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Revisión | Review

Use of erythrocytes in cytotoxicity and toxicity assays of medicinal plant extracts: analysis of their application and bibliometric study

[Utilización de eritrocitos en ensayos de citotoxicidad y toxidez de extractos de plantas medicinales: análisis de su aplicación y estudio bibliométrico]

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Abstract: Plant species have been used for therapeutic purposes since ancient times and are still in use today since these products represent a source of raw material for the production of phytotherapeutic formulations. Screening and investigation of plants with pharmacological potential require the evaluation of characteristics related to their action, efficacy and safety in different steps. Among these steps, pre-clinical trials are used to evaluate the properties of the test product in *in vitro* experiments, such as cytotoxicity assays. Within this context, this study consists of a bibliometric analysis of some *in vitro* cytotoxicity and toxicity assays in erythrocytes used during bioprospecting of medicinal plants. The results demonstrated the wide application of erythrocytes to evaluate the biological effects of medicinal plant extracts. The methods were found to be valid and effective for the preliminary investigation of the *in vitro* cytotoxicity and toxicity of plant products.

Keywords: Plant extracts; Bioprospecting; Toxicity assays; Erythrocytes.

Resumen: El uso de especies vegetales para fines terapéuticos es una práctica histórica y todavía bastante actual, ya que estos productos pueden representar una fuente de materia prima para la producción de formulaciones fitoterápicas. En investigación de plantas con potencial farmacológico requiere la evaluación de su acción, eficacia y seguridad, a través de diferentes etapas. Entre estas, en los ensayos preclínicos se evalúan las propiedades del producto-prueba en experimentos *in vitro*, tales como ensayos de citotoxicidad, entre otros. En este aspecto, el presente estudio consiste en un análisis bibliométrico acerca de algunas pruebas de citotoxicidad y toxicidad *in vitro* en eritrocitos realizados en los ensayos de bioprospección de plantas medicinales. Los resultados evidencian la amplia utilización de eritrocitos para la evaluación de los efectos biológicos de extractos de plantas medicinales, apuntándolos como métodos válidos y eficaces para la investigación preliminar de la citotoxicidad y toxicidad *in vitro* de productos vegetales.

Palabras clave: Extractos vegetales; Bioprospección; Pruebas de toxicidad; Sustitutos sanguíneos.

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INTRODUCTION

The use of medicinal plants for healing purposes is an ancient practice (Brasil, 2006; Migliato *et al.*, 2007; Pereira & Cardoso, 2012). Since the dawn of mankind, for millennia plants were the only source of substances available to humans for therapeutic purposes (Migliato *et al.*, 2007). The indication of plants for therapeutic purposes is mainly based on the cultural and popular knowledge passed from generation to generation (Albuquerque & Hanazaki, 2006; Brasil, 2006), with this practice continuing to be an important method for the approach, indication and selection of plants with pharmacological potential (Maciel *et al.*, 2002).

Within this context, the screening and investigation of plant species with active pharmacological potential require analyses to elucidate and provide scientific evidence of characteristics such as their action and efficacy, permitting their evaluation as a possible source of new therapeutic agents for the treatment of diseases (Albuquerque & Hanazaki, 2006). Thus, in studies on medicinal plant extracts, in addition to proving the existence of a certain biological activity, parameters that provide information about their quality, efficacy and safety must be investigated during the different steps of the study (Noldin *et al.*, 2003; Varanda, 2006; Migliato *et al*., 2007). These approaches should encompass different sequential steps that, in addition to elucidating some therapeutic activity, should include pre-clinical and clinical studies. Pre-clinical studies comprise the evaluation of the pharmacological properties of the test product by means of *in vitro* and *in vivo* experiments (Ferreira *et al.*, 2009). Among the several assays carried out during initial bioprospecting of medicinal plants, we can mention assays for the evaluation of their chemical constituents, biological activity and specific biological effects, particularly through some *in vitro* techniques (Maciel *et al*., 2002).

Therefore, the aim of this study was to perform a bibliometric analysis of scientific articles that use erythrocytes in cytotoxicity and toxicity assays of medicinal plant extracts, highlighting some conceptual and methodological aspects of the use of these cell lines for these purposes.

REVIEW OF THE LITERATURE

Conceptual and methodological aspects of some in vitro cytotoxicity and toxicity assays using erythrocytes

Evaluation of hemolytic potential

Different substances, including extracts of medicinal plants to be used in living organisms, can interact with cells and cell organelles, causing different types of biological effects such as cytotoxic effects (Vo *et al.*, 2016). Possible deleterious cellular effects include reactions that lead to lipid peroxidation and oxidation of proteins, damaging the lipids and proteins of the cell membrane. These reactions are potentially harmful to cell viability since they induce an osmotic imbalance in the cell membrane that can culminate in cell lysis (Reddy *et al*., 2007).

For the evaluation of the biological effects of substances, *in vitro* studies have used cellular models such as erythrocytes in order to evaluate the capacity of test substances to exert cytotoxic effects through the induction of hemolysis (Brasil, 2012; Pinto *et al*., 2012; Vo *et al*., 2016). In this context, the observation of hemolytic effects after the exposure of erythrocytes to a given substance reflects the occurrence of deleterious cytotoxic reactions resulting from a set of perturbations that induce the formation of pores in the cell membrane, leading to destabilization and rupture of the membrane and subsequent hemolysis (Arbos *et al*., 2008; Vo *et al*., 2016). Thus, the demonstration of damage such as hemolysis indicates the cytotoxic effect of a substance on the erythrocyte cell membrane (Sharma & Sharma, 2001).

Investigations of this type are indicated and used for the preliminary *in vitro* toxicological evaluation of substances with potential pharmacological or cosmetic application (Brasil, 2012; Pinto *et al*., 2012; Vo *et al.*, 2016).

Evaluation of erythrocyte osmotic fragility (or anti-hemolytic effect)

In *in vitro* cytotoxicity assays, the integrity of the erythrocyte cell membrane in response to a substance is a good indicator for evaluation of the biological effects induced by the compound (Sharma & Sharma, 2001). However, in addition to investigation of the cytotoxic effects in assays such as the hemolytic activity assay, it is also necessary to determine whether a test substance exerts a protective effect on the cell membrane after hemolysis induction (He *et al.*, 2009; Waczuk *et al*., 2015).

In erythrocytes, this analysis constitutes an important criterion for assessing their *in vitro*

functional behavior and stability (Chikezie *et al*., 2012) by exposing these cells to different concentrations of sodium chloride (NaCl) and consequent adverse osmotic conditions that pose a potential risk to their viability, inducing changes in cell membrane integrity and increasing their fragility (He *et al*., 2009).

The analysis is based on the observation of hemolytic effects produced by the exposure of erythrocytes to an osmotic challenge capable of causing cell lysis. These results of the effects observed by exposing erythrocytes to a substance that potentially induces osmotic effects are compared to the results obtained for the test substance, which is investigated for its capacity to reverse and/or attenuate these anti-hemolytic cytotoxic effects (Chikezie *et al*., 2012). Hence, these cytotoxicity assays investigate the possible protective or inducing effects of the test compound in response to cell membrane alterations that occur under adverse osmotic conditions (Chikezie *et al*., 2012; Duarte *et al.*, 2015).

Thereby, the use of erythrocyte osmotic fragility testing for the evaluation of cytotoxic effects is consolidated in studies on the biological effects of substances, providing evidence of *in vitro* toxicological effects on erythrocytes (Reddy *et al*., 2007; Chikezie *et al*., 2012; Duarte *et al*., 2015).

Reactive oxygen species, stress and oxidative damage and the role of antioxidant substances in the regulation of these effects

Reactive oxygen species (ROS) are produced during cell metabolism and include reactive compounds such as hydrogen peroxide (H_2O_2) and free radicals such as singlet oxygen ($^1O_2$), superoxide ion (O^{2-}), and hydroxyl ions (OH^-). These compounds are toxic and are highly reactive with cell structures and components such as the cell membrane, proteins, enzymes and genetic material (Suh *et al*., 2011; Wen *et al*., 2011; Mohamed *et al*., 2013a; Casagrande *et al.*, 2014; Félix-Silva *et al*., 2014; Gangwar *et al*., 2014; Harsha & Anilakumar, 2014; Phrueksanan *et al.*, 2014; Salini *et al*., 2016).

The accumulation of free radicals is due to the high production of ROS or to the counterbalance between their production and degradation (Suh *et al*., 2011; Félix-Silva *et al*., 2014; Gangwar *et al*., 2014; Ghosh *et al*., 2014; Phrueksanan *et al*., 2014; Salini *et al*., 2016). This accumulation can lead to a progressive increase in cell injuries through the oxidation of biomolecules (Alves *et al*., 2010; Suh *et al*., 2011; Wen *et al*., 2011; Mohamed *et al*., 2013a; Félix-Silva *et al*., 2014; Gangwar *et al*., 2014; Ghosh *et al*., 2014; Harsha & Anilakumar, 2014; Phrueksanan *et al*., 2014) and the installation of oxidative stress (Suh *et al*., 2011; Wen *et al*., 2011; Félix-Silva *et al*., 2014; Gangwar *et al*., 2014; Ghosh *et al*., 2014; Phrueksanan *et al*., 2014; Salini *et al*., 2016), which can progress to a state of oxidative damage (Quirantes-Piné *et al.*, 2013; Ghosh *et al*., 2014).

However, although the organism is capable of combating the effects of the accumulation of free radicals produced during metabolism through the action of antioxidant enzymes that reverse and/or prevent the harmful biological effects resulting from oxidative reactions and processes (Alves *et al*., 2010; Wen *et al*., 2011; Quirantes-Piné *et al*., 2013; Gangwar *et al*., 2014; Ghosh *et al.*, 2014; Salini *et al.*, 2016), these endogenous antioxidant mechanisms may in certain cases be insufficient and supplementation with antioxidant substances from exogenous sources is necessary (Arbos *et al*., 2008; Alves *et al*., 2010). Thus, considering the capacity of antioxidant substances to prevent and/or reverse the oxidative reactions of biomolecules, as well as the occurrence of oxidative cellular stress and damage (Alves *et al.*, 2010; Wen *et al*., 2011), the indication of exogenous supplementation with antioxidant compounds is an important auxiliary tool for the maintenance of cell viability and consequently for health and well-being (Harsha & Anilakumar, 2014).

Evaluation of the antioxidant potential of medicinal plant extracts

There is an evident need for studies designed to identify new sources of antioxidant substances in order to add them to already known and available compounds. Within this context, special attention has been given to the elucidation of substances of "natural" origin, such as those derived from extracts of medicinal plants (Alves *et al*., 2010; Mohamed *et al.*, 2013a; Félix-Silva *et al*., 2014).

The antioxidant effects of bioactive compounds have been related to their capacity of eliminating free radicals, participation in chelating and reducing processes, and protective effects on antioxidant enzymes (Mohamed *et al*., 2010; Harsha & Anilakumar, 2014).

The antioxidant capacity of medicinal plant extracts can be investigated by *in vitro* assays using erythrocytes (Arbos *et al*., 2008) since these cells possess a characteristic antioxidant system that allows them to react with free radicals and to reverse to some extent the potentially harmful intracellular oxidative reactions (Arbos *et al*., 2008; Pinto *et al*., 2012). A substance with an oxidizing effect can induce oxidative stress by triggering oxidative reactions in cellular structures and components (Reddy *et al*., 2007). In erythrocytes, these injuries can occur in the hemoglobin molecule, leading to the transformation of hemoglobin from the reduced state to the oxidized state (methemoglobin). The antioxidant capacity of the test substance will be demonstrated when their use is able to decrease the levels of methemoglobin formation by erythrocytes (Arbos *et al*., 2008; De Andrade *et al*., 2012)

METHODS

This is a bibliometric analysis of studies that use erythrocytes in cytotoxicity and toxicity assays of medicinal plant extracts. In addition, the application of these cells in the investigation of plant extracts was analyzed, addressing some conceptual and methodological aspects of hemolysis and osmotic fragility tests and investigation of antioxidant potential using erythrocytes.

For the study, the National Institutes of Health's National Library of Medicine (PubMed) and Science Direct was searched using the following search terms and Boolean operators: "erythrocytes AND hemolysis AND plant extracts","(osmotic fragility OR antihemolytic) AND erythrocytes AND plant extracts", and "antioxidant potential and erythrocytes and plant extracts". Articles published over the last 10 years (2009-2018) were first selected. The searches retrieved in PubMed and Science Direct databases retrieved, respectively 203 and 638; 48 and 37; 137 and 1685 articles for each search strategy.

After this step, the abstracts were read to determine whether they are related to the topic proposed. As inclusion criterion, only articles that used extracts and/or fractions of the extracts of medicinal plants alone or in combination with other substances were considered for full-text reading. Articles reporting experiments with already isolated or synthesized substances or compounds of medicinal plants were excluded. In addition, only articles published in English were included.

After application of these criteria, a large number of articles were found, showing a extensive scientific production involving the use of these cells in tests of evaluation of cytotoxicity of extracts of medicinal plants. To synthesize these findings, it was compiled and included in this study the articles repeated in both databases. Thus, 100 articles were been selected in the bibliometric analysis, presenting data of plants and their parts utilized, citotoxicity assay evaluated, type of blood utilized and type of experimental study.

RESULTS

Several studies using erythrocytes for evaluation of the biological effects of medicinal plant extracts in cytotoxicity and toxicity assays have been published in the recent literature. Thus, based on the above considerations, studies that has include in inclusion criteria are presenting in Table 1.

Table No. 1
Some studies that use erythrocytes to evaluate the cytotoxicity and toxicity of medicinal plant extracts

Reference	Plant	Part used	Method	Type of blood	Type of study
Adesanoye *et al*., 2013	*Vernonia amygdalina*	Leaves	Antihemolytic and hemolytic activity	Human	*In vitro*
Aguiar *et al*., 2012	*Erythroxylum caatingae* Plowman	Stem	Hemolytic activity	Rat	*In vitro*
Ahmed & Rahman 2016	*Callistemon citrinus* Curtis.	Leaves	EOF	Human	*In vitro*
Ahumibe & Braide, 2009	*Garcinia kola*	Seeds	EOF	Rat	*Ex vivo*
Alinezhad *et al*., 2012a	*Hyssopus angustifolius*	Stem, flowers, leaves	Antihemolytic activity	Rat	*In vitro*
Alinezhad *et al*., 2012b	*Primula heterochroma*	Leaves	Antihemolytic activity	Rat	*In vitro*
Alinezhad *et al*., 2012c	*Hyssopus angustifolius* M.	Flowers, leaves, stem	Antihemolytic activity	Rat	*In vitro*

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Alves *et al*., 2017	*Caryocar coriaceum* Wittm.	Bark, pulp of fruits	Hemolytic activity	Human	*In vitro*
Aouachria *et al*., 2017	*Reichardia picroide* L.	Entire plant	Antihemolytic activity	Rat	*In vitro*
Aralbaeva *et al*., 2017	65 plants (*)	Different plants	EOF	Rat	*In vitro*; *Ex vivo*
Assadpour *et al*., 2016	*Allium rotundum* L**.**	Leaves	Antihemolytic activity	Rat	*In vitro*
Atrooz, 2009	*Citrus sinensis*, *Hordeum sativum*, *Triticum sativum*, *Canna indica*, *Citrullus vulgaris*,*Capsicum annuum*	Seeds	Antihemolytic activity	Human	*In vitro*
Azeez *et al*., 2010	*Cnidoscolus aconitifolius*	Leaves	EOF	Rat	*Ex vivo*
Bag *et al*., 2012	*Eugenia jambolana*	Seeds	Antihemolytic activity	Human	*In vitro*
Belkhiri *et al*., 2017	*Salvia verbenaca* L.	Leaves	Antihemolytic activity	Rat	*In vitro*
Bhakta & Silva, 2012	*Morinda tinctoria* (Roxb.)	Roots	Antihemolytic activity	Human	*In vitro*
Bianchini *et al*., 2017	*Mentha pulegium*	NS	Hemolytic activity	Human	*In vitro*
Bonarska-Kujawa *et al*., 2014	*Lonicera caerulea* L. var. *kamtschatica Sevast.*	Leaves, fruits	Hemolytic activity and EOF	Pig	*In vitro*
Bonfanti *et al*., 2014	*Solanum guaraniticum* A. St.-Hil; *Syzygium jambos*(L.)	Leaves	Antihemolytic activity and EOF	Human	*In vitro*
Bors *et al*., 2011	*Uncaria tomentosa*	Bark, leaves	Antihemolytic and antioxidant activity	Human	*In vitro*
Botta *et al*., 2014	*Ulva lactuca*; *Castinea sativa*; *Sargassum muticum*	NS	Antihemolytic activity	Rat	*In vitro*
Brandelli *et al*., 2013	*Aloe arborescens* Mill.; *Bidens pilosa* L.; *Rhipsalis bacifera*;*Luehea divaricata*; *Trichilia* sp.;*Campomanesia xanthocarpa* O. Berg.;*Coix lacryma-jobi* Lin.; *Citrus limonium*; *Citrus reticulata*; *Verbena* sp.;	Respectively: leaves; leaves; leaves; bark; roots; leaves; leaves; leaves; leaves; leaves	Hemolytic activity	Human	*In vitro*
Campos *et al*., 2016a	*Senna velutina*	Leaves	Hemolytic and antihemolytic activity	Human	*In vitro*
Carvalho *et al*., 2010	*Juglans regia* L.	Leaves	Antihemolytic activity	Human	*In vitro*
Casagrande *et al*., 2014	*Jacaranda decurrens*	Leaves	Antihemolytic activity	Human	*In vitro*
Chaity *et al*., 2016	*Drynaria quercifolia* L.	Rhizome, leaves	Antihemolytic activity	Human	*In vitro*
Chan *et al*., 2011	*Radix Astragali*; *Radix Codonopsis*; *Cortex Lycii*	Roots	Antihemolytic activity	Rat	*In vitro*
Chen *et al*.,2011	*Litchi chinensis* Sonn.	Flowers	Antihemolytic activity	Human	*In vitro*
Chikezie & Uwakwe, 2011	*Anacardium Occidentale*; *Psidium guajava*; *Terminalia catappa*	Leaves	Hemolytic activity	Human	*In vitro*
Chitemerere & Mukanganyama, 2014	*Callistemon citrinus*; *Vernonia adoensis*	Leaves	Hemolytic activity	Sheep	*In vitro*
Costa *et al*., 2009	*Cydonia oblonga*	Leaves	Antihemolytic activity	Human	*In vitro*
Cyboran *et al*., 2014	*Actinidia arguta*	Leaves	EOF and hemolytic activity	Pig	*In vitro*
Da Cunha *et al.,* 2016	*Eugenia uniflora*	Leaves	EOF	Human	*In vitro*
De Andrade *et al*., 2012	*Petiveria alliacea* L.	Leaves, stem, roots	Antioxidant activity on hemoglobin	Human	*In vitro*

De Araújo *et al*., 2018	*Anacardium occidentale* L.; *Anadenanthera macrocarpa* (Benth.) Brenan	Stem bark	EOF, hemolytic activity, oxidant and antioxidant activity on hemoglobin	Human	*In vitro*
De Freitas Araújo *et al*., 2012	*Leiothrix spiralis*	Leaves	Hemolytic activity	Human	*In vitro*
De Lima-Saraiva *et al*., 2017	*Schinopsis brasiliensis* Engl.	Bark	Hemolytic activity	Human	*In vitro*
De Toledo *et al*., 2011	*Annona coriacea* Mart.; *Curatella americana* L.; *Himatanthus obovatus* (M. Arg.) Wood; *Kielmeyera lathrophyton* Saddi; *Plathymenia reticulata* Bth; *Pterodonemarginatus* Vogel; *Qualea grandiflora* Mart; *Sclerolobium aureum* (Tul.) Benth.	Respectively: leaves; bark; leaves; bark; bark; bark; leaves; bark	Hemolytic activity	Human	*In vitro*
Deepika *et al*., 2017	*Ailanthus excelsa* Roxb.	Leaves, bark	Hemolytic activity	Sheep	*In vitro*
Dinis *et al*., 2012	*Castanea sativa* Mill.	Fruits	Antihemolytic activity	Sheep	*In vitro*
Duarte *et al*., 2015	*Raphiodon echinus* (Nees & Mart.) Schauer	Leaves	EOF	Human	*In vitro*
Ebrahimzadeh *et al*., 2011	*Ferula gummosa Boiss*	Roots	Hemolytic and antihemolytic activity	Rat	*In vitro*
Ebrahimzadeh *et al*., 2014	*Vicia sojakii*	Leaves	Antihemolytic activity	Rat	*Ex vivo*
Elwej *et al*., 2016	*Punica granatum* L.	Bark	EOF	Rat	*Ex vivo*
Espindola *et al*., 2016	*Campomanesia adamantium* O. Berg	Roots	Hemolytic activity	Human	*In vitro*
Ezike *et al*., 2015	*Buchholzia coriacea* Engl.	Leaves	Antihemolytic activity	Cattle	*In vitro*
Félix-Silva *et al*., 2014	*Jatropha gossypiifolia* L.	Leaves	Hemolytic activity	Human	*In vitro*
García-Huertas *et al*., 2013	*Solanum nudum*	Stem, leaves, fruits	Hemolytic activity	Human	*In vitro*
Girish *et al*., 2012	*Vigna mungo* L.	Barks	Antihemolytic activity	Rat	*In vitro*
González *et al*., 2013	*Cytisus scoparius* L.	Branches	Antihemolytic activity	Rat	*In vitro*
Guha *et al*., 2011	*Cyanthillium cinereum*	Entire plant	Antihemolytic activity	Human	*In vitro*
Guo *et al*., 2016	*Bletilla striata* (Reichb. f.)	Rhizome	Hemolytic activity	Human	*In vitro*
Hao *et al*., 2017	*Marsdeniae tenacissimae*	Stem	EOF	Human	*In vitro*
He *et al*., 2009	*Ginkgo biloba*	Leaves	Antihemolytic activity, Antioxidant activity and EOF	Human	*In vitro*
Hossain *et al*., 2014	*Spilanthes paniculata*	Leaves	Antihemolytic activity	Human	*In vitro*
Jiang *et al*., 2010	*Nelumbo nucifera Gaertn*	Rhizome	Antihemolytic activity	Rat	*In vitro*
Kaiser *et al*., 2009	*Allium cepa*	Bulb	Antihemolytic activity	Rat	*In vitro*
Karou *et al.,* 2011	*Balanites aegyptiaca* L. Delile.; *Entada africana* Guill. & Perr.; *Parinari curatellifolia* Planch. Ex Benth.	Leaves	Hemolytic activity	Rat	*In vitro*

Khalili *et al*., 2014	*Orobanche orientalis*; *Cucumis melo*; *Albizia julibrissin*; *Galium verum*; *Scutellaria tournefortii*; *Crocus caspius*; *Sambucus ebulus*; *Danae racemosa*; *Rubus fruticsos*; *Artemisia absinthium*	Respectively: leaves; leaves and fruits; leaves and flowers; leaves; leaves; leaves and bulb; flowers; leaves; leaves; leaves	Antihemolytic activity	Rat	*In vitro*
Krishnamoorthy *et al*., 2011	*Bruguiera cylindrica* (L.) Blume; *Ceriops decandra* Perr	Bark	Antihemolytic activity	Cow	*In vitro*
Lopes *et al*., 2016	*Curatella americana* L.	Leaves	Antihemolytic activity	Human	*In vitro*
Magalhães *et al*., 2009	*Cydonia oblonga* Miller	Fruits	Antihemolytic activity	Human	*In vitro*
Mapfunde *et al*., 2016	*Combretum zeyheri*	Leaves	Hemolytic activity	Sheep	*In vitro*
Meczarska *et al*., 2017	*Amelanchier alnifolia* Nutt.	Fruits, leaves	Hemolytic activity and EOF	Pig	*In vitro*
Mehreen *et al*., 2016	29 plants (*)	Different parts	Hemolytic activity	Human	*In vitro*
Mendes *et al.,* 2011	*Arbutus unedo* L.	Leaves, fruits	Antihemolytic activity	Human	*In vitro*
Meshkini, 2016	*Prunus dulcis*	Seeds	Antihemolytic activity	Human	*In vitro*
Mohamed *et al*., 2013b	*Hibiscus sabdariffa* L.	Flowers	EOF	Rat	*Ex vivo*
Moghaddam *et al*., 2012	*Diospyros lotus* L.	Seeds	Antihemolytic activity	Rat	*In vitro*
Nabavi *et al*., 2012	*Primula heterochroma* Stapf.	Leaves	Antihemolytic activity	Rat	*In vitro*
Nurain *et al*., 2017	*Cajanus cajan*; *Zanthoxylum zanthoxyloides*; *Carica papaya*	Respectively: leaves and seeds; leaves; leaves	EOF	Human	*In vitro*
Olchowik *et al*., 2012	*Rhus typhina* L.; *Vitis vinifera* L.	Leaves; seeds	Antihemolytic activity, antioxidant activity and EOF	Pig	*In vitro*
Parvin *et al.,* 2015	*Crescentia cujete*	Leaves, stem bark	EOF	Human	*In vitro*
Pauline *et al*., 2013	*Zanthoxyllum heitzii* (Aubrev. & Pellegr)	Leaves, fruits, stem bark, roots	EOF	Human	*In vitro*
Petreanu *et al*., 2016	*Solanum capsicoides* All.	Seeds	Hemolytic activity	Rat	*In vitro*
Phrueksanan *et al*., 2014	*Clitoria ternatea*	Flowers	Antihemolytic activity	Dog	*In vitro*
Pieroni *et al*., 2011	*Miconia albicans* (Sw.) Triana	Leaves	Antihemolytic activity	Human	*In vitro*
Portela *et al*., 2017	*Ilex paraguariensis* A. St. – Hil	NS	Antihemolytic activity	Human	*In vitro*
Ramamurthy *et al*., 2012	*Solanum torvum* L.	Fruits	Antihemolytic activity	Sheep	*In vitro*
Ribeiro *et al*., 2015	*Vismia cauliflora* A.C.Sm	Stem bark, flowers	Antihemolytic and antioxidant activity	Human	*In vitro*
Rios *et al*., 2017	*Solanum paniculatum* L.	Fruits	Antihemolytic activity	Rat	*In vitro*
Rjeibi *et al*., 2017	*Pittosporum tobira*	Seeds	Antihemolytic activity	Human	*In vitro*
Rocha *et al*., 2018	*Schinus terebinthifolius* Raddi	Leaves	Hemolytic and antihemolytic activity	Human	*In vitro*
Saba *et al*., 2010	*Parquetina nigrescens*	Leaves	EOF	Rat	*Ex vivo*
Sabuncuoğlu & Söhretoğlu, 2012	*Geranium tuberosum* subsp. *tuberosum*	Leaves	Antihemolytic activity	Human	*In vitro*
Sahaa *et al*., 2013	*Musa sapientum* var.	Leaves	Antihemolytic activity	Human	*In vitro*

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Salami *et al*., 2012	*Allium cepa*; *Allium sativa*	Bulb	EOF	Rat	*In vitro*; *Ex vivo*
Salini *et al*., 2016	*Scutellaria colebrookiana*; *Scutellaria violacea*	Roots	Antihemolytic activity	Human	*In vitro*
Santos *et al.*, 2016	*Hancornia speciosa* Gomes	Leaves	Antihemolytic activity	Human	*In vitro*
Sureka *et al.,* 2015	*Sesbania grandiflora*	Flowers	EOF	Rat	*Ex vivo*
Suwalsky *et al*., 2017	*Buddleja globosa*	Leaves	Antihemolytic activity	Human	*In vitro*
Suwalsky & Avello, 2014	*Ugni molinae* Turcz.	Fruits	Antihemolytic activity	Human	*In vitro*
Taib *et al*., 2009	*Litsea elliptica* Blume	Leaves	EOF	Rat	*In vitro*
Thakur *et al*., 2016	*Berberis aristata*; *Camellia sinensis*; *Cyperus rotundus*; *Holarrhena antidysenterica*; *Andrographis paniculata*,	Respectively: stem bark; leaves, roots; stem; leaves	Antihemolytic activity	Rat	*In vitro*
Tupe *et al*., 2015	*Syzygium jambolanum*; *Cephalandra indica*	NS	Antihemolytic activity	Human	*In vitro*
Walter *et al*., 2014	*Pistacia atlantica*; *Pistacia terebinthus*; *Pistacia chinensis*	Leaves and seeds	Hemolytic and antioxidant activity	Horse	*In vitro*
Włoch *et al*., 2013	*Crataegus spp*.	Bark, leaves	EOF	Pig	*In vitro*
Włoch *et al*., 2016	*Fagopyrum esculentum* Moench	Bark, stem	EOF and hemolytic activity	Pig	*In vitro*
Zhou *et al*., 2012	*Salvia miltiorrhiza*	Roots	Antihemolytic activity	Human	*In vitro*
Zia-Ul-Haq *et al*., 2011	*Capparis decidua* (Forsk.) Edgew	Leaves, flowers, fruits	Antihemolytic activity	Cow	*In vitro*

Legend: NS = not specified; (*) List available in the supplementary material of the cited article.
EOF = Erythrocyte osmotic fragility

DISCUSSION

Plant substances have been widely investigated because they are the main "natural" source of biocompounds and medicinal plants are a potential source of raw material for the production of drugs and/or phytotherapeutic formulations (Maciel *et al*., 2002; Fenner *et al*., 2006; Chagas *et al*., 2015).

Numerous biological effects are associated with the presence of one or more groups of different groups of secondary metabolites (Alves *et al*., 2017; Belkhiri *et al*., 2017) like alkaloids, flavonoids, tannins, glycosides, phenolics (Parvin *et al*., 2015; Belkhiri *et al*., 2017) and saponins (Belkhiri *et al*., 2017) and others present in plant composition.

In the research and development of new agentes, is important that they do not cause cytotoxic effects to eukaryotic cells (Zipperer & Kretschmer, 2017), being important identification and preliminary characterization of the potentially harmful effects of the use of these substances in bioprospecting studies (Waczuk *et al*., 2015; Campos *et al*., 2016b).

For this, cell health can be monitored by various methods. Thus, alterations like plasmatic membrane integrity, DNA synthesis, enzyme activity are important indicators of cell viability and cell death. Thus, methods to analyze cytotoxic and hemolytic effects are important predictors of the safety and efficacy of nem medicinal compounds (Zipperer & Kretschmer, 2017).

Erythrocytes has polyunsaturated lipids in their cell membrane (De Lima Saraiva et al., 2017) and their structural and biochemical characteristics, especially those related to a higher susceptibility to oxidative reactions (Arbos *et al*., 2008; Mendes *et al*., 2011; Okoko & Ere, 2012; Alinezhad *et al*., 2012b; Girish *et al*., 2012; Olchowik *et al*., 2012; Adesanoye *et al*., 2013; Khalili *et al*., 2014; Phrueksanan *et al*., 2014; Lira *et al*., 2018) make this cells suscetible to free radicals, causing damage in proteins, lipids, nucleus,mitochondrial membranes (De Lima Saraiva *et al*., 2017). Thus highlighting the execution of preclinical *in vitro* cytotoxicity assays with

erythrocytes, as demonstrated by the studies in the literature observed and in the examples discuted here.

Some phytochemicals compounds like saponins cause cytotoxicity effects (Casagrande *et al*., 2014) with haemolytic activity, showing that cytotoxicity effects of plant extracts depends on their chemical composition (Mapfunde *et al*., 2016). On the other hand, secondary metabolites like anthocyanins (Phrueksanan *et al*., 2014), tannins (Belkhiri *et al*., 2017), carotenoids (Alimi *et al*., 2012; Ribeiro *et al*., 2015; Espindola *et al*, 2016; Alves *et al*., 2017; Aralbaeva *et al*., 2017), polyphenols and flavonoids shows antioxidant and cytoprotective activities (Alimi *et al*., 2012; Ribeiro *et al*., 2015; Espindola *et al*., 2016; Alves *et al*., 2017; Aralbaeva *et al*., 2017; Belkhiri *et al*., 2017) on lipid oxidation and hemolysis in erythrocytes (Alimi *et al*., 2012; Phrueksanan *et al*., 2014; Ribeiro *et al*., 2015; Salini *et al*., 2016; Włoch *et al*., 2016). Thus, the presence of different phytochemicals compounds indicate that evaluation of antioxidant assays allows to determine the presence or absence of this important biological property in plant species (Mohamed *et al*., 2013a; Harsha & Anilakumar, 2014).

In this context, the validity of the cited *in vitro* assays has been mainly demonstrated by the marked use of erythrocytes, applying easy and relevant methods to predict whether or not the test product exerts cytotoxic effects on the cell membrane (Brasil, 2012; Pinto *et al*., 2012; Vo *et al*., 2016) and hemoglobin (Arbos *et al*., 2008; De Araújo *et al*., 2018) of erythrocytes.

These assays permit to perform spectrophotometric analysis (Magalhães *et al*., 2009; Carvalho *et al*., 2010; Mendes *et al*., 2011; Meczarska *et al*., 2017) in the evaluation of parameters observed by the studies in the literature and demonstrated here, such as hemolytic activity,once cell damage like hemolytic effects can be attributes to the dynamic and sensitive caracter of cell membrane, which undergoes significant changes when exposed to the action of exogenous toxic substances (Sharma & Sharma, 2001; Vo *et al*., 2016). On the other hand, investigations of erythrocyte osmotic fragility in situations of osmotic stress is also important to investigate markers of the susceptibility of cells to potentially hazardous osmotic changes (Reddy *et al*., 2007). Lastly, the investigation of antioxidant effects such as quantification of hemoglobin and methemoglobin can be justified once oxidant effects can increase the levels of methemoglobin formation (Arbos *et al*., 2008; De Andrade *et al*., 2012), causing potentially harmful intracellular oxidative reactions (Arbos *et al*., 2008; Pinto *et al*., 2012).

Taken together aspects discuted here can to elucidate that the use of the *in vitro* citotoxicity methodologies may provide preliminary data that permit to ensure or not the possibility of safe use for future indication and therapeutic use of products under pharmacological evaluation (Noldin *et al*., 2003; Varanda, 2006; Migliato *et al*., 2007; Waczuk *et al*., 2015), for example medicinal plants. However limitations of these assays can be related to the fact that although represents a good model for investigations and research of *in vitro*, specifically in biological cell membrane (Włoch *et al*., 2016), permiting an alternative quick, reproducible and cheap to carry out, enabling the evaluation of parameters before preliminary investigations *in vivo*, determination of citotoxicity effects like hemolysis activity and others are only an alternative screening process for evaluating simple toxicity (Mapfunde *et al*., 2016). Thus, although positive results for *in vitro* citotoxicity assays can sugest aditional investigations (Casagrande *et al*., 2014), further investigations are always necessary for the characterization of extracts and *in vivo* studies should be always performed to he sreening of new pharmaceutical products (Alves *et al*., 2017).

CONCLUSION

The investigation of plants for therapeutic purposes represents an important source of natural resources for the approach and selection of substances with pharmacological potential. The initial steps of bioprospecting of these substances should include the *in vitro* evaluation of parameters related to their safe use by analysis of specific actions and biological effects. Among them, evaluation of *in vitro* cytotoxic effects on cell lines such as erythrocytes in assays determining their hemolytic activity, osmotic fragility and antioxidant potential is widely used in the literature. In this respect, the evidence from studies that employ these methods confirms their validity and importance for the investigation of the *in vitro* biological effects of plant species with pharmacological potential.

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