of MEG (Microemulsion Gel), and formulations 14M and 18M showed SF (Phase Separation), these 3 samples being excluded from the next analyzes. Formulations 6M, 7M, 9M, and 13M showed SF characteristics after the stability test and were also discarded from the study.

### 3.5. Rheological Characterization

Figure 3 shows the flow curves of the points selected for studying the SP/TW/BT system before (3a) and after (3b) the stability test. Figure 4 shows

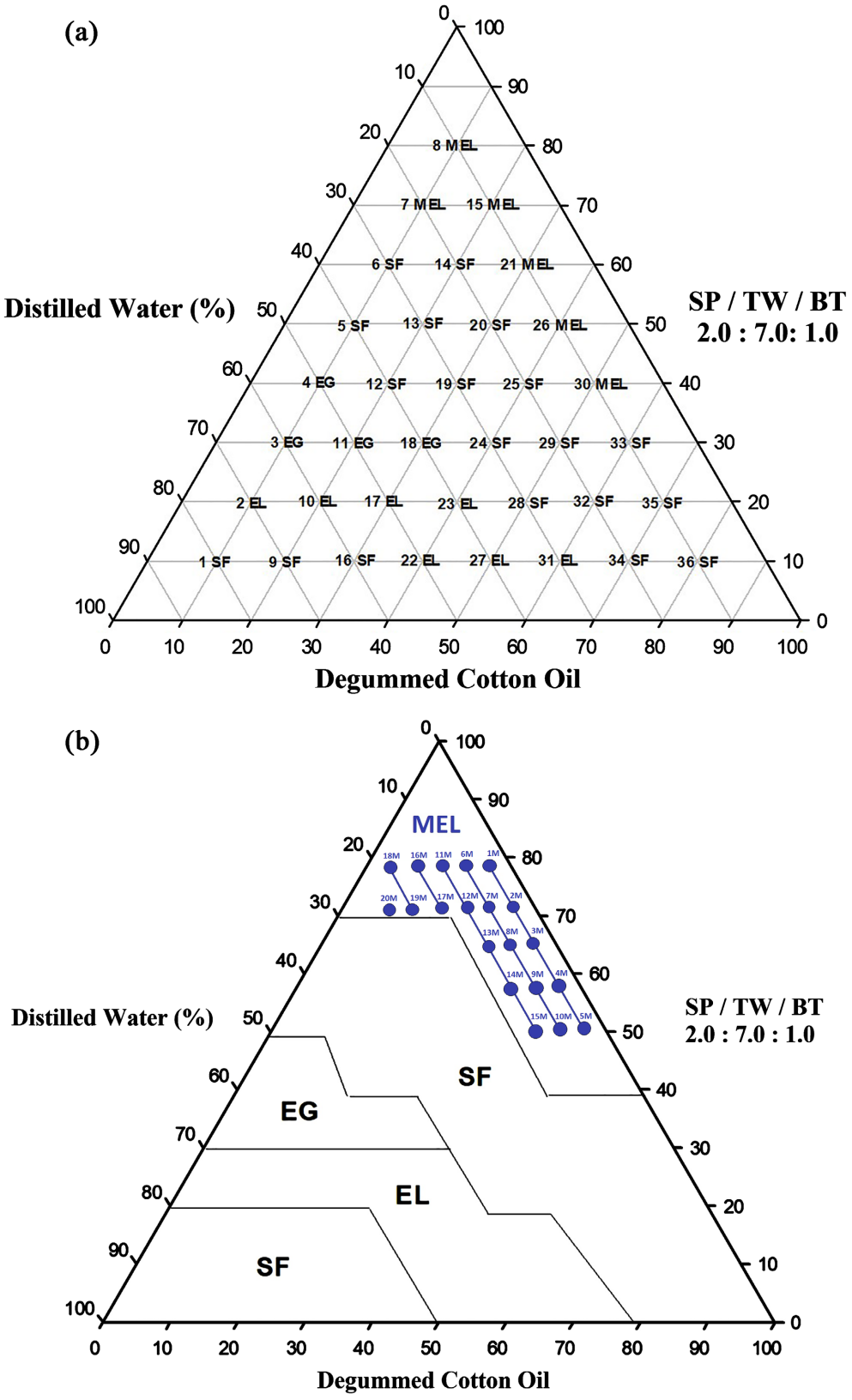


**Figure 2.** Pseudoternary diagrams for classifying points (a) and region domains with the selected microemulsions (b) from the SP/TW/BT system. Obs: Liquid Microemulsion (MEL), Liquid Emulsion (EL), Gel Emulsion (EG) and Phase Separation (SF).

Table 2. Physical-chemical characterization of microemulsion before and after the stability test.

| Points | Visual Aspects | | Heterogeneity Analysis | | Electric Conductivity (>1.3 μS·cm$^{-1}$) | | pH | |
|---|---|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After | Before | After |
| 1M | MEL | MEL | MEL | MEL | 62.2 | 44.4 | 4.4 | 7.0 |
| 2M | MEG | MEG | MEG | MEG | - | - | - | - |
| 3M | MEL | MEL | MEL | MEL | 20.2 | 20.3 | 6.6 | 6.8 |
| 4M | MEL | MEL | MEL | MEL | 14.6 | 6.1 | 5.7 | 2.5 |
| 5M | MEL | MEL | MEL | MEL | 7.1 | 6.1 | 6.5 | 7.9 |
| 6M | MEL | SF | MEL | SF | 3.3 | - | 6.6 | - |
| 7M | MEL | SF | MEL | SF | 35.8 | - | 6.2 | - |
| 8M | MEL | MEL | MEL | MEL | 23.8 | 12.8 | 4.7 | 7.6 |
| 9M | MEL | SF | MEL | SF | 10.5 | - | 6.4 | - |
| 10M | MEL | MEL | MEL | MEL | 7.0 | 4.1 | 5.7 | 7.3 |
| 11M | MEL | MEL | MEL | MEL | 6.5 | 1.4 | 5.3 | 3.2 |
| 12M | MEL | MEL | MEL | MEL | 8.4 | 21.5 | 5.8 | 6.6 |
| 13M | MEL | SF | MEL | SF | 14.9 | - | 6.4 | - |
| 14M | MEL | SF | SF | SF | - | - | - | - |
| 15M | MEL | MEL | MEL | MEL | 2.2 | 1.9 | 5.7 | 5.3 |
| 16M | MEL | MEL | MEL | MEL | 3.0 | 7.6 | 6.3 | 6.8 |
| 17M | MEL | MEL | MEL | MEL | 6.1 | 10.9 | 6.2 | 7.3 |
| 18M | MEL | SF | SF | SF | - | - | - | - |
| 19M | MEL | MEL | MEL | MEL | 4.1 | 5.2 | 6.2 | 8.8 |
| 20M | MEL | MEL | MEL | MEL | 2.1 | 1.7 | 6.7 | 8.6 |

MEL = Liquid microemulsion, EL = Liquid emulsion, EG = Gel emulsion and SF = Phase separation.

the points' viscosity curves before (4a) and after (4b) the stability test. Table 3 shows the average viscosity of each formulation before and after the stability study, respectively. The description of the rheological parameters of the degreased cotton oil microemulsion under the study conditions was carried out using the mathematical model of Ostwald-de-Waele (Power Law) and is summarized in Table 4.

## 3.6. Biological Assay

### Cells Viability Index

Table 5 shows the cell viability index in the presence of cotton oil microemulsion. Degummed cotton oil incorporated or not into the microemulsion did not

change the viability index.

**Superoxide Anion Release**

MN phagocytes showed greater release of superoxide anion ($p < 0.05$) when treated with the ME, in the presence or not of EPEC in comparison to phagocytes not treated with ME (Table 5).

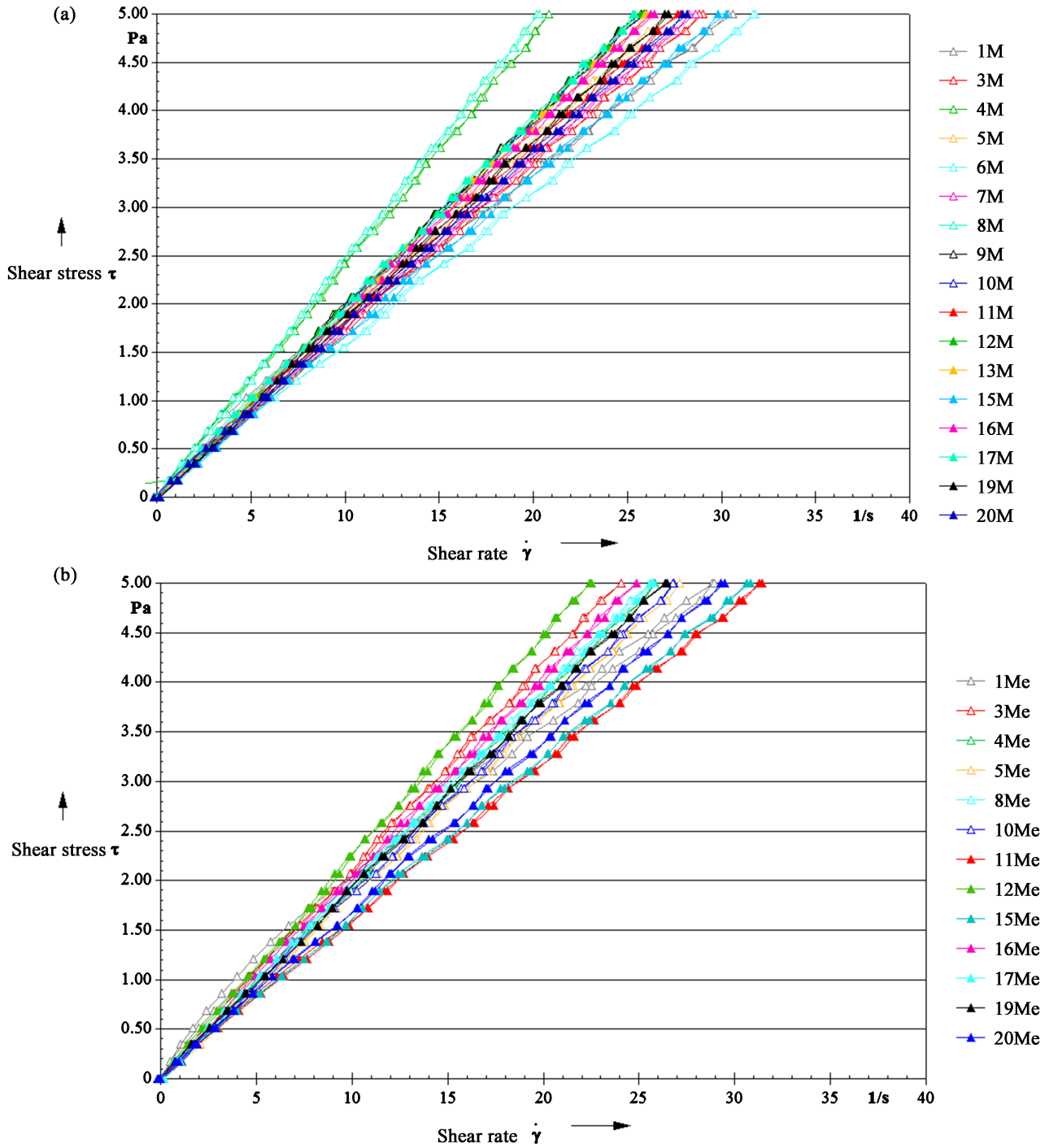


**Figure 3.** Flow Curve of pre-selected points (a) and selected points after 14 days of stability test (b) of the SP/TW/BT system. *Me = microemulsion after stability study.

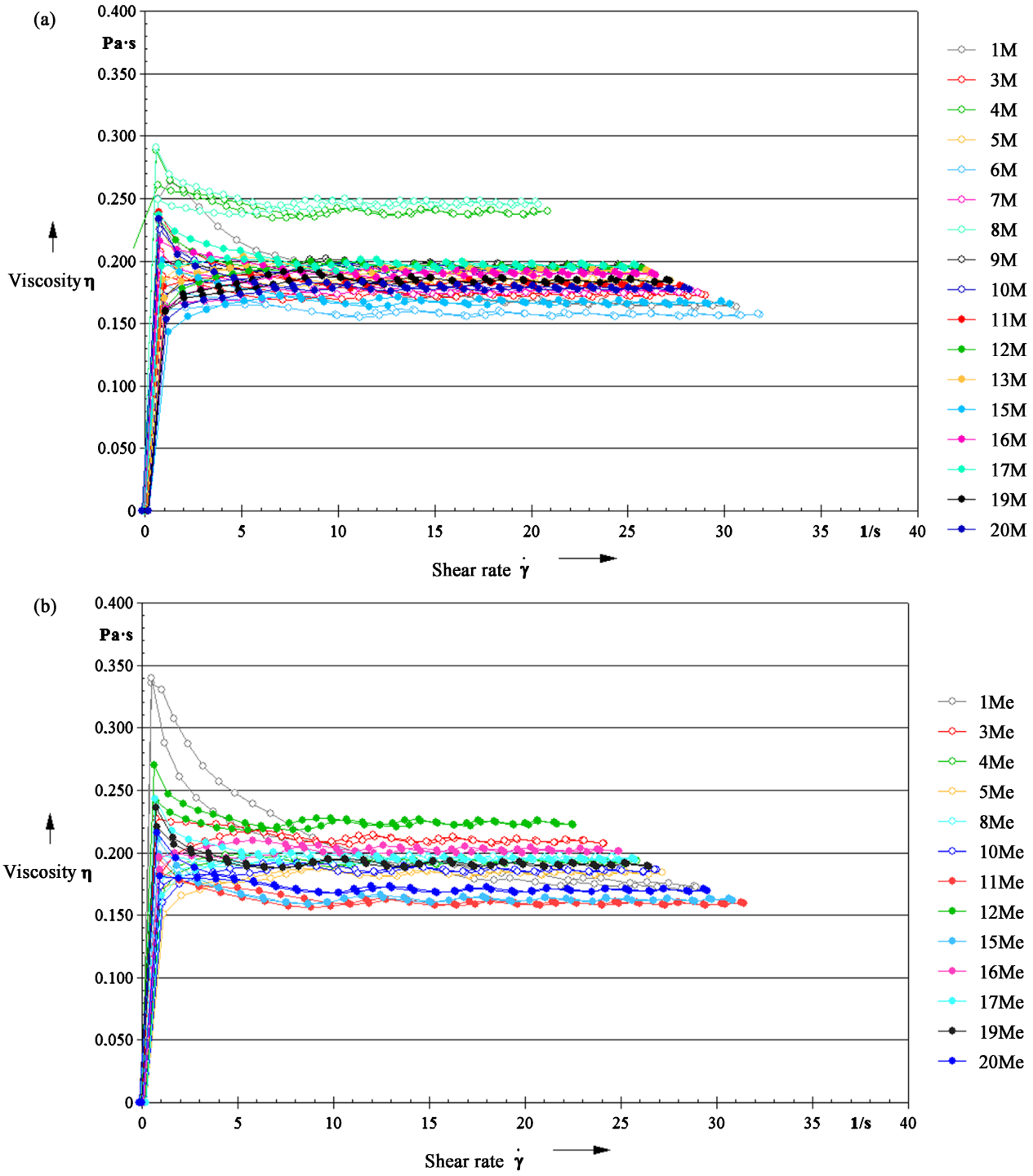


**Figure 4.** Viscosity curve of pre-selected points (a) and selected points after 14 days of stability test (b) of the SP/TW/BT system. *Me = microemulsion after stability study.

### Phagocytosis and Microbicide Index

The phagocytosis index and microbicidal activity of cells in the presence of EPEC and treated with ME are shown in Table 5. Phagocytic activity was higher in cells treated with ME. The microemulsion increased the microbicidal activity of MN phagocytes ($p < 0.05$).

**Table 3.** Viscosity of microemulsion before and after the stability test.

| Point | Average Viscosity (Pa·s) | |
|---|---|---|
| | Before | After |
| 1M | 0.1769 ± 0.0415 | 0.2031 ± 0.0576 |
| 3M | 0.1689 ± 0.0322 | 0.2042 ± 0.0387 |
| 4M | 0.2346 ± 0.0448 | 0.1859 ± 0.0351 |
| 5M | 0.1802 ± 0.0344 | 0.1754 ± 0.0333 |
| 6M | 0.1545 ± 0.0293 | - |
| 7M | 0.1704 ± 0.0324 | - |
| 8M | 0.2388 ± 0.0454 | 0.1885 ± 0.0354 |
| 9M | 0.1884 ± 0.0355 | - |
| 10M | 0.1870 ± 0.0356 | 0.1792 ± 0.0338 |
| 11M | 0.1789 ± 0.0348 | 0.1572 ± 0.0303 |
| 12M | 0.1887 ± 0.0362 | 0.2179 ± 0.0415 |
| 13M | 0.1863 ± 0.0352 | - |
| 15M | 0.1628 ± 0.0314 | 0.1601 ± 0.0313 |
| 16M | 0.1842 ± 0.0353 | 0.1949 ± 0.0366 |
| 17M | 0.1925 ± 0.0368 | 0.1890 ± 0.0365 |
| 19M | 0.1765 ± 0.0335 | 0.1869 ± 0.0359 |
| 20M | 0.1736 ± 0.0339 | 0.1673 ± 0.0322 |

The data represent the mean ± standard deviation, with $p > 0.05$.

## 4. Discussion

In this study, we developed a liquid microemulsion formulation from degummed cotton oil classified as oil-to-water. When subjected to temperature variations, we maintained its homogeneity, viscosity, and electrical conductivity biocompatible pH, non-toxic, stable, and capable of interacting with blood cells.

The stability of microemulsion depends intrinsically on interphase interactions of the emulsifier component and the immiscible phases. The characteristic ratio and selection of surfactant used in the preparation are provided by verifying the HLB [hydrophilic-lipophilic balance] of substances for evaluation of the behavior polar and nonpolar of compost [17], and the HLB values for a surfactants mixture maybe indicates that the systems present classification phase, O/W or W/O [5] [14].

The HLB value for vegetable oils can vary between 5.73 and 16.7 [18] [19] [20] [21]. In this work, the HLB resulting from the mixture of surfactants for the microemulsified system SP/TW/BT in a ratio of 7.0:2.0:1.0 presents an HLB value (12.62) close to that required by cotton oil (in the range of 5.73 to 16.7).

High HLB values indicate hydrophilic characteristics, thus providing a smaller

growth of the core and decrease the final particle size [22]. Due to this reduction in particle size, there are materials with greater thermodynamic stability [23]. In pseudoternary diagrams, frequency is observed in different regions and levels of stability, varying only the proportion of the components of the formulation [14] [24] [25] [26].

The variation stability increases the proportion of the oil phase. It results in a phase transition from translucent systems to opaque systems and, consequently, an increase in the phase separation region [14]. The same can be observed in the present study, in addition to the occurrence of non-microemulsified systems.

Other studies with microemulsions have shown that in addition to increasing the proportion of the oil phase, high proportions of the aqueous phase (approximately 80%) cannot form thermodynamically stable systems [24], which was found in this work, considering that translucent and homogeneous systems were found in the formulations with a maximum percentage of 30% of the aqueous phase.

Centrifugation is considered a screening test and does not necessarily indicate the formulations' actual physical stability. It increases the collision between the particles, increasing their sizes and destabilizing the formulations [27]. In the heterogeneity analysis performed after the stability test, it is observed that of the 17 formulations used, 13 remained stable even after 14 days subjected to variations in temperature conditions.

According to **Table 2**, all points were classified as O/W (oil in water) because they exhibit higher electrical conductivity values than distilled water, which is 1.3 μS/cm [28]. The pH values obtained before and after the 14 days of the stability test did not vary significantly. Point 1M with initial pH of 4.4 and points 4M and 11M with final pH of 2.5 and 3.2, respectively, were excluded from the study. The other points varied between 4.7 and 8.8, with the best result being the 15M point, with a final pH of 5.3, indicating biocompatibility. The human organism has values ranging from 4.5 to 7.0, depending on the tissue's anatomical region [29].

From this conclusion, the 15M sample was submitted to a new test. For 15 days, the pH stability was verified, and an average value of 5.76 was obtained, confirming the biocompatibility characteristics of this formulation.

The formulations have also been subjected to rheological analysis since this analysis monitors changes in the physical-chemical stability of microemulsions resulting from destabilization processes [30].

According to the flow curve of the selected points (**Figure 3**), it is observed that for most of the treatments evaluated, the behavior is characteristic of a non-Newtonian profile because it does not present linearity between shear stress and strain rate.

Microemulsions' rheological properties depend on the type, shape, density, number of components present in the system, and their interactions. Thus, microstructural changes caused by destabilizing agents such as time, heat, humidity, pressure, sedimentation, coalescence, and chemical reactivity are reflected in

the microemulsion rheology, directly affecting the viscosity and stability of the final products [31] [32]. Such factors may explain that the cotton oil-based microemulsion has a non-Newtonian behavior since changes in the structure are reflected in the microemulsions' rheological behavior.

Other factors can also modify the rheological properties of microemulsions, such as pH, a fraction of the dispersed phase, particle size, nature and concentration of the emulsifying agents, and viscosity of the continuous phase concentration of solids, mixing conditions, agitation, mixing devices, among others [33] [34].

Viscosity was similar between most points at the end of the stability test, remaining between the values of 0.16 Pa·s and 0.19 Pa·s (**Table 3**), presenting gradual and homogeneous reduction in viscosity and characteristic of resistance to temperature changes. According to the viscosity curves (**Figure 4**), there was an increase in the shear rate, reducing viscosity values, except for points 4M and 8M.

This reduction in viscosity with the increase in the shear rate can be justified by the change in the sample's molecular structure due to the generated force and temperature cycles, causing the particles to rearrange in parallel directions, causing the breaking of large particles into particles. Smaller ones, and these, in turn, can flow more easily in the direction of the applied tension [35] [36].

The rheological behavior can be described through mathematical models used to relate the shear stress to the strain rate. Among the most used equations to describe the non-Newtonian behavior of fluids is the Ostwald-de-Waele model that provides values to adapt the control of production lines, the design and dimensioning processes [37].

In the Ostwald-de-Waele model (Power Law), the flow behavior index (n) classifies the fluid as Newtonian when $n = 1$, and non-Newtonian pseudoplastic when $0 < n < 1$, and as non-Newtonian dilating when $1 < n < \infty$ [38] [39] [40]. In this study was observed that the degummed cotton oil microemulsion behaved as a pseudoplastic fluid ($n < 1$) in most formulations, as a dilator ($n > 1$) for the 5M, 8M and 17M, and as Newtonian ($n = 1$) for the 3M, 10M e 16M (**Table 4**). However, only the 1M, 4M, 11M, 12M, 15M and 20M formulations maintained the rheological behavior at the end of the thermal stability cycle. For all formulations, the correlation coefficient was higher than 0.99, indicating that the data was adjusted to Power Law model [41].

According to the physical-chemical tests, viscosity, and rheological parameters, it was possible to observe that only the 15M formulation was efficient in all analyses, maintaining homogeneity, viscosity, pH, and conductivity subjected to temperature variations. Therefore, biological tests were conducted with this formulation.

Several studies have demonstrated immunomodulatory, anti-inflammatory, antiseptic, and anthelmintic effects in medicinal plants [25] [42] [43] [44]. In this study, the microemulsified system did not alter the viability of blood cells (**Table 5**). The viability index was greater than 90%, indicating that degummed cotton

**Table 4.** Rheological parameters of cotton oil microemulsified before and after the stability test.

| Point | Parameters | | | | Fluid Classification | | | |
|---|---|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After | Before | After |
| | n | | K | | $R^2$ | | | |
| 1M | 0.8319 | 0.7859 | 280.3391 | 340.9378 | 0.9990 | 0.9990 | N-N:P | N-N:P |
| 3M | 0.9761 | 1,0010 | 185.0267 | 209.5998 | 0.9996 | 0.9993 | N-N:P | N |
| 4M | 0.9723 | 0.9953 | 257.0318 | 195.3514 | 0.9997 | 0.9996 | N-N:P | N-N:P |
| 5M | 0.9821 | 1.0178 | 194.2216 | 174.1819 | 0.9997 | 0.9997 | N-N:P | N-N:D |
| 6M | 0.9631 | - | 176.5669 | - | 0.9996 | - | N-N:P | - |
| 7M | 0.9700 | - | 191.7706 | - | 0.9995 | - | N-N:P | - |
| 8M | 0.9737 | 1.0160 | 262.9069 | 187.1855 | 0.9995 | 0,9996 | N-N:P | N-N:D |
| 9M | 1.0000 | - | 196.3699 | - | 0.9997 | - | N | - |
| 10M | 0.9861 | 1.0055 | 201.7643 | 183.4056 | 0.9995 | 0.9996 | N-N:P | N |
| 11M | 0.9464 | 0.9585 | 212.215 | 180.6389 | 0.9993 | 0.9996 | N-N:P | N-N:P |
| 12M | 0.9776 | 0.9749 | 207.7842 | 238.0784 | 0.9994 | 0.9994 | N-N:P | N-N:P |
| 13M | 0.9803 | - | 204.0367 | - | 0.9996 | - | N-N:P | - |
| 15M | 0.9557 | 0.9637 | 190.9287 | 180.2600 | 0.9994 | 0.9987 | N-N:P | N-N:P |
| 16M | 0.9659 | 1.0099 | 210.1665 | 196.1148 | 0.9997 | 0.9998 | N-N:P | N |
| 17M | 0.9584 | 1.0285 | 221.8053 | 180.0978 | 0.9997 | 0.9990 | N-N:P | N-N:D |
| 19M | 1.0265 | 0.9826 | 172.3625 | 199.8566 | 0.9999 | 0.9995 | N-N: D | N-N:P |
| 20M | 0.9615 | 0.9597 | 199.2181 | 191.1006 | 0.9992 | 0.9992 | N-N:P | N-N:P |

The data represent the mean ± standard deviation, with $p > 0.05$.

**Table 5.** Cell viability index, superoxide release ($O_{2^-}$), phagocytosis, and microbicidal activity of blood mononuclear phagocytes (MN) in the presence of the cotton oil microemulsified system.

| Grupos | Cell Viability (%) | Superoxide Release (nmol) | Phagocytosis (%) | Microbicidal Index (%) |
|---|---|---|---|---|
| Phagocytes | 97.83 ± 3.06 | 20.46 ± 2.48 | - | - |
| Phagocytes + ME | 94.00 ± 2.23 | 37.86 ± 4.65* | - | - |
| Phagocytes + EPEC | - | 19.83 ± 1.52 | 53.67 ± 2.16 | 40.08 ± 5.32 |
| Phagocytes + EPEC + ME | - | 40.90 ± 4.22* | 64.83 ± 3.06* | 52.28 ± 4.93* |

The data represent the mean ± standard deviation, with *$p < 0.05$. EPEC = Enteropathogenic *Escherichia coli*; ME = Microemulsion.

oil microemulsion does not have a toxic effect and confirm the biocompatibility characteristics previously studied in the stability tests. Similar results were found for babassu oil incorporated into microemulsion [5].

Several authors relate cell viability to functional activity, demonstrating the increased release of superoxide anion from blood and colostrum phagocytes through the study with medicinal plants [45] [46] [47]. During oxidative stress, cells can produce large amounts of superoxide radicals [48] [49]. The formation of free radicals has been associated with the functioning of the body's defense during infectious processes, mainly in intestinal infections [15] [16] [50], as well as the intensity of ROS production by phagocytes may act on the sensitivity of the tumor to some drugs [51]. In this study, the cotton oil microemulsion activated blood phagocytes. Furthermore, when treated by the microemulsion, these cells increased in superoxide anion, regardless of the bacteria's presence.

Increased release of superoxide anion by MN phagocytes has been associated with phagocytosis and microbicidal activity to eliminate microorganisms [16] [52] [53], including EPEC [54]. In this study, the release of superoxide anion by MN phagocytes treated with ME was reflected in phagocytosis and microbicidal activity.

In the presence of the microemulsified system containing degummed cotton oil, it was observed that the peripheral blood increased phagocytosis and microbicidal activity. Oils in plants such as babassu and buriti have also been reported as important phagocyte modulators capable of increasing microbicidal activity [5] [6]. In addition, these natural products have shown functionalities in the immune system capable of treating several infections [55].

Here, the immunomodulatory effects of degummed cottonseed oil incorporated into the microemulsion may be related to the presence of vitamin E. The literature has shown that vitamin E, taken in high doses in supplementation, can improve the immune response and increase bactericidal activity [56] [57]. However, it is necessary to carry out further studies to prove the immunomodulatory effect of vitamin E, abundantly present in degummed cotton oil, on human peripheral blood phagocytes' functional activity.

The present study is the first to report the increased activity of blood phagocytes associated with a degreased cotton oil microemulsion system, showing efficacy in the phagocytic and microbicidal activity. Therefore, it is an alternative to a potential biomaterial for future therapeutic applications.

## 5. Conclusion

In conclusion, the cottonseed oil microemulsion system had an immunostimulating effect on blood phagocytes, increasing the functional activity of the cells. In addition, since cottonseed oil is a natural and easily obtained product and is part of the cuisine of many countries, it can be used in the diet to improve the functional activity of cells. Furthermore, with the increased release of superoxide anion by blood phagocytes stimulated by the microemulsion, this system may be an alternative for future immunotherapy applications, especially for infectious diseases.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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