In vitro erythrocyte oxidative damage of *Morinda citrifolia* L. (noni) leaves extract.

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**Abstract**

Slight decrease of hemoglobin and erythrocyte count was observed previously after subchronic oral dosing of Morinda citrifolia L leaves extract in rats. Induction of erythrocyte membrane damage could be the cause for these effects.

**Aims:** The objective of this investigation was to assess the in vitro cytotoxicity of Morinda citrifolia L leaves extract and fractions on rat erythrocytes.

**Methods:** Hemolytic damage was assayed in rat erythrocytes. Oxidative stress was assessed by measuring methemoglobin formation, thiobarbituric acid reactive substances (TBARS) and enzyme antioxidant activities, superoxide dismutase (SOD) and catalase (CAT).

**Results:** Morinda citrifolia L extract caused no hemolysis and induced oxidative damage to red cells in vitro. Methemoglobin increase was observed at concentration between 2 and 8 mg/ml of the extract. Lipid peroxidation was increased and CAT and SOD activities were depleted indicating a possible increase of hydrogen peroxide and superoxide radicals in erythrocytes. Ethyl acetate, dichloromethane and butanol fraction did not cause methemoglobin formation while water fraction increased methemoglobin level at doses up to 6 mg/ml.

**Conclusions:** We concluded that high doses of Morinda citrifolia L extract promote erythrocyte oxidative damage due to metabolites present in water fraction. These could be the cause of decreased erythrocyte and hemoglobin levels observed.

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**INTRODUCTION**

*Morinda citrifolia* Linn, popularly known as “noni,” has been used in traditional Polynesian medicine for over 2,000 years. *M. citrifolia* (Rubiaceae) is native from southeast Asia to Australia and is cultivated in Polynesia, India, the Caribbean region, and central and northern South America [1, 2]. Cultures native to these regions favored using *M. citrifolia* for treating major diseases and used it for nourishment in times of famine. Noni fruit has been recognized by cultures as an excellent source of nutrition [3]. Noni leaves have been consumed as a vegetable by many cultural groups. For this reason, it is included in the World Health Organization’s and Food and Agriculture Organization’s food composition tables for East Asia and the Islands of the Pacific [4].

Whereas noni juice and fruit have been well characterized chemically and pharmacologically [1-3], few data are available regarding the properties of *M. citrifolia* leaves. Despite the lack of experimental data on potential therapeutic properties, the use of noni for different purposes is still widespread in many parts of...
the world. However, infusions prepared with the aerial parts (stems and leaves) of noni are used in folk medicine to treat painful conditions and as a sedative.

Previous evaluation of oral toxicity of *M. citrifolia* leaves extract in rats show slight variations in few hematological parameters after subchronic oral dosing. Hemoglobin and differential leukocyte count were significantly affected and erythrocyte count was marginally affected after *M. citrifolia* subchronic exposure. Hematological varations were within or close to normal range and reversibly. The extract was non-toxic and non-genotoxic according to the results of subacute and genotoxicity assays [5]. A possible explanation for the erythrocyte and hemoglobin reduction in treated animals could be the induction of oxidative erythrocyte membrane damage. The objective of this investigation was to assess the in vitro cytotoxicity of *Morinda citrifolia* L leaves aqueous extract and fractions on rat erythrocytes.

**MATERIALS AND METHODS**

**Test substances**

Leaves of *M. citrifolia* were collected in April in the Medicinal Plant Experimental Station “Dr. Juan Tomás Roig” (Güira de Melena, Artemisa, Cuba). Voucher specimen (Nº 4741) was deposited at the "Dr. Juan Tomás Roig" (Güira de Melena, Artemisa, Cuba). Voucher specimen (Nº 4741) was deposited at the "Dr. Juan Tomás Roig" herbarium in the cited Experimental Station. The leaves were dried in a recycled air stove at 45°C for two days. Dried *M. citrifolia* leaves were extracted with demineralized water at 100°C for one hour with agitation. The extract obtained was dried with spray drier equipment as described previously [10]. A possible explanation for the erythrocyte and hemoglobin reduction in treated animals could be the induction of oxidative erythrocyte membrane damage. The objective of this investigation was to assess the in vitro cytotoxicity of *Morinda citrifolia* L leaves aqueous extract and fractions on rat erythrocytes.

To detect the presence of various chemical constituents in *M. citrifolia* extract, phytochemical screening was performed according to the method described by Garcia [7]. The extract was qualitatively analyzed for the presence of essential oils, terpenoids, flavonoids, glycosides, amines, amino acids, oligosaccharides, alkaloids, anthraquinone compounds, and coumarins. The phytochemical screening of the extract showed the presence of terpenoids, flavonoids, amines, amino acids, and anthraquinone compounds. The extract was standardized in accordance with the content of anthraquinone compounds and total anthracen-derived. Anthraquinone compounds were performed by quantification of colored phenols obtained by chemical reaction of alkali and anthracen-derived. Total anthracen-derived content was determined by quantification of colored phenols obtained by anthracen-derived phenols oxidation with ferric chloride in acid medium. Quantification was performed by using a spectrophotometer at 525 nm. Reference substance used was cobalt chloride 1% in ammonium alkaline solution equivalent to 0.43 mg of oxianthraquinone. Results were expressed as % w/v from calibration curve ($r^2=0.999$) [8]. *M. citrifolia* total extract with 2.09% of anthraquinone compounds and 11.21% of total anthracen-derived was used in the studies [6].

**Animals**

Animal care was performed in conformity with Canadian Council for Animal Care guidelines [9]. Healthy male Wistar (Cenp:Wistar) rats, 200-250 g of body weight, were used for obtaining blood. Animals were obtained from the Laboratory Animal National Centre (CENPALAB), Havana, Cuba and were housed together in polycarbonate cages in a light- and humidity-controlled biohazard suite (24 ± 2 °C; 55 ± 5% relative humidity), with a 12-hour light-dark cycle, and free access to drinking water and a standard laboratory diet CMO1000 (CENPALAB).

**Evaluation with M. citrifolia total extract**

**Hemolysis test**: Rat blood, containing heparin as anticoagulant, was centrifuged and the plasma and buffy coat discarded. The cells were washed four times with isotonic phosphate buffer (pH 7.4) and adjusted via oxyhemoglobin concentration (0.125 mmol/l) corresponding to about 8x10⁹ cells/ml for hemolysis test. Hemolysis assay was performed as described previously [10]. Five equidistantly increasing concentrations of the test sample and sodium dodecyl sulfate (SDS) as positive control were assayed. Percentage of hemolysis was determined by comparing the absorbance (560nm) of the supernatants with that of hemolyzed control samples.

For erythrocyte oxidative damage measure, the red blood cell (RBC) suspension was resuspended in isotonic phosphate buffer to a hematocrit of 5%. Test materials were added as solutions in purified water at concentration between 0 and 8 mg/ml; control suspensions received purified water alone. Additionally, a control vial with high dose of the test solution without RBC suspension was tested to discard color interference. The cell suspensions were incubated with test solutions at 37°C in a shaking water-bath. All experiments were performed in triplicate.

**Methemoglobin formation**: Methemoglobin level in erythrocytes was determined by the method of Evelyn and Malloy [11] in which the conversion of methemoglobin into cyan-methemoglobin is monitored spectrophotometrically at 635nm following the
addition of sodium cyanide.

**Measurement of lipid peroxidation**: Oxidative damage in erythrocytes was assessed by measuring the rate of lipid thiobarbituric acid reactive substances (TBARS) which were determined by a method based on the reaction of thiobarbituric acid with malondialdehyde (MDA) or MDA-like substances to produce a pink pigment with an absorbance maximum at 532nm [12].

**Measurement of antioxidant enzyme activities**: Catalase (CAT) activity was determined by the kinetic assay following the method of Beers and Sizerin [13] in which the disappearance of peroxide is monitored spectrophotometrically at 240nm. Catalase activity was expressed as unit of CAT / mg of protein.

Superoxide dismutase (SOD) activity was determined indirectly by a method based on inhibition of pyrogallol autoxidation [14]. Autoxidation of pyrogallol was recorded spectrophotometrically at 420 nm during 1 min after addition of pyrogallol (0.1 mM final concentration) in medium containing 50 mM TRIS buffer (pH 8.2). The linear slope (with $r^2>0.99$) was calculated, and the increase in absorbance after this time was taken as the initial rate of autoxidation. Linear slope inhibition was indicative of SOD activity.

**Evaluation with M. citrifolia fractions**

Dichloromethane, ethyl-acetate, n-butanol, and aqueous fractions were tested for methemoglobin formation as described above to identify the fractions with erythrocyte toxicity *in vitro*. Dichloromethane, ethyl-acetate, and n-butanol fractions were re-suspended in dimethyl sulfoxide, and dissolved in purified water up to concentration of 10 mg/mL. Fractions were tested at concentrations between 0 and 0.8 mg/mL. Upper concentrations were not tested for solubility. Water fraction was tested in purified water at concentration between 0 and 14 mg/mL.

**Statistics**

Results were expressed as the mean ± SEM. All statistical analysis was assessed using the GraphPad Prism Version 5 (GraphPad Software, San Diego, California, USA). Each test group was compared with control. One-way analysis of variance (ANOVA) and the Tukey-Kramer Multiple Comparisons Test were performed. Statistical significance was considered at $p<0.05$.

**RESULTS**

**Evaluation with M. citrifolia total extract**

No hemolysis was observed when *M. citrifolia* extract was incubated with rat erythrocytes (Fig. 1). *M. citrifolia* extract induced a significant and dose dependent increase in methemoglobin levels at concentrations between 2 and 8 mg/ml (Fig. 2).

![Figure 1](http://www.jicep.com)  Relationship between logarithm of test samples concentration and % of hemolysis. The data are expressed as mean ± SEM of 3 determinations.

![Figure 2](http://www.jicep.com)  Effect of *M. citrifolia* extract on rat erythrocyte methemoglobin (g/100ml) level. The data are expressed as mean ± SEM of 3 determinations. **$p < 0.01$ (significantly different from control).**

*M. citrifolia* extract induced an increase in TBARS levels of rat erythrocytes at doses between 2.0 and 8.0 mg/ml, which was significantly different at doses of 8.0 mg/ml (Fig. 3). Catalase activity was significantly reduced after incubated *M. citrifolia* extract with rat erythrocytes. Inhibition of spontaneous oxidation of pyrogallol was observed similarly in untreated and treated erythrocytes at 0.5-2.0 mg/ml of *M. citrifolia* extract (Fig. 4). The doses of 4.0 and 8.0 mg/ml of *M. citrifolia* extract affected the spontaneous oxidation of pyrogallol. This effect was statistically significant at dose of 8.0 mg/ml ($p < 0.05$).
Dichloromethane, n-butanol and ethyl acetate fractions did not increase methemoglobin formation. Increase of methemoglobin formation was observed with water fraction at doses up to 6 mg/ml (Fig. 5). These results were similar to the cytotoxicity observed with total extract.

**DISCUSSION**

Some changes were observed in the previous study [5] carried out to evaluate the subchronic 13-week repeated oral dose toxicity of *M. citrifolia* extract. Hemoglobin and differential leukocyte count were significantly affected and erythrocyte count was marginally affected after *M. citrifolia* subchronic exposure. Hematological variations were within or close to normal range and reversibly [5]. A possible explanation for the erythrocyte and hemoglobin reduction in treated animals could be the induction of erythrocyte membrane damage. In our study we evaluated the in vitro cytotoxicity of *M. citrifolia* leaves aqueous extract and fractions on rat erythrocytes. Membrane rupture was not observed after incubation of *M. citrifolia* extract with rat erythrocytes. Dose dependent increase of methemoglobin level at concentration between 2 and 8 mg/ml of the extract was observed. This result indicates the hemoglobin oxidation in erythrocytes after exposure with the extract probably due to oxidative damage of RBC. Lipid peroxidation was increased and CAT and SOD activity were depleted at dose dependent manner indicating a possible increase of hydrogen peroxide and superoxide radicals in erythrocytes.

The reactive oxygen species (ROS) generation in tissues is efficiently scavenged by the enzymatic and nonenzymatic antioxidants. The decrease in the activities of antioxidant enzymes is in close

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**Evaluation with Morinda citrifolia fractions**

Cytotoxicity was not observed on erythrocytes with all fractions at doses between 0.1 and 0.8 mg/ml.
relationship with the induction of lipid peroxidation [15]. The components of the defense system, which have evolved to reduce and contain the injury from free-radical attack, include several enzymes and a few free-radical scavenger molecules [16]. SOD plays an important role in protecting the cells from oxidative damage by converting superoxide radicals into hydrogen peroxide, which is further metabolized by CAT to molecular oxygen and water. SOD is the first enzyme of the scavenger enzyme series to ameliorate the damage caused to cells by free radicals [17], while CAT is one of the several cellular antioxidant enzymes that provide a defense system for the scavenging of reactive oxygen metabolites.

The inhibition of pyrogallol autoxidation brought about by superoxide dismutase can be employed in a rapid and convenient method for the determination of the enzyme. Results suggested that SOD activity could be depleted at 4.0 and 8.0 mg/ml M. citrifolia extract treated erythrocytes probably due to superoxide radical generation. Dimitrova et al. [18] suggested that superoxide radicals and/or their transformation to hydrogen peroxide cause a cysteine oxidation in the enzyme and decrease SOD activity.

M. citrifolia leaves extract has been reported to possess antioxidant activity. Serafini et al. [19] reported the significant antioxidant effect of aqueous extracts from M. citrifolia leaves by protection against lipid peroxidation, hydroxyl radical–scavenging capacity and nitric oxide–scavenging activity at doses between 1 µg/ml and 1mg/ml. However, the authors suggested that the dosage used could be high, and recommended additional studies to evaluate the potential toxicity of the extract.

In the present study we evaluated the toxicity of M. citrifolia leaves extract. Doses over 2 mg/ml of M. citrifolia leaf extract produced in vitro hemoglobin oxidation with increase of H₂O₂ and O₂•− generation. Antioxidative activity of the methanol crude extract and ethyl acetate extract of leaves, fruit and roots of M. citrifolia was report by Zin et al. [20]. The antioxidative activities were measured using ferric thiocyanate (FTC) and thiobarbituric acid (TBA). The FTC method was used to measure the peroxide level during the initial stage of lipid oxidation. In this method, low absorbance values would indicate high levels of antioxidative activity. Results showed that the methanol extracts of both fruit and leaf of M. citrifolia had negligible antioxidative activities, and were not significantly different from the control. However, the results showed significant increase in absorbance values for leaf and fruit extract was observed too. The authors did not discuss this finding but increased peroxide generation could be occurring with leaves and fruit extracts.

Findings of this study showed that high concentrations of M. citrifolia extract could induce erythrocytes toward oxidative damage. Oxidative stress has been suspected in several pathologies including intoxication, genotoxicity and cancer development [21, 22]. Reductions in the erythrocyte count of treated rats observed previously [5] could be a consequence of oxidative stress complication which is incriminated to induce hemolysis by shortening RBC survival and increasing their fragilities. Results of fractions evaluation on hemoglobin oxidation of rat erythrocytes suggest that responsible metabolite is content in water fraction.

CONCLUSIONS

M. citrifolia leaves extract at high dose could be provoke hemoglobin oxidation in rat erythrocytes in vitro with lipid peroxidation and increase of H₂O₂ and O₂•− generation. Components responsible for this effect could be found in water fraction of the extract. These effects could be the cause of erythrocyte and hemoglobin decrease observed previously in rats.

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REFERENCES


