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Scientific research article

Toxicity of flavone and its hydroxylated derivatives: a Study *in silico*, *in vitro* and *in vivo*

Camila de Albuquerque Montenegro[1]*, Abrahão Alves de Oliveira Filho[2], Andressa Brito Lira[3], Ingrid Louise Lins de Albuquerque Evaristo[4], Marcela Lins Cavalcanti de Pontes[3], Natanael Teles Ramos de Lima[5], Thays Thyara Mendes Cassiano[5], Ivana Maria Fechine[5], Francisco Patricio de Andrade Júnior*[6], José Maria Barbosa-Filho[3], Margareth de Fátima Formiga Melo Diniz[3] & Hilzeth Luna Freire Pêssoa[3]

[1] Academic Unit of Health, Education and Health Center, Federal University of Campina Grande, Paraiba – Brazil.

[2] Academic Unit of Biological Sciences, Health Center and Rural Technology, Federal University of Campina Grande, Paraiba, Brazil.

[3] Program in Natural and Synthetic Bioactive Products, Federal University of Paraiba, Paraiba-Brazil.

[4] Department of Molecular Biology, Science and Nature Center, Federal University of Paraiba

[5] Center of Biological Sciences and Health, Department of Pharmacy, State University of Paraiba.

[6] Undergraduate student in Medicine, Pharmacist and Doctor in Pharmacology, State University of Piauí, 2335 Olavo Bilac Street, Centro (South), Teresina, Piauí, 64001-280, Brazil. E-mail: juniorfarmacia.ufcg@outlook.com

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SUMMARY

Purpose: The present study investigated the toxicity and theoretical pharmacokinetics, cytotoxicity, and genotoxicity of flavone and its hydroxylated derivatives: 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone. **Methods**: The *in silico* study was conducted using OSIRISsoftware; cytotoxicity was determined through models of hemolysis and Erythrocyte Osmotic Fragility (EOF) using a 0.5 % solution of human erythrocytes with blood types A, B and O, having positive (+) and negative (-) Rh factors; and genotoxicity was evaluated using the micronucleus test. **Results**: Flavone and its hydroxylated derivatives do not present significant toxicity risks (theoretically), yet they do have good oral bioavailability. Flavone, 5-hydroxyflavone and 6-hydroxyflavone showed moderate hemolytic potential at higher concentrations. The hydroxylated flavones protected cells types A and O from osmotic stress at a minimum concentration of 500 μg/mL, in blood types A and O. In the genotoxicity evaluation, orally administered flavone did not show genotoxicity compared to cyclophosphamide, a proven genotoxic agent. **Conclusion**: It is concluded that the flavones have low theoretical toxicity, good bioavailability, moderate cytotoxicity in higher concentrations and nogenotoxicity, suggesting a considerable margin of safety for future pharmacological use, and showing the importance of including computational chemistry techniques to guide biological protocols.

Keywords: Flavonoids; cytotoxicity; hemolysis; erythrocyte osmotic fragility; micronucleus test.

RESUMEN

Toxicidad de la flavona y sus derivados hidroxilados: un estudio *in silico*, *in vitro* e *in vivo*

Propósito: El presente estudio investigó la toxicidad y farmacocinética teórica, citotoxicidad y genotoxicidad de la flavona y sus derivados hidroxilados: 3-hidroxiflavona, 5-hidroxiflavona y 6-hidroxiflavona. **Métodos**: El estudio in silico se llevó a cabo utilizando el software OSIRIS; la citotoxicidad se determinó mediante modelos de hemólisis y fragilidad osmótica de los eritrocitos (EOF) utilizando una solución al 0,5% de eritrocitos humanos de los tipos sanguíneos A, B y O, con factores Rh positivos (+) y negativos (-); y la genotoxicidad se evaluó mediante el test de micronúcleos. **Resultados**: La flavona y sus derivados hidroxilados no presentan riesgos significativos de toxicidad (teóricamente), pero tienen buena biodisponibilidad oral. La flavona, 5-hidroxiflavona y 6-hidroxiflavona mostraron potencial hemolítico moderado en concentraciones más altas. Los flavonoides hidroxilados protegieron a los tipos celulares A y O del estrés osmótico a una concentración mínima de 500 μg/mL, en los tipos sanguíneos A y O. En la evaluación de genotoxicidad, la flavona administrada oralmente no mostró genotoxicidad en comparación con la ciclofosfamida, un agente genotóxico comprobado. **Conclusión**: Se concluye que las flavonas tienen baja toxicidad teórica, buena biodisponibilidad, citotoxicidad moderada en concentraciones más altas y ninguna genotoxicidad, sugiriendo un margen de seguridad considerable para su uso farmacológico futuro, y destacando la importancia de incluir técnicas de química computacional para orientar protocolos biológicos.

Palabras clave: Flavonoides; citotoxicidad; hemólises; fragilidad osmótica eritrocitária; test de micronúcleos.

RESUMO

Toxicidade da flavona e seus derivados hidroxilados: um estudo *in silico*, *in vitro* e *in vivo*

Propósito: O presente estudo investigou a toxicidade e farmacocinética teórica, citotoxicidade e genotoxicidade da flavona e seus derivados hidroxilados: 3-hidroxiflavona, 5-hidroxiflavona e 6-hidroxiflavona. **Métodos**: O estudo *in silico* foi conduzido utilizando o *software* OSIRIS; a citotoxicidade foi determinada através de modelos de hemólise e fragilidade osmótica dos eritrócitos (EOF) usando uma solução a 0,5% de eritrócitos humanos dos tipos sanguíneos A, B e O, com fatores Rh positivos (+) e negativos (-); e a genotoxicidade foi avaliada pelo teste do micronúcleo. **Resultados**: A flavona e seus derivados hidroxilados não apresentam riscos significativos de toxicidade (teoricamente), mas têm boa biodisponibilidade oral. A flavona, 5-hidroxiflavona e 6-hidroxiflavona mostraram potencial hemolítico moderado em concentrações mais elevadas. Os flavonóides hidroxilados protegeram os tipos celulares A e O do estresse osmótico a uma concentração mínima de 500 μg/mL, nos tipos sanguíneos A e O. Na avaliação de genotoxicidade, a flavona administrada oralmente não mostrou genotoxicidade em comparação com a ciclofosfamida, um agente genotóxico comprovado. **Conclusão**: Conclui-se que as flavonas têm baixa toxicidade teórica, boa biodisponibilidade, citotoxicidade moderada em concentrações mais elevadas e nenhuma genotoxicidade, sugerindo uma margem de segurança considerável para uso farmacológico futuro, e destacando a importância da inclusão de técnicas de química computacional para orientar protocolos biológicos.

Palavras-chave: Flavonoides; citotoxicidade; hemólise; fragilidade osmótica eritrocitária; teste do micronúcleo.

1. INTRODUCTION

Plants and herbs have played an important role in healthcare since ancient times; today it is known that 80% of the world's population depends on plant-derived medicines as the first choice of treatment in primary healthcare. In the last century alone, traditional knowledge obtained from many sources has made the formulation of roughly 121 pharmaceutical products possible. During the 1950s until the 1970s, approximately 100 new drugs based on plants were

commercialized. It is also well known that approximately 80% of cardiovascular, antimicrobial, immunosuppressive, and antineoplastic drugs have their origins in plants. It is also accepted that most drug ingredients are either derived or developed from natural products and compounds. In United States, roughly 25% of all pharmaceutical prescriptions contain at least one plant-derived ingredient. Speaking in broader terms, about 50% of pharmaceuticals are developed from active ingredients first identified or isolated from herbs, plants, animals, or insects [1, 2].

In this scenario, for future medicines, flavonoids stand out. Phenolic compounds are one of largest groups of secondary plant constituents, and flavonoids figure importantly in this group. Flavonoids are found in in numerous specimens of the plant kingdom, mainly concentrated in fruit skin, in vacuoles of plant cells, and in epidermic pigments. Flavonoids are classified into six major subgroups: chalcones, flavones (e.g., flavone, apigenin, and luteolin), flavonols (e.g. quercetin, kaempferol, myricetin, and fisetin), flavandiols, anthocyanins, and proanthocyanidins (condensed tannins). These substances are important to plants because they are involved in processes like UV (Ultra Violet) protection, pigmentation, stimulation of nitrogen-fixing nodules, and plant disease resistance. Their popularity is due to the fact they are indeed bioactive, and can provide health benefits to human body such as their anti-allergic, anti-cancer, antioxidant, anti-inflammatory, anti-viral, nutritional and functional activities [3].

Among flavonoids, natural flavones and their synthetic derivatives have demonstrated several biological activities, like neuroprotective, cardioprotective and antimicrobial, including activities mentioned above, antioxidant, anti-inflammatory, antitumor and anti-allergic [4]. Flavones are natural products of the benzopyran class constituting an important group of oxygen heterocycles, they exhibit biological activities which are dependent on the nature and position of the substituents on the flavone skeleton. The flavones have a unique ability to modulate various enzyme systems, these compounds are active against metabolic and infectious diseases and also demonstrate anti-estrogenic and cytotoxic activities [5].

It is prudent, before any investigation into the mechanism of action of a molecule, to realize toxicity tests. There are different cytotoxicity assays, and the Red Blood Cell (RBC) assay is a simple, fast and effective way to observe injury that a xenobiotic substance might produce. The method is based on measurement of hemoglobin efflux from erythrocytes exposed to a toxic substance [6, 7]. *In vivo* genotoxicity assays investigate substances toxic to DNA and the carcinogenetic potential of chemical or physical agents. Such data can be evaluated by methods such as comet assay, micronuclei, apoptosis, and phagocytosis counts. The micronucleus test seeks to quantify small individual nuclei called micronuclei (MN), these nuclei appear when chromosome fragments or whole chromosomes are not coupled to chromosome groupings in a cell [8].

Computational models of prediction, also called *in silico* predictive tools, play a major role in the repertoire of methodological alternatives to guide pharmaceutical research, there are of course *in vitro* and *in vivo* models. These tools are used to study both existing and hypothetical substances and deliver fast results [9].

Software like Osiris has been developed and used to provide theoretical toxicological risk assessments according to the molecular structure of the substance, giving predictions regarding mutagenicity, tumorigenicity, influence on the reproductive system, and data such as: cLog*P* value (partition coefficient between n-octanol and water), druglikeness (similarity to other molecules available in the market), and "drug-score" (combination of druglikeness, clog*P*, molecular mass, and toxicological risk results) [9].

Given these assumptions, the present study set out to investigate the theoretical toxicological characteristics of flavone (Figure 1) and its hydroxylated derivatives 3-hydroxyflavone (Figure 2), 5-hydroxyflavone (Figure 3) and 6-hydroxyflavone (Figure 4) to test their cytotoxicity and genotoxicity. While seeking toxicological safety evidence for their use, we hope to contribute to the expanding range of effective alternatives for pharmacological evaluation, and disease treatment.

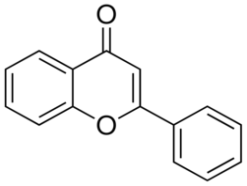


Figure 1. Flavone

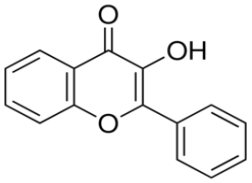


Figure 2. 3-hydroxyflavone

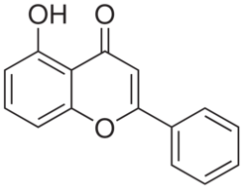


Figure 3. 5-hydroxyflavone

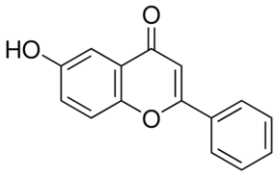


Figure 4. 6-hydroxyflavone

2. MATERIALS AND METHODS

2.1. Flavones

The flavones investigated were kindly provided by Prof. Dr. José Maria Barbosa Filho from the Phytochemical Laboratory of Natural Products of the Postgraduate Program in Natural and Synthetic Bioactive, Center of Biological Sciences and Health, Department of Pharmacy, State University of Paraiba Products– CCS/UFPB, having been purchased from Sigma, USA. These included: flavone, 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone.

To perform the experimental protocol for determination of antibacterial activity, solutions with a concentration of 10 mg/mL of flavonoid, using DMSO as a vehicle were prepared.

2.2. Human erythrocytes

Human erythrocytes of blood types A, B, and O, with positive and negative Rh factors were obtained from bags containing erythrocyte concentrate (that would no longer be used for transfusion), from the Transfusion Unit of the University Hospital Lauro Wanderley/UFPB.

Manipulation and disposal of the erythrocytes followed the Safety Standards standardized by said Unit.

The project was approved by the Research Ethics Committee of the Health Sciences Center, Federal University of Paraíba, Protocol: 0184/14 and CAEE: 30652314.0.0000.5188.

2.3. Animals

To perform the micronucleus assay, *Mus musculus* mice of the Swiss strain from the Prof. Dr. Thomas George *Vivarium,* and weighing between 20-30 g were used. The mice were kept at a temperature of 21 ± 1 °C, light and dark cycle of 12 h, and fed with pellet-type feed and water *ad libitum*.

This experimental protocol was approved by the Committee on Ethics in Animal Use (CEUA), of the Biotechnology Center (CBiotec) of UFPB, with registration N°. 3306/13.

2.4. *In silico* assay

OSIRIS

The chemical structures were submitted to *in silico* study for theoretical analysis of flavone toxicity and pharmacokinetic parameters. These parameters were ADME (Absorption, Distribution, Metabolism, and Excretion) and toxicity, using the Osiris Property Explorer program.

In this analysis we determined the potential druglikeness and drug-score that are related to topological descriptors, and other properties such as cLog*P* and molecular mass [10]. *In silico* evaluation of molecular toxicity also included theoretical analysis of mutagenic effect, tumorigenicity, irritant properties, and effects on reproduction as described by Abreu [11].

Finally, since the flavones were to be administered orally, requiring good absorption in the gastrointestinal tract, we used the Lipinski "Rule of Five".

2.5. Cytotoxicity assays

2.5.1. Hemolysis assay

A human blood sample was mixed with 0.9% NaCl at a ratio of 1:30 and centrifuged at 2500 rpm for 5 minutes, the procedure was repeated twice more and 500 μL of the pellet of the last centrifugation was resuspended in 100 ml of 0.9% NaCl, resulting in a 0.5% solution. Samples of the flavones at concentrations of 1, 10, 100, 500 and 1000 μg/mL were added to 2 mL of the erythrocyte solution. Tubes containing the erythrocyte solution alone formed the negative control group (0% hemolysis), and erythrocyte solution plus 1% Triton X-100 (as a hemolyzing agent) was used as the positive control (100% hemolysis). The samples were incubated for 1 hour at 25 ± 2°C under slow and constant (100 rpm) shaking. After this, centrifugation was performed at 2500 rpm for 5 minutes, and hemolysis was quantified by reading an aliquot of the supernatant using spectrophotometry at a wavelength of 540 nm [12].

The experiments were performed in triplicate and the results expressed in % hemolysis as compared to the negative control group.

2.5.2. Evaluation of osmotic fragility in human erythrocytes in the presence of flavones

A solution of each flavone and its derivates at concentrations of 1, 10 and 100 μg/ml was incubated in tubes containing 2 ml of a 0.5% solution of erythrocytes for 1 hour at 25 ± 2 °C. After this, the preparations were centrifuged at 2500 rpm for 5 minutes and the supernatant discarded. The erythrocytes were then resuspended in a hypotonic sodium chloride solution (0.24%) solution, and shaken at 100 rpm for 20 minutes at 25 ± 2 °C. After this period, the

samples were centrifuged at 2500 rpm for 5 minutes and hemolysis was quantified by means of an aliquot of the supernatant read using spectrophotometry at a wavelength of 540 nm [13].

A solution of erythrocytes was used as a negative control (0% hemolysis) and a solution of erythrocytes in the presence of a 0.24% sodium chloride solution was used as a positive control (100% hemolysis).

The experiments were performed in triplicate and the results expressed as % hemolysis as compared to the positive control group.

2.6. Genotoxicity assays

2.6.1. Investigation of Flavonoid clastogenic and aneugenic potential in mouse erythrocytes

The experimental procedures were performed according to Resolution No. 90, of the National Agency of Sanitary Surveillance - ANVISA (RE 90/2004) [14]. Swiss mice were divided into groups composed of three males and three females who were given oral flavone solution doses at 200 mg/kg. Another group, the positive control, was treated with cyclophosphamide, a proven mutagenic agent, at a dose of 50 mg/kg. After 24 hours, the animals were euthanized and a blood sample was collected for the preparation of slides, which were stained with panopticus and observed under an optical microscope at 1000x magnification to count micronuclei. Slides were analyzed for presence or absence of micronuclei in the erythrocytes of each animal. About 2000 erythrocytes per animal were counted [15].

Results were expressed as the percentage of mean ± standard deviation compared to the positive control group.

2.7. Statistical analysis

The results obtained in the experiments were analyzed using GraphPad Prism 5.0® software, San Diego, CA, USA, using the ANOVA test followed by Dunnett's post-test or the unpaired Student t-test for two-column analysis. The values were expressed as mean ± standard error of the mean (SEM) or standard deviation of the mean (SD) and considered significant when $p < 0.05$.

3. RESULTS

3.1. *In silico* assay

The OSIRIS analyses indicated low theoretical risk of toxicity for the flavonoids, except one flavone exhibiting mutagenic potential (chart 1).

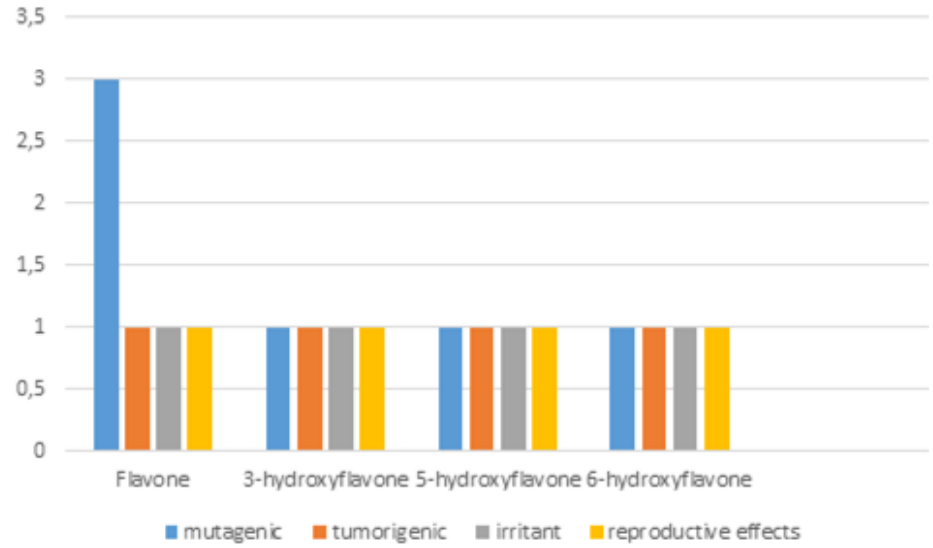


Chart 1. Theoretical risk of toxicity for Flavone and its derivatives calculated using the OSIRIS Property Explorer software.

In addition, OSIRIS presented considerable values for "druglikeness" and "drug-score" (drug-score = correlation between "druglikeness", cLog*P*, molecular weight, and risk of toxicity), generating important data, and revealing possible future medicinal use for the tested substances (table 1).

Table 1. Evaluation of theoretical toxicity risks.

Parameters / **Flavones**	**cLog*P*** **(2 – 5)**	**"Druglikeness"** **(≤1)**	**"Drug-score"** **(≤1)**
Flavone	3.74	1.85	0.45
3-hydroxyflavone	3.45	1.62	0.76
5-hydroxyflavone	3.47	1.33	0.75
6-hydroxyflavone	3.23	1.35	0.75

cLog*P* = Partition coefficient octanol: water - confers solubility/Druglikeness = similarity with other structures available on the market/Drug-score = combination of results of "druglikeness", cLog*P*, molecular mass and toxicological risks.

As for theoretical bioavailability with oral administration, all of the flavonoids assessed met the "rule of five" Lipinski standards in which each substance must possess at least three of the four requirements listed (table 2). In this way, the result obtained demonstrated that flavone, 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone will be bioavailable after oral administration.

Table 2. Theoretical analysis of the physicochemical properties involved in the oral bioavailability of flavones according to Lipinski's "Rule of Five".

Parameters / Substance	nDLH (≤ 5)	nALH (≤ 10)	Da (≤ 500)	cLog*P* (≤ 5)
Flavone	0	2	222	3.74
3-hydroxyflavone	1	3	238	3.45
5-hydroxyflavone	1	3	238	3.47
6-hydroxyflavone	1	3	238	3.23

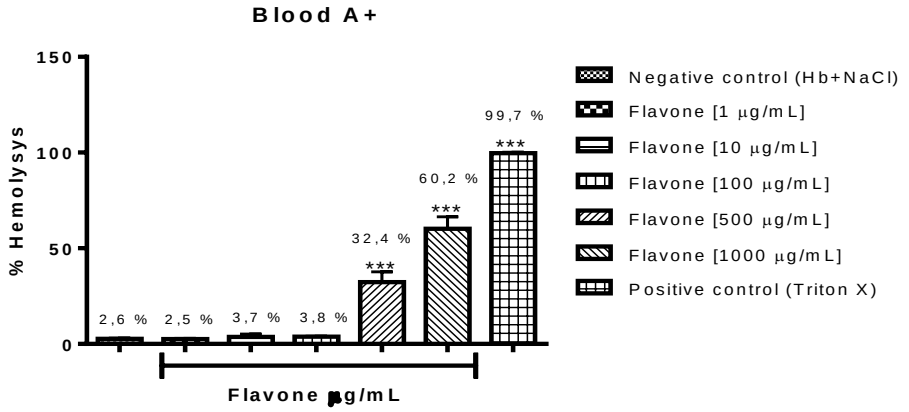
*nDLH: Number of hydrogen bond donors; *NALH: number of hydrogen bonding acceptors; *Da: molecular weight; *cLog*P*: partition coefficient octanol: water.

3.2. Cytotoxicity assays

3.2.1. Evaluation of Hemolytic potential in human erythrocytes

In this assay it was found that flavone induced hemolysis only at concentrations of 500 μg/mL and 1000 μg/mL as compared to the negative control group, and as expressed in charts 2a to 4b.

a)



b)

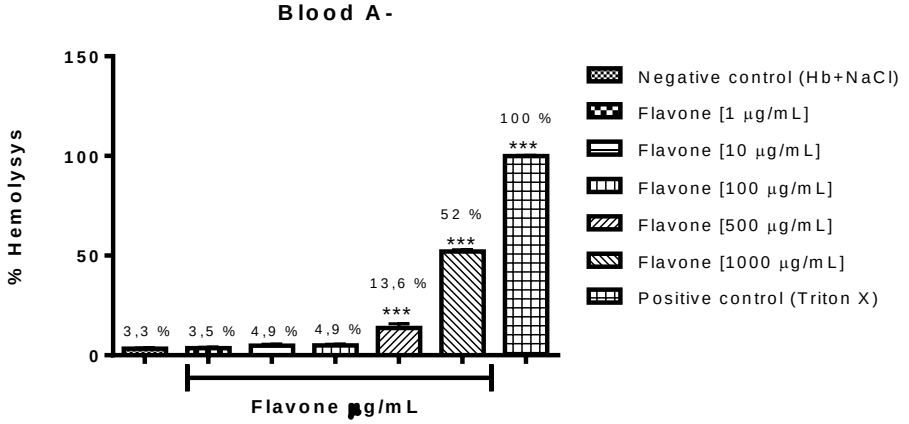
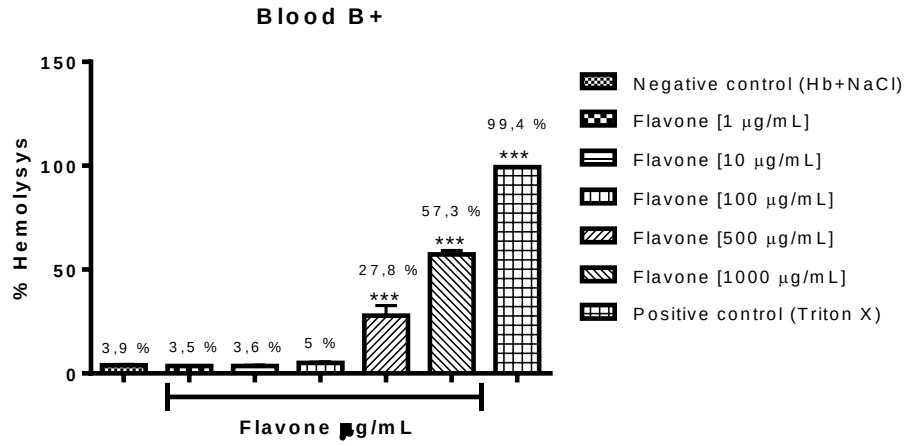


Chart 2. Evaluation of cytotoxicity in erythrocytes type A, Rh+ (a) and A, Rh- (b) induced by flavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***$p < 0.001$ ($n = 3$).

a)



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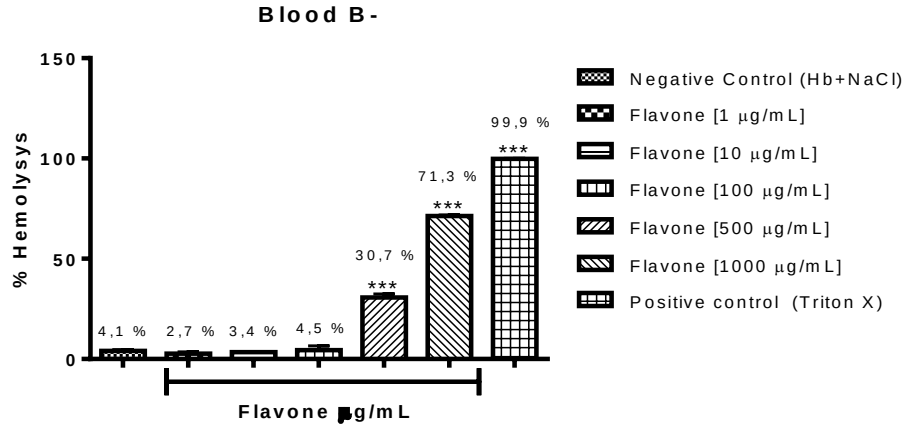
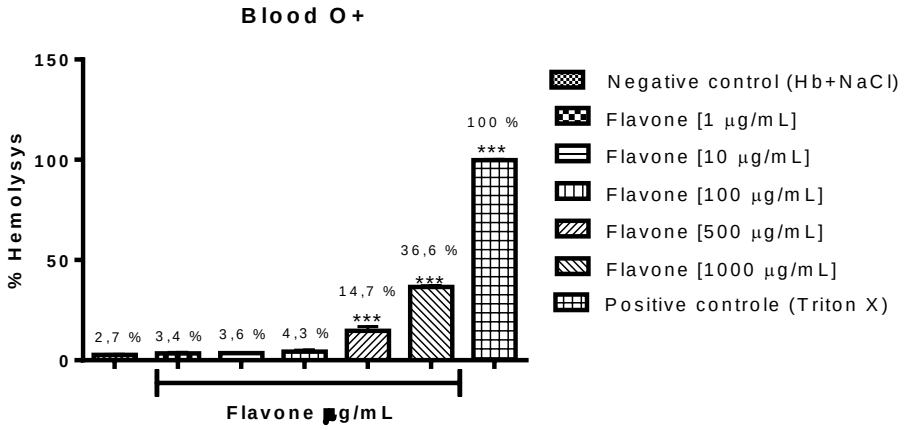


Chart 3. Evaluation of cytotoxicity in erythrocytes type B, Rh+ (a) and B, Rh- (b) induced by flavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***$p < 0.001$ ($n = 3$).

a)



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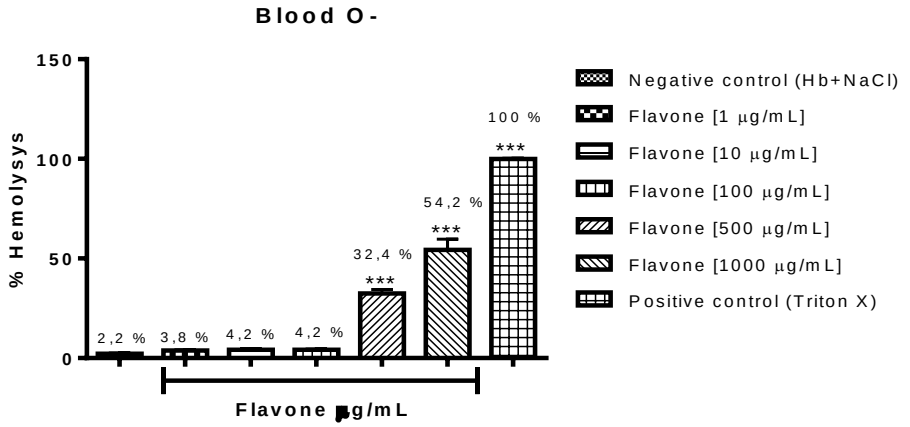
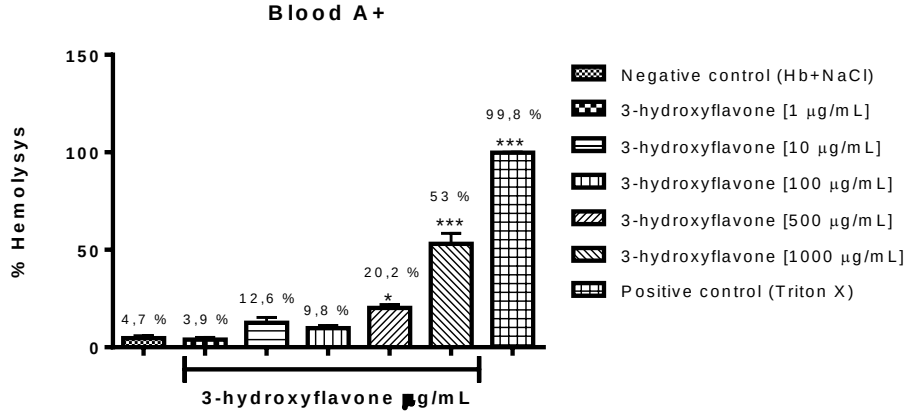


Chart 4. Evaluation of cytotoxicity in erythrocytes type O, Rh+ (a) and O, Rh- (b) induced by flavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***p < 0.001 (n = 3).

3-hydroxyflavone promoted cytotoxicity at concentrations of 500 µg/mL and 1000 µg/mL in erythrocytes type A+ and A-, B- and O+. Only B- cells showed greater sensitivity to 3-hydroxyflavone, since lysis occurred upon incubation from the concentration of 100 µg/mL, as compared to the negative control group (charts 5a to 7b).

a)



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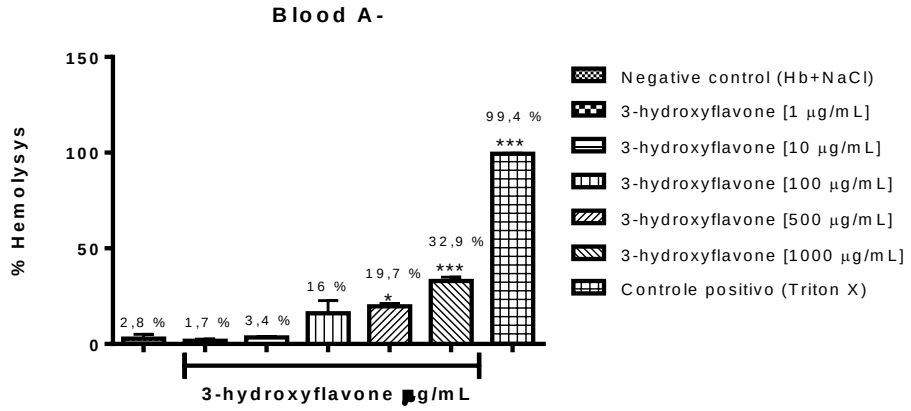
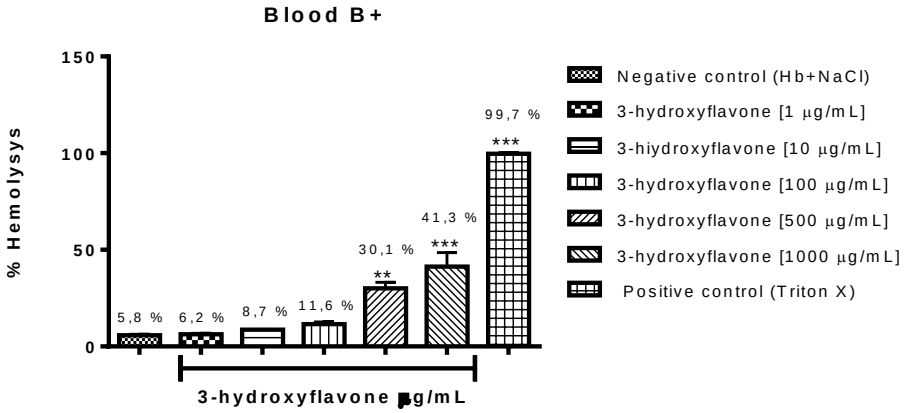


Chart 5. Evaluation of cytotoxicity in erythrocytes type A, Rh+ (a) and A, Rh- (b) induced by 3-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. *$p < 0.05$, ***$p<0.001$ (n=3).

a)



b)

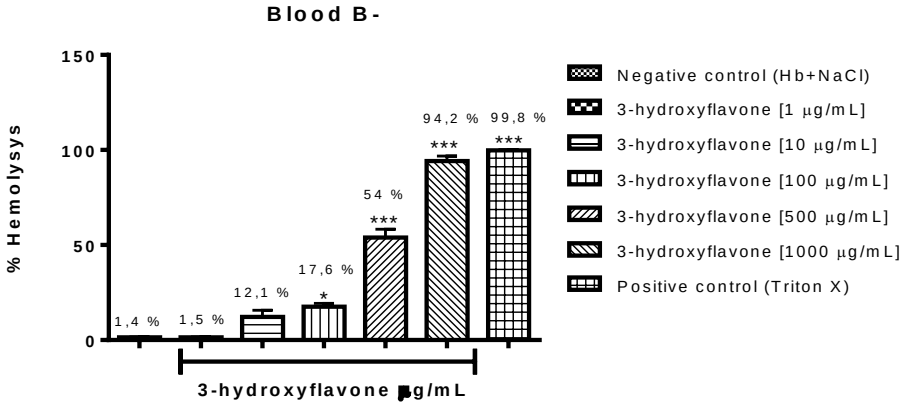
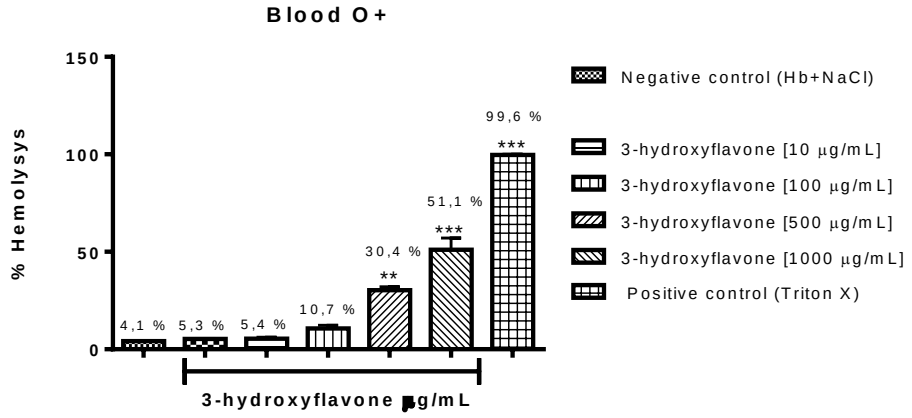


Chart 6. Evaluation of cytotoxicity in erythrocytes type B, Rh+ (a) and B, Rh- (b) induced by 3-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. *$p < 0.05$, ***$p<0.001$ (n=3).

a)



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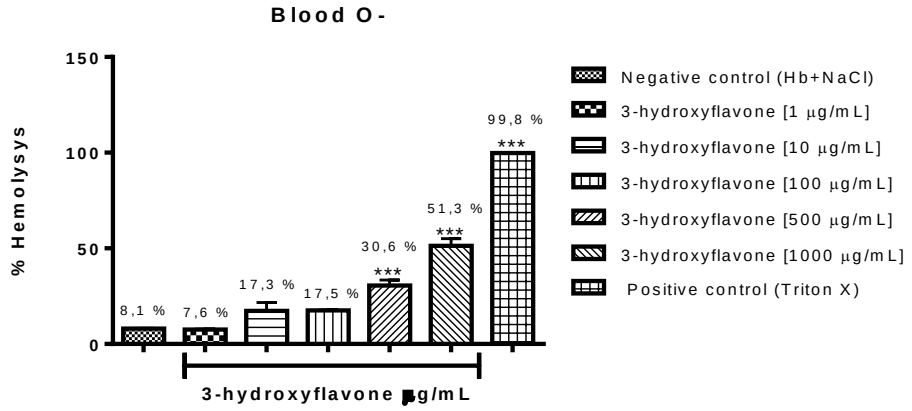
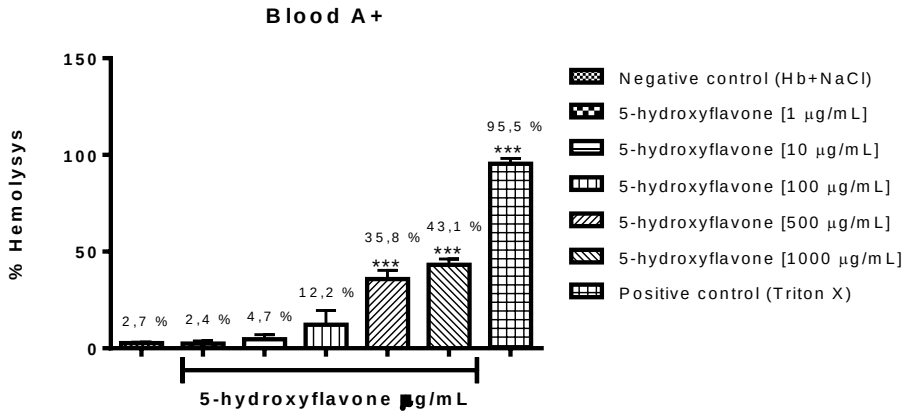


Chart 7. Evaluation of cytotoxicity in erythrocytes type O, Rh+ (a) and O, Rh- (b) induced by 3-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p < 0.01, ***p<0.001 (n=3).

The flavone 5-hydroxyflavone, at concentrations of 500 µg/mL and 1000 µg/mL was toxic for all erythrocyte types tested, as compared to the negative control, as can be seen in charts 8a to 10b.

a)



b)

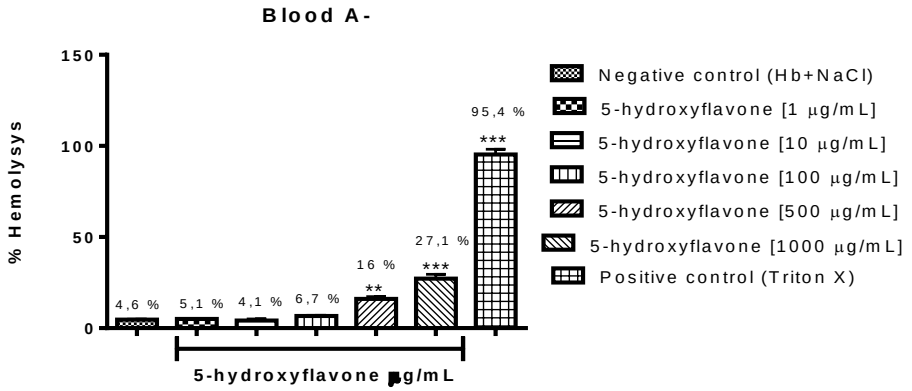
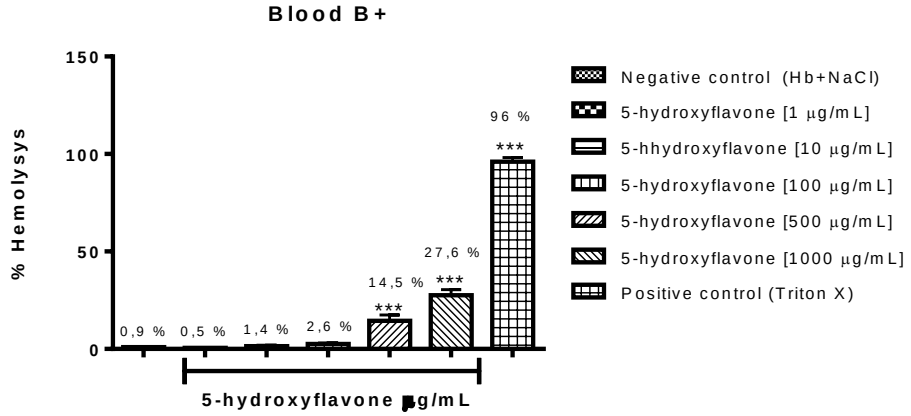


Chart 8. Evaluation of cytotoxicity in erythrocytes type A, Rh+ (a) and A, Rh- (b) induced by 5-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p < 0.01, ***p<0.001 (n=3).

a)



b)

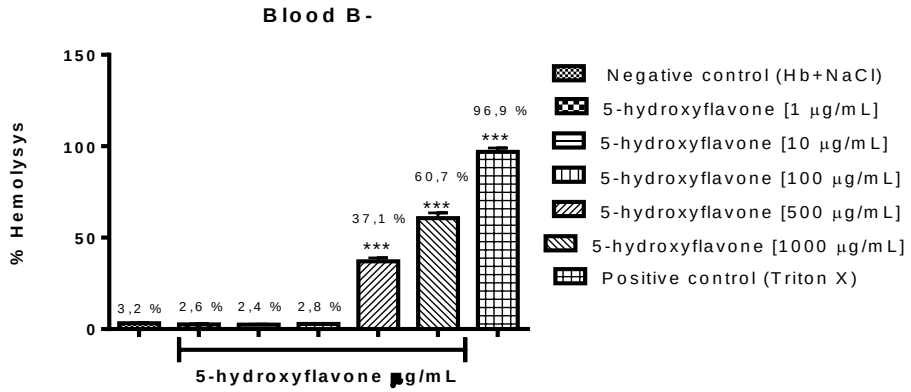
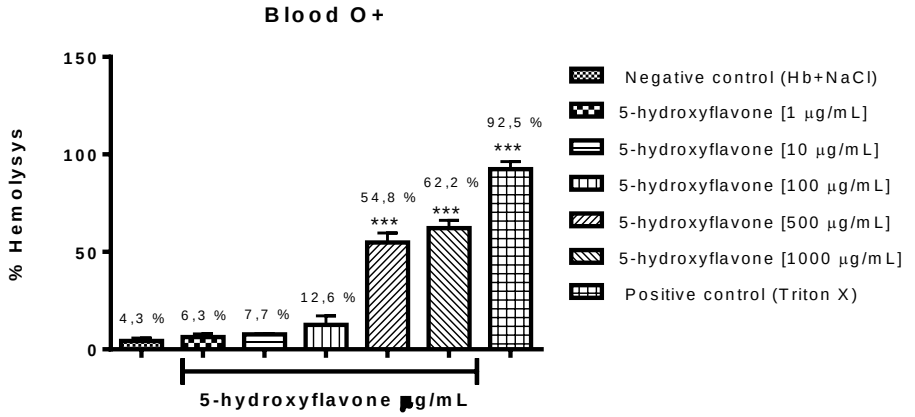


Chart 9. Evaluation of cytotoxicity in erythrocytes type B, Rh+ (a) and B, Rh- (b) induced by 5-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***p<0.001 (n=3).

a)



b)

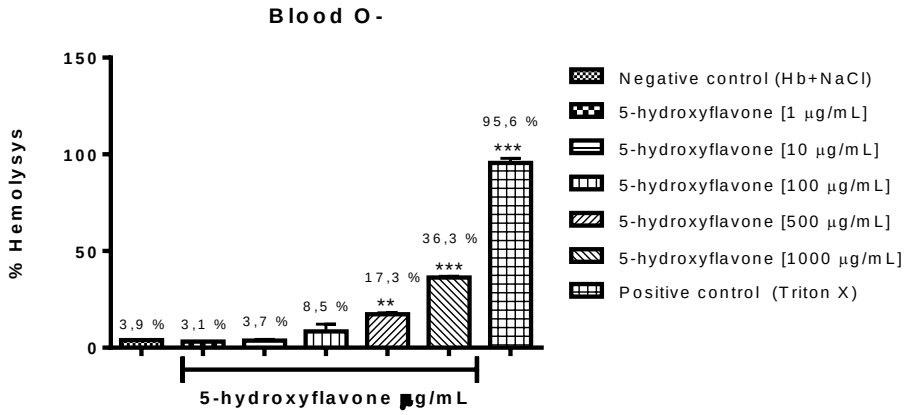
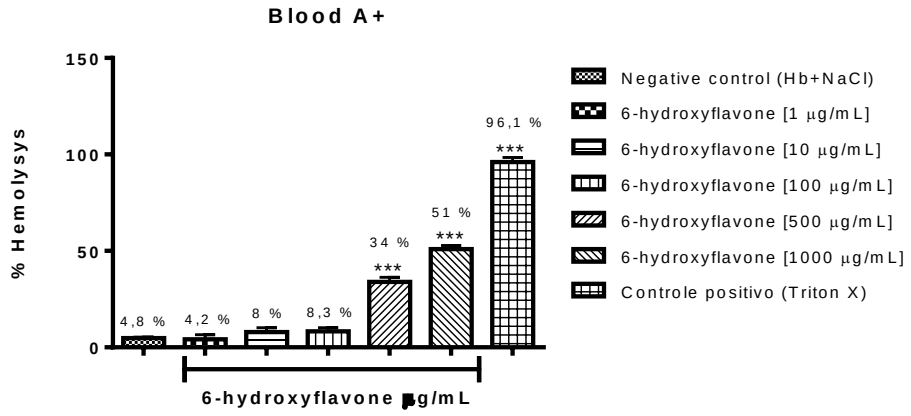


Chart 10. Evaluation of cytotoxicity in erythrocytes type O, Rh+ (a) and O, Rh- (b) induced by 5-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p < 0.01, ***p<0.001 (n=3).

Finally, 6-hydroxyflavone, similar to flavone and 5-hydroxyflavone, was shown to have potential to induce hemolysis at concentrations of 500 µg/mL and 1000 µg/mL in types A, B, and O; both Rh+ and Rh-, when compared to the negative control. These results are shown in charts 11a and 11b, 12a and 12b and 13a and 13b.

a)



b)

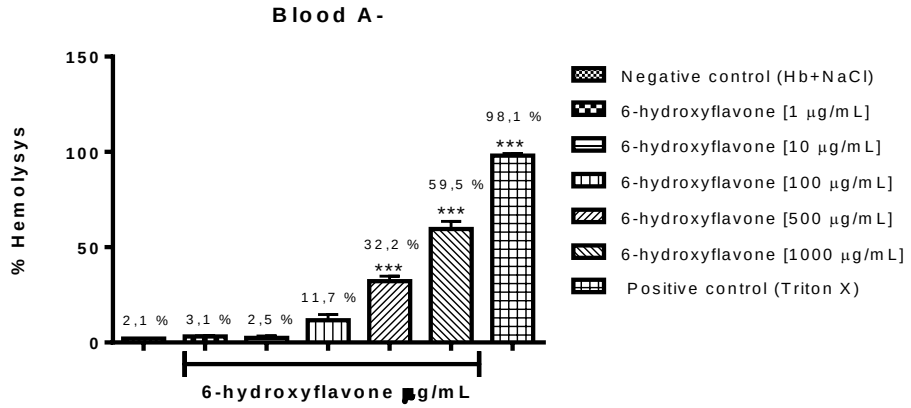
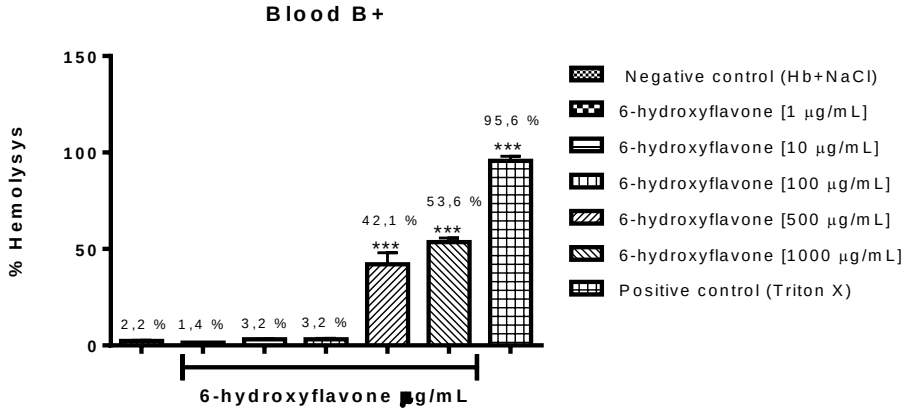


Chart 11. Evaluation of cytotoxicity in erythrocytes type A, Rh+ (a) and A, Rh- (b) induced by 6-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***$p<0.001$ (n=3).

a)



b)

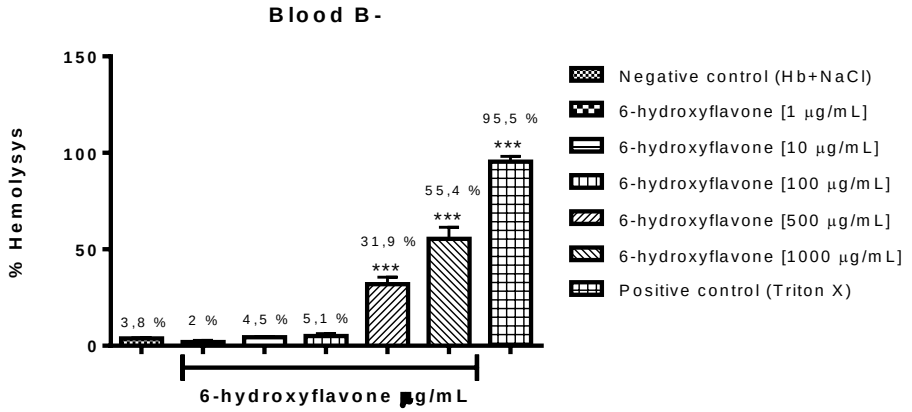
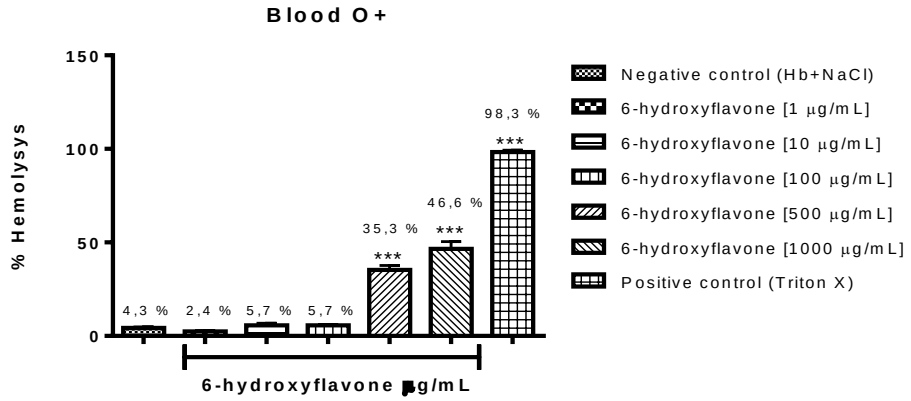


Chart 12. Evaluation of cytotoxicity in erythrocytes type B, Rh+ (a) and B, Rh- (b) induced by 6-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***p<0.001 (n=3).

a)



b)

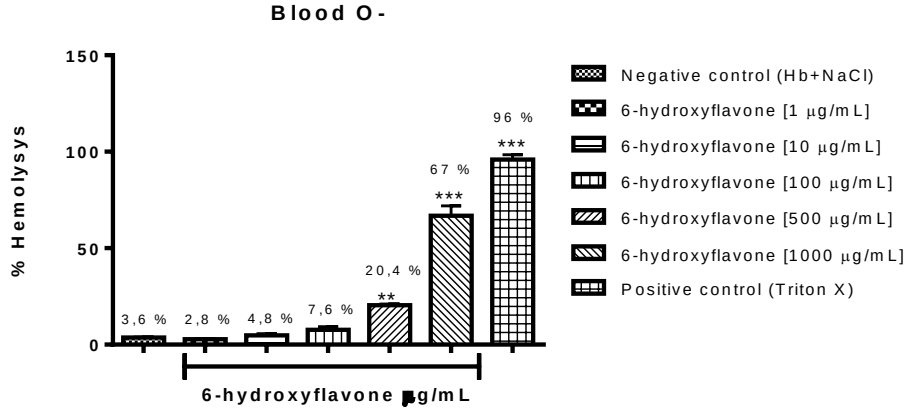


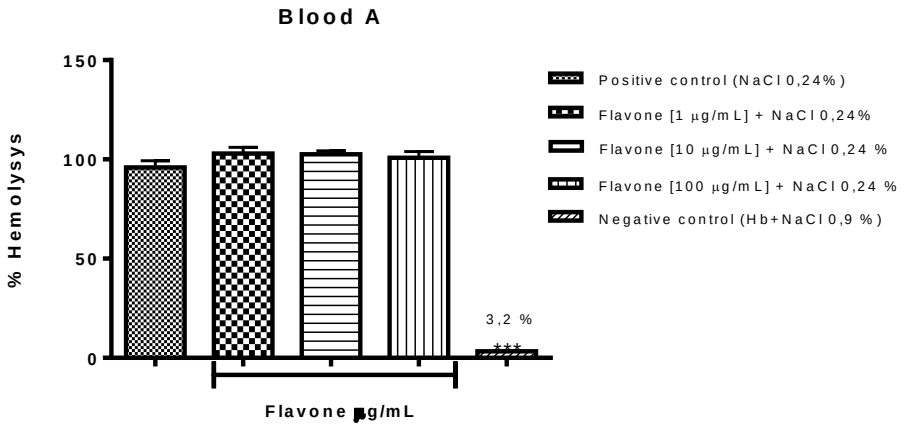
Chart 13. Evaluation of cytotoxicity in erythrocytes type O, Rh+ (a) and O, Rh- (b) induced by 6-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p<0.01, ***p< 0.001 (n=3).

The results present moderate flavonoid cytotoxicity in higher concentrations (500 and 1000 µg/mL) in all blood types A, and B; without Rh involvement.

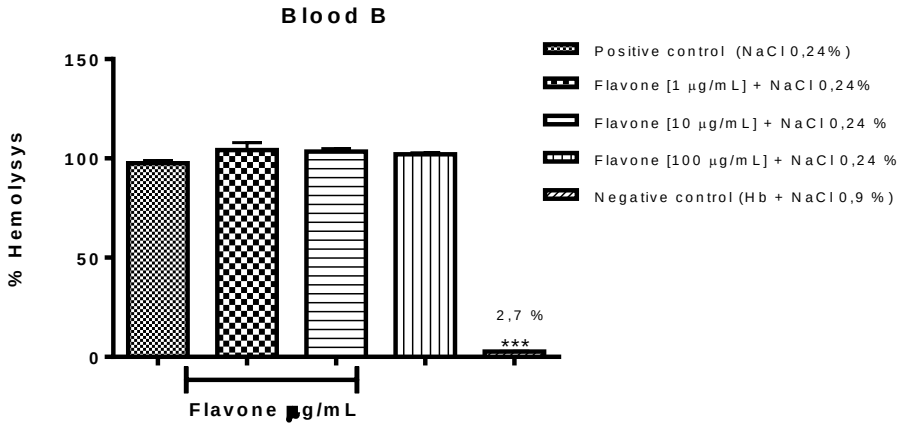
3.2.2. Evaluation of osmotic fragility in human erythrocytes in the presence of Flavones

When the degree of hemolysis in hypotonic medium (0.24% NaCl solution) was analyzed, flavone did not promote reductions for any of the tested concentrations (1, 10 and 100 µg/mL); in erythrocytes types A, B, or O; this, as compared to the positive control group (NaCl solution 0.24%), in which maximum membrane lysis was observed (chart 14).

a)



b)



c)

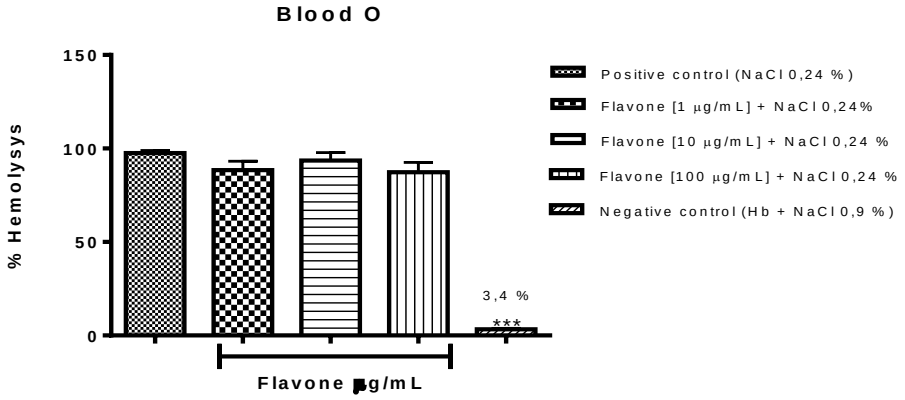
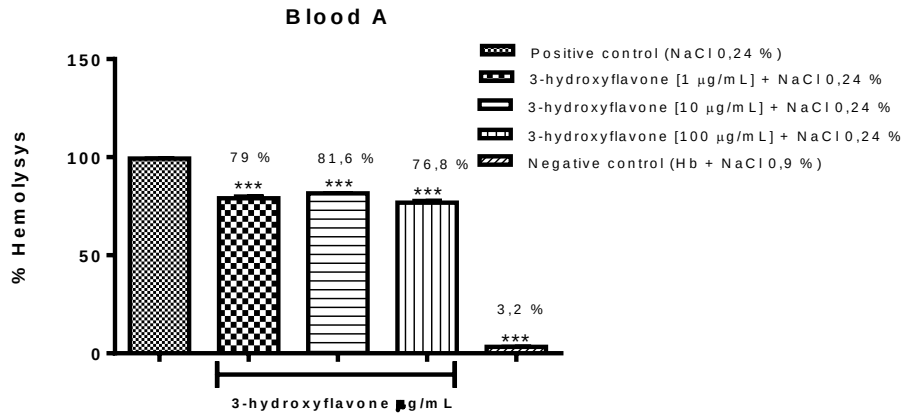


Chart 14. Antihemolytic activity of flavone on blood types A (a), B (b) and O (c) when in hypotonic solution (NaCl 0.24%). The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p<0.01, ***p< 0.001 (n=3).

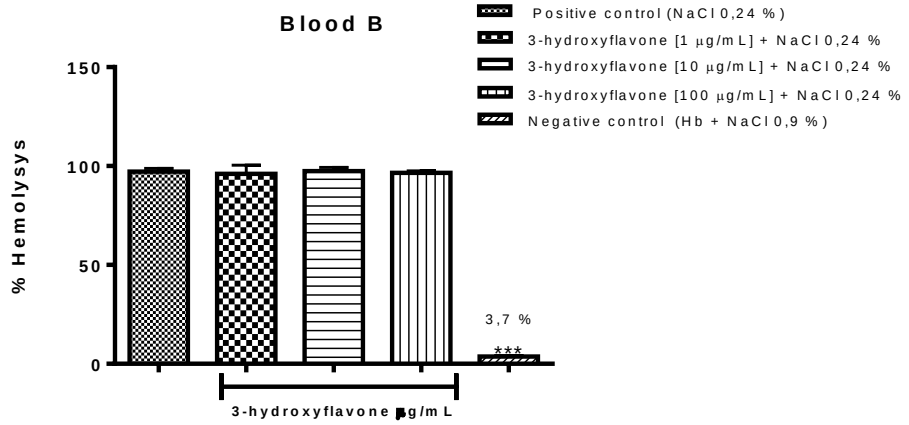
The flavonoid 3-hydroxyflavone, when incubated with blood type A, protected the erythrocytes from osmotic stress at concentrations of 1, 10 and 100 µg/mL, as compared to the positive control group (NaCl 0.24%), thus reducing the respective hemolysis percentages to 79%,

81.6% and 76.8%. This result differed from that observed for blood types B and O, where none of the concentrations used offered protection against hemolysis (chart 15).

a)



b)



c)

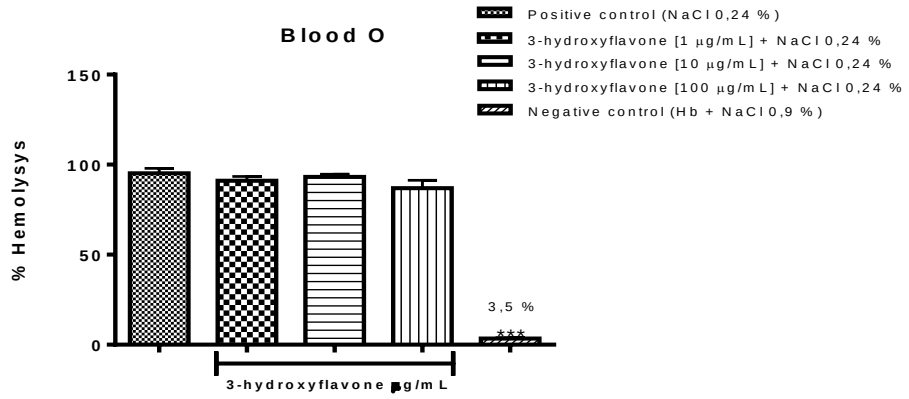
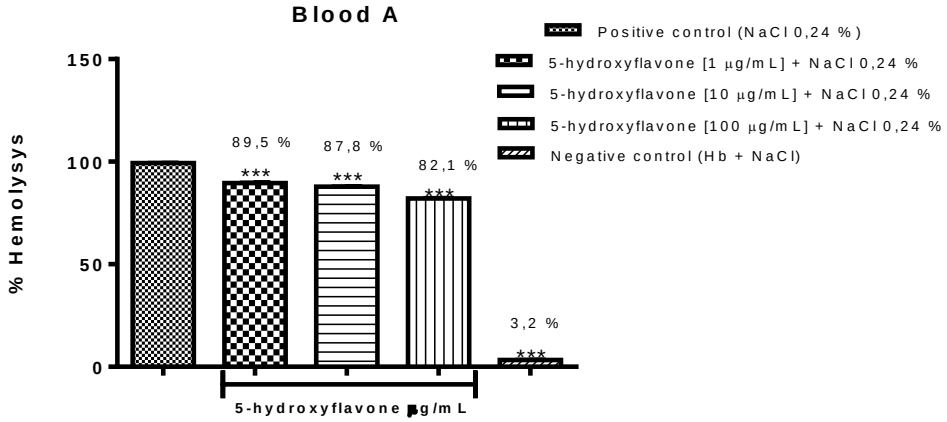


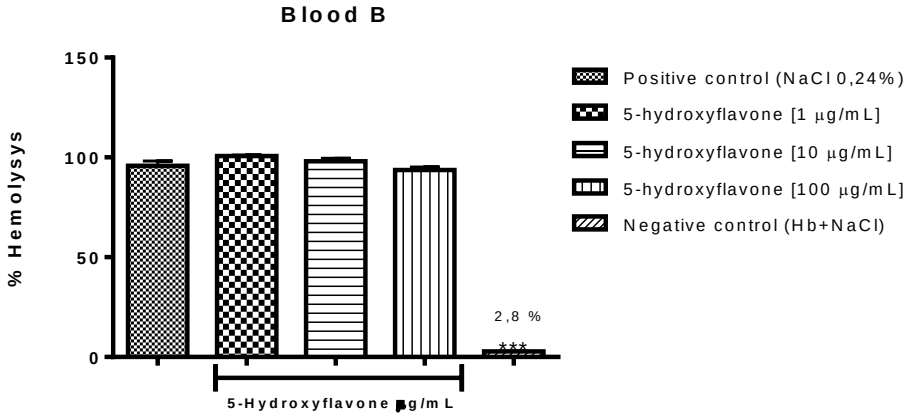
Chart 15. Antihemolytic activity of 3-hydroxyflavone on blood types A (a), B (b) and O (c) when in hypotonic solution (NaCl 0.24%) The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***p< 0.001 (n=3).

The concentrations of 1, 10 and 100 μg/mL of 5-hydroxyflavone protected types A and O erythrocytes, reducing respective hemolysis to 89.5%, 87.8% and 82.1% (blood A), and 86%, 92.7% and 86.3% (blood O), as compared to the NaCl control group 0.24% (99.3% hemolysis), and as observed in chart16.

a)



b)



c)

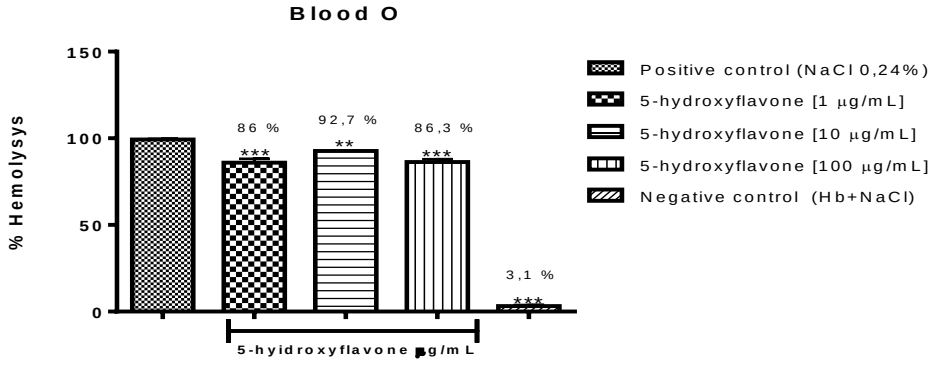
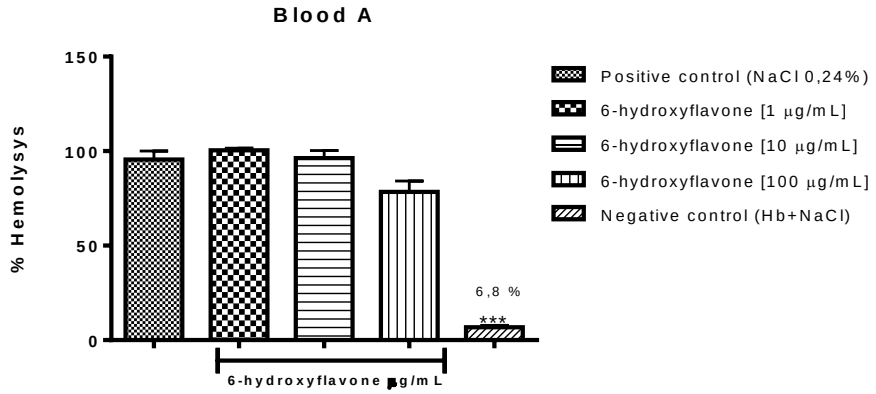


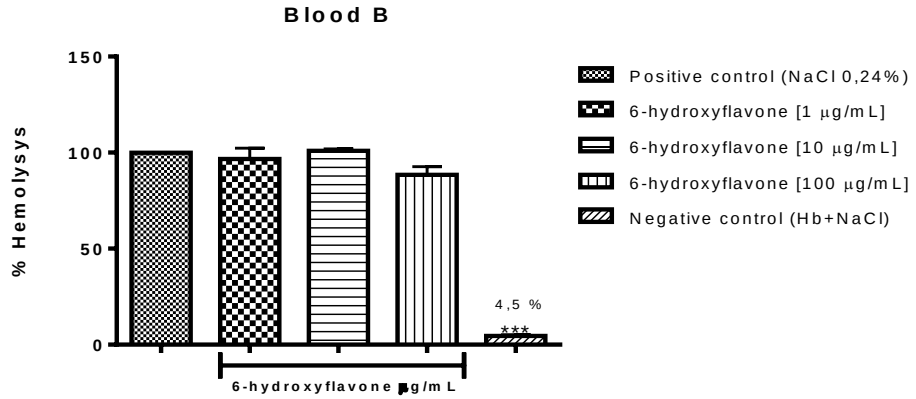
Chart 16. Antihemolytic activity of 5-hydroxyflavone on blood types A (a), B (b) and O (c) when in hypotonic solution (NaCl 0.24%). The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p<0.01, ***p< 0.001 (n=3).

The flavonoid 6-hydroxyflavone was effective against the osmotic stress caused by the hypotonic solution in blood type O, at which concentrations of 1, 10 and 100 μg/mL significantly reduced hemolysis to 75.6%, 74.7%, and 68 %, Compared to the positive control group (NaCl 0.24%), as we can see in chart 17.

a)



b)



c)

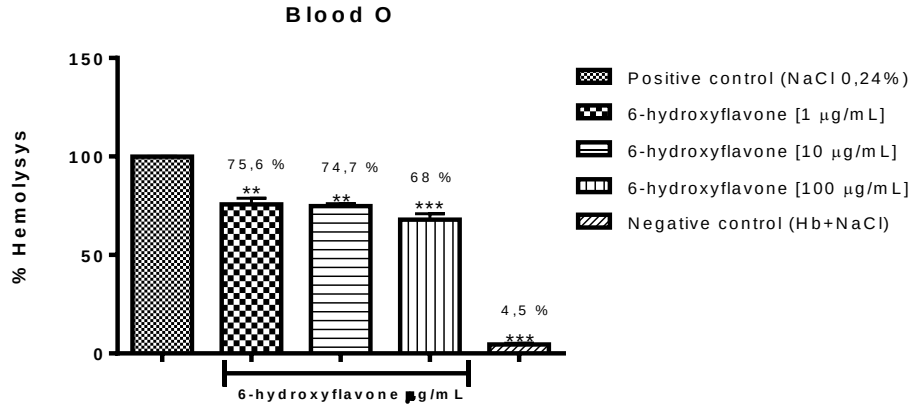


Chart 17. Anti-hemolytic activity of 6-hydroxyflavone on blood types A (a), B (b) and O (c) when in hypotonic solution (NaCl 0.24%). The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p<0.01, ***p< 0.001 (n=3).

From these results, antihemolytic activity can be conferred to the flavonoids 3-hydroxyflavone with blood type A, 5-hydroxyflavone with blood type A and O, and 6-hydroxyflavone with blood type O in the presence of osmotic stress caused by the hypotonic solution (NaCl 0.24%).

3.3. Genotoxicity assay

3.3.1. Investigation of the clastogenic and aneugenic potential of Flavone in mouse erythrocytes

To analyze the genotoxicity of flavone, the micronucleus protocol was performed in order to detect the frequency of formation of these structures in peripheral blood red cells of mice. As shown in Table 3 and figure 5, flavone at a dose of 200 mg/kg administered orally significantly reduced (1.0 ± 0.6 %) the frequency of micronucleus formation compared to the positive control group (9.8 ± 1.5%) represented by the drug cyclophosphamide at 50 mg/kg.

Table 3. Frequency of micronucleated erythrocytes in peripheral blood of male and female Swiss mice.

Tested substances	**Micronucleated erythrocytes (%) (average ± SD)**
Cyclophosphamide (50 mg/kg)	9.8 ± 1.5
Flavone (200 mg/kg)	1.0 ± 0.6***

Results are expressed as a percentage of the mean compared to the positive control group (Cyclophosphamide 50 mg/kg). Test analysis t *** p <0.001 (n = 6). SD = Standard Deviation.

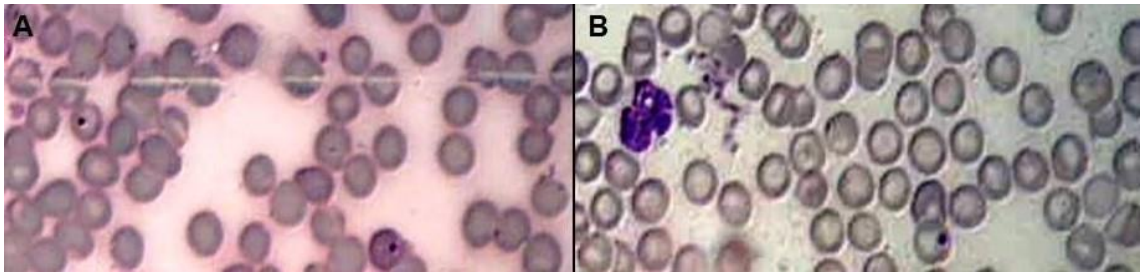


Figure 5. Erythrocytes in mice treated with cyclophosphamide 50 mg/kg (positive control) (A) and flavone 200 mg/kg (B).

The results show that flavone exhibits low genotoxicity at dose of 200 mg/kg administered orally.

4. DISCUSSION

The development of new compounds can be influenced by computational method predictions of ADME/Tox properties; either to avoid potential related problems or to warn that these same problems may well appear experimentally [16].

In silico study of the metabolism, through the application of molecular modeling techniques, is on the rise for developing new drugs [17, 18] and is recommended by various regulatory agencies for technological development studies in order to verify theoretical toxicities of pharmaceuticals in the mammalian metabolic environment [19]. *In silico* toxicological eval-

uations bring a new paradigm to assessing toxicity in which predictions are made using computational tools such as: QSAR (Quantitative Structure-Activity Relationship), REA models (Structure-activity), statistical models, and others [20].

The flavonoid molecular structure analyses performed using the Osiris software made it possible to theoretically characterize the flavone derivates (excluding flavone) as low toxicity risks. Flavone presented considerable mutagenicity and which may be associated to the lower value "Drug-score" (a combination of druglikeness, clog*P*, molecular weight and risk of toxicity) (Chart 1, and Table 1).

The results obtained from the Lipinski "Rule of five" tests demonstrate that all four flavonoids will likely be biologically available on oral administration having met all of the requirements imposed by this rule (table 2). The rule recommends that most "drug-like" molecules have a log*P* value of≤5, a molecular weight of≤ 500u, a hydrogen bond acceptor number of≤10, and a hydrogen bond donor number of ≤5. Molecules violating more than one of these parameters may have problems with bioavailability [21].

As for the *in vitro* toxicological flavonoid analyses, preliminary toxicity tests are an excellent tool to help reduce the use of animals of concern to animal ethics committees [22]. Mammalian erythrocytes represent a good model for evaluating organic and inorganic, and natural or synthetic molecular cytotoxicity, using cellular damage as a measure. Hemolysis, consequent loss of hemoglobin, is the principal signal of blood cell membrane instability [7].

Flavone, 5-hydroxyflavone and 6-hydroxyflavone presented hemolytic potential in types A, B, and O erythrocytes with Rh factor positive (+) or negative (-) in concentrations of 500 μg/mL and 1000 μg/mL as compared to the negative control group (Hb + NaCl 0.9%). The flavonoid 3-hydroxyflavone presented a similar profile, that is, concentrations of 500 μg/mL and 1000 μg/mL induced hemolysis in erythrocytes of types A+, A-, B+, O+ and O- excepting blood type B-, which in a concentration of 100 μg/mL, caused cell lysis as compared to the negative control (charts 2 to 13).

In general, from the results obtained, low to moderate hemolytic activity may be attributed to these flavonoids, since according to Rangel et al. [12] a percentage of hemolysis between 0 and 40% can be considered as low, from 40 to 80% as moderate, and as high, greater than 80%.

The next step was to verify if in a hypotonic solution (0.24% NaCl) flavones would be able to protect erythrocytes from hemolysis in the osmotic fragility test, thus verifying their role in the physiology of erythrocyte membrane function. It is important to point out that in this experiment, concentrations of 500 and 1000 μg/mL are not considered, having led to hemolysis.

The results expressed in charts 14, 15, 16 and 17 confer antihemolytic activity to 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone in blood types A and O, this in view of the osmotic stress caused by the hypotonic medium used. 3-hydroxyflavone at concentrations of 1, 10 and 100 μg/mL demonstrated this potential for type A blood alone; 5-hydroxyflavone protected erythrocytes of blood types A and O; and 6-hydroxyflavone was effective for type O blood cells. By associating the reduced hemolysis data (up to the concentration of 100 μg/mL), low cytotoxicity can be attributed to said flavonoids, suggesting a considerable safety margin for future pharmacological use.

Evidence of low *in vitro* cytotoxicity corroborates the *in silico* results that predicted a possible flavonoid action as a membrane integrity agonist, this was verified by the hemolysis protocol. This applies as well to potential membrane permeability inhibition as detected for 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone in blood types A and O; which act on the functionality of the lipid bilayer. The difference in results can be justified by secondary

metabolite structural diversity which evidences influence of both the presence and hydroxyl-C-ring position variations on the flavonoids, this, besides respective antigen variations (type A), or absence (type O) in the erythrocyte membranes.

Genotoxicity assays to predict possible genotoxic or carcinogenic activity are also important to the development of new drugs, these tests carried out in the early stages; can provide information that may lead to less toxic structures. Genotoxic analysis detects irregularities in eukaryotic organisms, applied to the detection of agents that might cause disorders in the process of chromosome to spindle microfibrils binding, and might also induce chromosomal breaks. An increase in the number of micronucleated cells indicates chromosome damage [8, 23].

Analysis of flavone by micronuclei method indicated significant reduction (1.0 ± 0.6 %) in the frequency micronuclei observed for the oral dose of 200 mg/kg, when compared to the positive control (9.8 ± 1.5%), seen in table 3.

In a study conducted by Resende [24], the findings regarding flavone were similar to those obtained in this work, flavone promoted reductions in micronucleos formation. Resende's study tested the flavonoids quercetin, kaempferol, luteolin, fisetin, chrysin, galangin, flavone, 3-hydroxyflavone, 5-hydroxyflavone and 7-hydroxyflavone for antimutagenic activity using the Ames test, in bacterial strains TA98, TA100 and TA102 in combination with direct and indirect mutagens. The difference between their structures reflects the number and position of hydroxyl groups present in the molecule. All of the compounds showed a protective effect against both direct and indirect action mutagenic agents, and antimutagenic potential in more than one bacterial strain.

In a study by Souza *et al.* [8] the data demonstrated that flavone metabolites are also non-mutagenic and the same data did not demonstrate dose-response correlation, since the increase in flavone dosage did not alter the micronuclei frequency of groups treated with flavone.

In accordance with the *in silico* assays, all of the evaluated flavones met the requirements of Lipinski's Rule of Five, thus presenting good bioavailability with oral administration. Low theoretical toxicity can be attributed to 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone, excepting flavone which showed mutagenic potential. Flavonoids presented ideal values of "druglikeness" and "drug-score", which makes them candidates for future medications.

In the *in vitro* assays, the flavones presented low cytotoxicity, inducing moderate hemolysis at the highest concentrations (500 and 1000 μg/mL) and, except for flavone, protected erythrocytes in the presence of hypotonic solution (NaCl 0.24%), indicating that phenols do not compromise the structure or function of the erythrocyte membrane.

The genotixicity test showed that 200 mg/kg flavone did not induce either aneugenic or clastogenic effects in mice erythroblasts. Certain pharmacological effects were influenced by the electron donating group (OH) as well as variation of its position on the flavonoids' benzopyran rings. As flavone significantly reduced the formation of micronuclei in cells, we suggest further research regarding cancer inhibition as promoted by this compound.

CONFLICT OF INTEREST

All authors report that they do not have any conflicts of interest.

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