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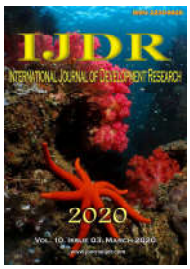

RESEARCH ARTICLE OPEN ACCESS

RED BLOOD CELL HEMOLYTIC ASSAY: AN ALTERNATIVE TO ASSESS CYTOTOXICITY OF ESSENTIAL OILS

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ABSTRACT

Given the growing ethical questioning regarding the use of animal testing in the analysis of toxicity research and *in vitro* assay validation is a viable alternative to animal replacement. In this sense, this study evaluated the hemolytic activity of four essential oils extracted from *Syzygiumjambolanum, Lippiagracilis*, *Myracrodruonurundeuva* and *Bixaorellana*plants, by applying the red blood cell hemolytic assay, since it is a simple technique, low cost, and effective to screening natural extracts cytotoxicity. With this purpose, a hemolytic assay was carried out in micro-centrifuge tubes containing 10% sheep erythrocytes suspension using samples in different concentrations previously established, at 37 °C/60 min. The hemolysis percentage was determined by spectrophotometer reading at 540 nm. Assays were performed in triplicate. The results showed values below 10% indicating that the essential oils did not show any hemolytic activity against the erythrocytes suspension, i.e. no toxicity was observed. These promising results are important for the use of the substances as well as to confirm the applicability and relevance of the test.

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INTRODUCTION

The constant reports of microbial resistance to antibiotics has led the pharmaceutical industry to search for new sources to develop new antibiotics(Tacconelli; Carrara; Savoldi; Harbarth; Mendelson; Monnet; Carmeli, 2018). Essential oils from plant materials such as leaves and flowers are known for possessing antimicrobial activity against various microorganisms, therefore, they may be an alternative for effective new antibiotics (Bučková; Puškárová; Kalászová; Kisová; Pangallo, 2018; Lingan, 2018). However, before releasing a new product for human use it is necessary to determine its efficacy and safety, and for that it must go through a few steps of pharmacological and toxicological analysis (Bowes, Brown, Hamon, & Jarolimek, 2012). Such analyses are performed in a series of preclinical and clinical trials.

The first tests are based on *in vivo* and *in vitro* models and the other ones are performed in humans (Anvisa, 2010; FDA, 2018). The safety assessment includes toxicity tests that guide regulatory agencies about the risks to health from exposure to these substances. Despite the fact that *in vivo* tests are essential for future research in human beings, these still causes death and suffering for a large number of laboratory animals, making it necessary to search for alternative practices in non-clinical studies (Jain; Singh; Dubey; Maurya; Mittal; Pandey, 2018; Moura *et al*., 2012; Brasil, 2013). *In vitro* cytotoxicity assays are accepted as the first toxicological tests in the biocompatibility evaluation of any material for biomedical or cosmetic use; some known alternatives are bacteria, crustaceans, tissue culture, cells and cell suspensions. Current research and discussions are intended to develop and validate alternative *in vitro* methods, which along with existing methods, may create an association that would allow a

Table 1. Hemolytic activity (% hemolysis ± standard deviation) of the four tested essential oils

Concentrations (µg/ml)	*Syzygiumjambolanum*	*Lippiagracilis*	*Myracrodrunurundeuva*	*Bixaorellana*
	%Hemolysis±SD	%Hemolysis±SD	%Hemolysis±SD	%Hemolysis±SD
25	0.00±0.005	0.00±0.001	-*	1.65±0.001
50	0.16±0.001	0.00±0.005	0.50±0.002	1.42±0.001
100	0.66±0.001	0.00±0.001	1.01±0.002	1.18±0.001
250	0.66±0.001	1.84±0.004	3.03±0.002	1.89±0.002
500	0.66±0.002	2.04±0.003	4.54±0.002	3.08±0.006
1000	1.33±0.004	2.25±0.006	6.56±0.005	4.50±0.008

*No significant activity were observed in this concentration ($p<0,05$).

Table 2. Essential oils concentrations capable of causing 50% of red blood cell death (HC_{50})

Essential Oils	*Syzygiumjambolanum*	*Lippiagracilis*	*Myracrodrunurundeuva*	*Bixaorellana*
HC50 (mg/ml)	45.25	19.91	6.68	14.77

reduction in the number of experimental animals (Jain; Singh; Dubey; Maurya; Mittal; Pandey, 2018; Bednarczuk; Verdam; Miguel, 2011; Kandárová & Letašiová, 2011). Easily assessed by spectrophotometry, a simple and low cost *in vitro* alternative is the test of hemolytic activity or hemolysis test. Since blood cells are exposed to any substance absorbed and/or injected intravenously, the determination of hemolysis caused after contact with such agents represents a potential for assessing the toxicity of new compounds (Bednarczuk *et al*., 2011; Bloom, 1993). Therefore, this test is used as a predictive toxicology screening method for different types of surfactants with the power to cause damage to the erythrocyte membrane (Alves, 2003; Selin *et al*., 2015). Thus, by evaluating the hemolytic potential of essential oils of *Syzygiumjambolanum*, *Lippiagracilis*, *Myracroduonurundeuva,* and *Bixaorellana*, known as possessors of some antimicrobial activity, this study aimed to use and assess the hemolytic test as an applicable and alternative method for*in vivo* screening assays of new substances for biomedical application thereby resulting in possible reduction of these tests.

MATERIALS AND METHODS

For the purpose of this paper, four essential oils extracted from *Syzygium jambolanum* (Department of Microbiology and Parasitology, UFRN)*, Lippia gracilis* (Plant Physiology and Biochemistry Laboratory, UERN), *Myracrodruon urundeuva* and *Bixa orellana* (Laboratory of Isolation and Synthesis of Organic Compounds, UFRN) were kept in the molecules collection of the Immunologic, Antimicrobial and Cytotoxicity Assays Laboratory of Federal University of Rio Grande do Norte. The methodology used, with some modifications, is an integration of some techniques (Selin *et al*., 2015) (Tariku, Hymete, Hailu, & Rohloff, 2011) according to some recommendations of the World Health Organization (WHO, 2011). Thus, it was initially prepared a 10% sheep erythrocytes suspension was prepared initially by centrifuging 500 µL of blood at 4000 rpm / 5min followed by three washes with removal of the buffy coat followed by addition of 1 ml PBS. After repeated centrifugations, when no hemolysis was visible, the red blood cells precipitate was suspended in PBS to 5 ml to obtain a 10% suspension. Subsequently, into 1.5 mL polypropylene microtubes containing the samples extract in varying concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, 1000 ug/ml) were added 200 uL of the erythrocytes suspension, which were then incubated at 37 °C/1h. As positive control, corresponding to 100% hemolysis, incubation was carried out under the same conditions with 1% Triton-X; as negative control PBS was used.

As solvent control, varying concentrations of DMSO were used. Each sample and control were done in triplicate. After incubation, the samples were cooled on ice (5 min) and subsequently centrifuged at 4000 rpm / 5 min. Then 100 µL of supernatant were transferred to a 96 well plate and the absorbance was measured at 540 nm on an ELISA reader (Spectra Max 340PC384, Molecular Devices). To determine the hemolysis percentage the control solution absorbance was subtract from the sample absorbance and the total was divided by the positive control absorbance. The final result was multiplied by 100. Dose-response curves were calculated as concentrations causing 50% hemolysis (HC_{50}) by linear regression.

RESULTS

To determine hemolytic activity of the four tested samples, the results obtained in this study were compared to the values determined by some authors(Kannan, Arumugam, Iyapparaj, Thangaradjou, & Anantharaman, 2013; Nofiani, Kurniadi, & Ardiningsih, 2011). Thus, the values that coincide with the range of 5-10% hemolysis were rated as low hemolytic activity and the ones above 40% as high activity. The four tested oils did not show any hemolytic activity against sheep erythrocytes at the used concentrations, 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, 1000 ug/ml. Table 1 shows that the average percentage of hemolysis caused by the substances in the higher concentration (1000 ug/ml), was below 10%, which is considered a non-significant reduced hemolytic activity (Nofiani *et al*., 2011). When charts were plotted in the dose-response curves (Figure 1), it was possible predict that the concentrations capable of causing 50% of cells death (HC_{50}) were low, as shown in Table 2 and visible in the charts as the outlier value.

DISCUSSION

In the search for newer alternative methodologies the hemolytic assay was used to assess the toxicity potential of some plant extracts (*JambolanumSyzygium, Lippiagracilis, Myracrodruonurundeuva, Bixaorellana*) known for their antimicrobial potential (Dantas *et al*., 2015; Loguercio, Battistin, Vargas, Henzel, & Witt, 2005; Michelin, Moreschi, Lima, Paganelli, & Chaud, 2005). Many aspects of this assay supported its use as an alternative for assessing *in vitro* toxicology. First, the variety of different methodologies, as the blood agar plates assay(D. M. S. Oliveira *et al*., 2013), the tube based hemolytic assay where hemolysis is detect by visual

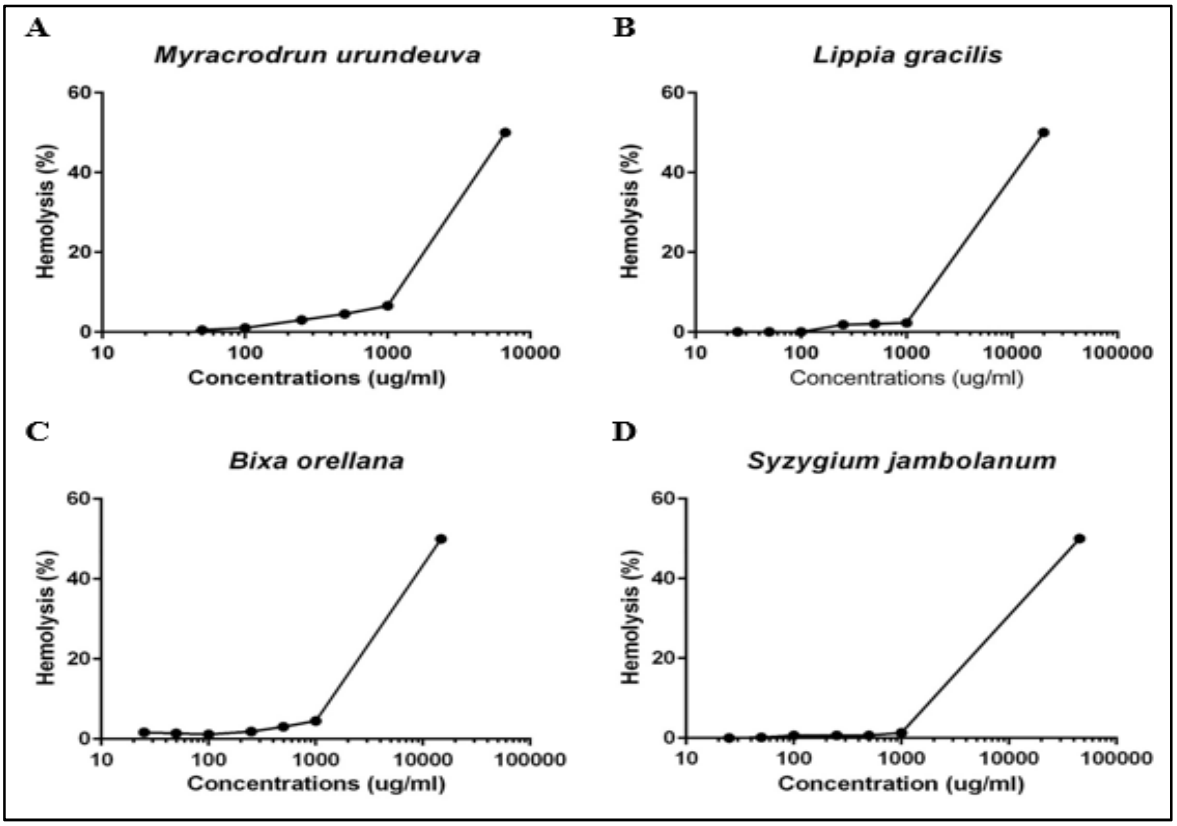

Figure 1. Percentage of Hemolytic activity dose-response (p<0,05). (A) Hemolytic activity dose-response in curve graph of *Myracrodrunurundeuva*oil.(B) Hemolytic activity dose-response in curve graph of *Lippiagracilis*oil. (C) Hemolytic activity dose-response in curve graph of *Bixaorellana*oil. (D) Hemolytic activity dose-response in curve graph of *Syzygiumjambolanum*oil

Reading (D. M. S. Oliveira *et al*., 2013) (D. M. S. Oliveira *et al*., 2013), thehemolyticassay in micro tubes based on spectrophotometricreading (Selin *et al*., 2015), and the microplate technique (Tariku *et al*., 2011). This test diversity allowedchoosing the best method suitable to the laboratory material resource. Another facet is that this test can be used as an screening method for different types of agents, as some cosmetics (Alves, 2003; Oliveira *et al*., 2013), drugs(Yamamoto, Tsurumaki, Takei, Hosaka, & Oomori, 2001), synthetic substances (Selin *et al*., 2015), nanoparticles (Dobrovolskaia *et al*., 2009), heavy metals (Ribarov & Benov, 1981), and especially plant extracts (DE Oliveira *et al*., 2009; Manetti *et al*., 2010; Desoti *et al*., 2011; Carvalho; Oliveira, 2012). Sensitivity is also a characteristic that make the hemolysis test a great option, for red blood cells are typically sensitive to the slightest toxic actionwhich would leads to disruption of the cell, due to their membrane fragility, resulting in hemolysis (Alves, 2003; Barreiro, 2009; DE Oliveira *et al*., 2009). Additionally, since red blood cells have direct contact with any substance absorbed and / or injected intravenously, they can be primary targets of toxicological actions (Bednarczuk *et al*., 2011; Bloom, 1993). Similar to the assay sensitivity is the simple technical process, which is based on the tested sample incubating with a blood suspension and subsequent measurement of released hemoglobin by spectrophotometry (Alves, 2003; Selin *et al*., 2015). Other points that may be considered in the technique choice as a solution for screening toxicity is the rapid execution, especially in this methodology where the test took an average time of 60 minutes in which the samples that present toxicity front red blood cells could be discarded, avoiding the unnecessary use of more refined technical resources (Blaauboer, 2002); the low cost, unlike some alternative *in vitro* tests as *Artemia salina*, *Caenorhabditis elegans* tests, and even some cell cultures, that are relatively simple and quick but require more refined care, the hemolytic assay has a simple and low cost maintenance requirements. Regarding the technical relevance and reliability of the results for the tested samples in this study, is important to notice that the plant samples used are commonly identified by having compounds called saponins, surfactants and responsible for the toxic action of most plants. Although the presence of these compounds may produces erythrocyte plasma membrane solubilisation, and subsequent cell lysis (Pequeno; Soto-Blanco, 2006; DE Oliveira *et al*., 2009; Carvalho; Oliveira, 2012), there are examples where some plant samples presenting saponins in their chemical composition, when evaluated by hemolysis test, showed no hemolytic activity. This fact was reported before when researchers assessed the hemolytic activity of different concentrations of *Myracrodruonurundeuva*(mastic of the hinterland), which was similar to the results obtained in our study for the same plant species(Carvalho & Oliveira, 2012). Another aspect showing the reproducibility and relevance of the test is present in the hemolysis values obtained for the *Bixaorellana*oil (Urucum/Annatto). Here, the Urucum (in English: annatto) sample, despite having presented the higher hemolytic activity percentage among other samples at the highest concentration (6.56% (±0.005)), it did not produce toxicity comparable to the values set as low hemolytic activity (<10%).These results of no toxic effect matches the data reported by some authors who when whensearching for data available about Urucum (annatto) found no record of genotoxic or carcinogenic related to it, and no toxicity was possible to be elucidate by acute toxicological studies (Silva; Braga; Da Silva, 2006). Some of the oils tested here when evaluated by other cytotoxicity methods showed similar results

to those observed in this study, for example, when the toxicity of *Syzygiumjambolanum*fruits was assessed front*Artemia salina*larvae, and no significant toxic effects at 1000 µg/ml were found (Barbosa *et al*., 2016). To establish the relevance of the technique it is essential to highlight its main strengths, by considering the graphical analysis (Figures 1-4) or concentration versus percentage of hemolysis.The test employed in this study was able to generate a forecast of the concentration capable to cause hemolysis in 50% of red blood cells. This prediction can help design further cytotoxicity preclinical tests by excluding potential harmful concentrations and / or molecules (Dobrovolskaia *et al*., 2009). Certainly, so far the evaluation of hemolytic activity is presented as an easy and reproducible technique, however this may have some limitations, such as the presence of substances with color and / or turbidity, which can interfere with the reading; the mixture of components with different toxic potential; different type of erythrocyte donors, such as rabbit, sheep or human, which make difficult the comparison of results. Nonetheless, the hemolysis test has been shown effective in routine use as a screening test for toxicity in pre-clinical evaluations, which means a significant reduction and refinement of animal testing (Pape *et al*., 1999; Alves, 2003; DE Oliveira *et al*., 2009).

Conclusion

The hemolysis test has many steps to achieve to be validating for regulatory purposes. However this technique has emerged as an excellent toxicological screening tool giving an initial guidance on the risk assessment of potential biomedical products. The technique has advantages that go beyond its limitations, and the results obtained to date confirm its promising role in the replacement and reduction in the number of animals used in scientific research.

Conflict of interest: The authors declare no conflict of interest regarding this work.

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