In silico, in vitro and ex-vivo Toxicological Profiling of 5,7,4'-Trihydroxyflavone-8-C-β-Glucopyranoside - Vitexin

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Abstract

This study aimed to evaluate the in silico, in vitro, and ex-vivo toxicity of vitexin, the flavonoid 5,7,4'-trihydroxyflavone-8-C-β-glucopyranoside from Waltheria viscosissima. The chemical structure and predicted bioactive properties were also in silico analyzed. The in vitro and ex-vivo assays were performed according to the Ethics Code of the World Medical Association and were approved by the Ethics Committee of University Center of Patos (protocol number: 3.621.284). In silico analysis suggested that the molecule presents good oral bioavailability and good absorption; penetrating biological membranes. The toxicity tests revealed the potential effectiveness of the molecule in cellular protection against free radicals, in addition to possible antimutagenic, anticarcinogenic, antioxidant, antineoplastic, anti-inflammatory, anti-hemorrhagic and apoptosis agonist activity. Hemolytic and genotoxic assessment detected low hemolysis rates in human red blood cells and no cellular toxicity against oral mucosa cells. The data suggest that vitexin is a safe molecule for possible therapeutic application and its toxicity profile indicates viability for future studies.

Keywords: Hemolytic. Genotoxicity. Toxicity. Waltheria viscosissima.

INTRODUCTION

The development of medicines originating from naturally-derived products involves study of a wide variety of plant species, identifying their constituents (phytochemistry), and analyzing the bioactive efficacy of the isolated substances through their interactions with target molecules, to elucidate possible pharmacological mechanisms1.

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The genus *Waltheria* (family Malvaceae) is native to both Asia, and to North, Central, and South America. Various species of this genus are used in popular medicine for antimicrobial, antitussive, and expectorant applications. Various studies have identified the presence of quinoline alkaloids, triterpenes, phenolic compounds, and flavonoids being the main constituents responsible for its biological properties.

*Waltheria viscosissima* A. St. Hil., commonly known as “malva branca” and “malva viscosa” (“viscous mallow”), is endemic to Northeast Brazil where it is used as an antitussive and expectorant. Previous studies have reported that flavonoids are the main components found in extracts from the aerial parts of this species. Flavonoids are well-known for their antioxidant, antibacterial, anti-inflammatory activities, and for protecting against free radicals generated by ultraviolet light. Further, both aerial parts and roots have been shown to be active against *Aedes Aegypti* larvae.

Flavonoids present an ability to repair cell damage, increase enzyme activity in endothelial cells, and inhibit free radical formation. In particular, vitexin has been related as exerting antiviral, antioxidant, and antineoplastic activity. This study aimed to investigate the toxicity of vitexin or (5,7,4’-trihydroxyflavone-8-C-β-glucopyranoside), as isolated from *W. viscosissima* using *in silico*, *in vitro*, and *ex-vivo* testing. Assessments/predictions of theoretical molecular toxicity, chemical structure, toxicity and bioactive properties, hemolytic effect, and genotoxicity against human oral mucosa cells were performed.

**MATERIAL AND METHODS**

**Plant Material**

*W. viscosissima* was collected in August 2013 in Santa Rita (Paraíba State, Brazil) and identified by Prof. Maria de Fátima Agra (Federal University of Paraíba - UFPB). A voucher specimen was deposited in the Herbarium Prof. Lauro Pires Xavier (# MF Agra 21709) at UFPB (João Pessoa, Brazil), and the study was registered in the National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen—A568B8A).

**Sample preparation**

Aerial parts of the plant were dried in an oven at 40°C, powdered and macerated with 95% ethanol (5 L) for 72 hours. The extract solution was dried under reduced pressure at 40°C to obtain the crude ethanolic extracts (CEE). Afterwards, liquid-liquid chromatography separation was conducted with hexane, chloroform (CHCl₃), ethyl acetate, and n-butanol yielding the respective fractions. The hydroalcoholic fraction was also obtained. The CHCl₃ fraction underwent chromatographic and spectroscopic analysis for isolation and structural characterization of vitexin (Figure 1). Vitexin was used in the experiments described in this study, more information on the obtention procedures and structural determination is described in a previous study.

![Figure 1. (5,7,4’-trihydroxyflavone-8-C-β-glucopyranoside) - vitexin](image-url)

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Human erythrocyte and oral mucosa cell collection

The assays were performed according to the Ethics Code of the World Medical Association and were approved by the Ethics Committee of University Center of Patos (protocol number: 3.621.284). The blood samples (A, B, and O) and the oral mucosa smears were collected from healthy young adults of both sexes between 18 and 40 years of age.

In silico analysis

The predictive bioactive properties were analyzed using the PASS online® software (http://www.pharmaexpert.ru/passonline/). This software predicts the biological potential of a compound taking into account its activity spectrum as probable activity (Pa) or probable inactivity (Pi), whose values range between 0.000 and 1.000. If Pa is greater than Pi, the compound is believed to be experimentally active13,14. The theoretical toxicity evaluation was performed using the AdmetSAR® software (http://lmmd.ecust.edu.cn/admetsar1/) with the analysis of the following parameters: Gene Inhibition (GI), Ames Toxicity (AT), Potential Carcinogens (CP) Acute Oral Toxicity (AOT) and Carcinogenicity (Car). Pubchem® (https://pubchem.ncbi.nlm.nih.gov) was used to determine the chemical structure of vitexin.

In vitro analysis

Hemolytic activity

Aliquots of human blood (types A, B and O) were mixed with 0.9% NaCl (1:30) and centrifuged at 2500 rpm for 5 min. After repeating this procedure twice, the pellet from the last centrifugation was re-suspended in 0.9% NaCl to produce a 0.5% suspension of red blood cells (RBC) free of leukocytes and platelets. 2 mL of RBC suspensions were treated with vitexin at the concentrations of 50, 100, 500, and 1000 µg/mL. The samples were incubated for 1 h at 22 ± 2ºC and kept in slow and continuous agitation (100 rpm). Afterwards, they were centrifuged at 2500 rpm for 5 min. Hemolysis was quantified by spectrophotometry at the maximum wavelength absorbance (540 nm)15. An RBC suspension was used as negative control (0% hemolysis), and another RBC suspension with 1% Triton X-100 was used as positive control (100% hemolysis). Each test was performed in triplicate, and the data were expressed as percentages representing the arithmetic average of three measurements.

Ex vivo analysis

Evaluation of the genotoxic effect on oral mucosa cells

Epithelial cells were collected from the left and right sides of the oral mucosa using a cytobrush (endocervical sample/cell collector), which is considered the most suitable instrument for obtaining exfoliated cells from the oral mucosa16. The cells were kept in a tube with 5 mL 0.9% NaCl (cell preservation medium) until preparation of the slides. Control cells were divided into two groups: those treated with hydrogen peroxide (0.0005%) (positive control), and those receiving no treatment (negative control).

The cells were washed twice in saline solution, centrifuged for 10 min at 1500 rpm, and then kept in 5 mL saline solution. The supernatant was removed. The samples were washed once more and exposed ex-vivo to vitexin (50, 100, 500, and 1000 µg/mL) for 30 min, each test was performed in triplicate. Afterwards, they were centrifuged and the supernatant was discarded. Before the preparation of the smears, the slides were preheated to 37ºC and the cells were homogenized in a vortex mixer. They were placed on three slides at each concentration analyzed, dried at room temperature, and fixed in methanol: acetic acid (3:1) for 15 min 17. The triplicate slides were maintained at room temperature for 12 h, after which they were immersed in distilled water for 1 min and stained in 2% Giemsa for optical microscopy analysis18. Cell toxicity can be assessed through the presence of cellular indicators.
such as micronuclei, bi-nucleation, karyolysis, karyorrhexis, and macronuclei\textsuperscript{19,20}. Approximately 1000 cells were analyzed per slide, and the data were expressed as percentages representing the arithmetic average of three measurements.

Statistical analysis

The experiments were carried out in triplicate and the results were expressed as percentages representing the arithmetic average of three measurements. The data were analyzed using One-way Analysis of Variance (ANOVA) and the Bonferroni post-hoc test. The tests were performed using GraphPadPrism software (version 6.0 for Windows, San Diego, CA-USA). Differences were considered significant when \( P \leq 0.05 \).

RESULTS AND DISCUSSION

Vitexin has a molar mass of 432.38 g/mol and a total of 31 atoms. According to its theoretical physico-chemical properties, it presents good oral bioavailability. It also follows the Lipinski's rule of 5 (Ro5) which specifies molecular properties significant for the pharmacokinetics of compounds in the living organism. Ro5 determines the criteria for good oral bioavailability: molar mass (MM) \( \leq 500 \) g/mol, number of hydrogen acceptors (nON) \( \leq 10 \), number of hydrogen bond donors (nOHNH) \( \leq 5 \) and the number of violations (nViolations) \( \leq 1 \)\textsuperscript{21}. The results revealed that vitexin presents: nON: 10, nOHNH: 7, and nViolations: 1.

Other theoretical bioavailability aspects included: the lipophilicity coefficient (LogP: 0.52), the aqueous solubility coefficient (LogS: -2.39), and the topological polar surface area (TPSA: 181.04 Å\(^2\)). These suggested that vitexin presents a robust absorption index when crossing cell membranes. If the LogP: \( \leq 5.00 \) and LogS: \( \leq -4.00 \), the molecule is considered to be soluble with lipophilic properties, as stipulated in the Ro5 \textsuperscript{21}). The indexes justify a higher polar surface than the parameter adopted for indication in TPSA for good membrane permeability: \( \leq 140 \) Å\(^2\)\textsuperscript{22} (Table 1).

Table 1. Physical-chemical and theoretical bioavailability

| Parameter   | Vitexin |
|-------------|---------|
| nON         | 10      |
| nOHNH       | 7       |
| nViolations | 1       |
| LogP        | 0.52    |
| LogS        | -2.39   |
| TPSA        | 181 Å\(^2\) |

nON: numbers of hydrogen acceptors, nOHNH: number of hydrogen bond donors, nViolations: number of violations, LogP: lipophilicity coefficient, LogS: aqueous solubility coefficient, TPSA: topological polar surface area.

\textit{In silico} toxicity predictions investigate whether vitexin induces or inhibits mutagenicity. The gene inhibition analysis (GI: 0.736) indicated that vitexin did not affect gene inhibitors. In addition, Ames toxicity (AT: 0.723), and carcinogens (C: 0.955) showed no evidence of carcinogenic activity. Evaluation of carcinogenicity (Car: 0.725) was therefore not required in this investigation. For acute oral toxicity (AOT: 0.374), vitexin was placed in category IV (practically non-toxic and non-irritant); under this classification, an LD\(_{50}\) greater than 5000 mg/kg is required to present a toxic effect on the organism\textsuperscript{23} (Table 2). In a study developed by Auniq et al.\textsuperscript{24} using flavonoids isolated from Vitex Peduncularis Wall, vitexin was considered safe and displayed good oral bioavailability. Other physical-chemical parameters also indicated good lipid and aqueous solubility levels, corroborating the data described in the present study. The vitexin values described for LogS, LogP, and TPSA suggest \textit{in vivo} route viability, whether intramuscular, cutaneous, or intravenous. Further, its performance in
toxicity parameters (GI, C, AOT, and Car) indicate a potential for cellular protection against free radicals.

Table 2. Vitexin in silico toxicity analysis

| Parameter | Non-Inhibitor | 0.736 |
|-----------|---------------|-------|
| GI        | AMES Toxic    | 0.723 |
| AT        | Non-carcinogens | 0.955 |
| C         | IV            | 0.374 |
| AOT       | Non-required  | 0.725 |

GI: Gene Inhibition; AT: AMES Toxicity; C: Carcinogens; AOT: Acute Oral Toxicity; Car: Carcinogenicity

Predictive analysis for bioactive properties provided information regarding the activation probability (Pa) - inactivation probability values (Pi), as well as revealing that vitexin has a high probability of exerting a wide range of activities, including anticarcinogenic, antineoplastic, antimutagenic, antioxidant, and anti-hemorrhagic activity, and as an apoptosis agonist (Table 3). In silico and in vitro studies with V. Peduncularis (in isolated flavonoids) evidenced possible antinociceptive, anthelmintic, anti-eczema, anti-protozoan, anti-hypercholesterolemic, hepatoprotective, anti-ulcerative, anti-arthritis, antipruritic, and anti-seborrheic activities. Potential antimutagenic (Pa: 0.820 - Pi: 0.004) and anti-hemorrhagic (Pa: 0.826 - Pi: 0.002) effects were also reported, giving further support for our initial results.

Table 3. The bioactive properties of vitexin

| Pa    | Pi    | Property         |
|-------|-------|------------------|
| 0.826 | 0.002 | Antihemorrhagic  |
| 0.606 | 0.030 | Anti-inflammatory |
| 0.866 | 0.003 | Anticarcinogenic |
| 0.836 | 0.008 | Antineoplastic   |
| 0.820 | 0.004 | Antimutagenic    |
| 0.780 | 0.004 | Antioxidant      |
| 0.737 | 0.012 | Apoptosis agonist|
| 0.251 | 0.066 | Apoptosis antagonist|

Pa: Probability active, Pi: Probability inactive

RBCs are considerably susceptible to free radical damage. In this context, our findings are consistent with the proposal that oxidative stress causes RBC hemolysis. Analysis of hemolysis revealed that vitexin was not significantly toxic to RBC of the ABO system in any of the tested concentrations (from 50 to 1000 µg/mL). In accordance with the hemolytic index (percentage of hemolysis) is low when between 0 and 40%, moderate when between 40 and 80%, and high when above 80%. The percentage of hemolysis observed after exposure to vitexin varied from low to moderate (from 1% to 75%). The hemolytic rate (1000 µg/mL) was <75% for blood type O; <63% for blood type B, and <61% for blood type A. Thus, all blood types suffered moderate hemolysis and the hemolytic ranking was: A<B<O (Figure 2).
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The data evidenced a slightly different interaction between the test substance and the differing types of human RBC. The RBC membrane is composed of distinct glycoprotein types and the composition will make it either more susceptible or resistant to interactions with various molecules\textsuperscript{25}. Hemolysis potential is a measurement related to destruction of the RBC plasma membrane, and reveals the cytotoxic effect of a substance on the cell. Research conducted for investigating the ability of flavonoids to inhibit MRP1 (multidrug resistance-associated protein 1)-like efflux and induce hemolysis in human RBC revealed low to moderate hemolytic activity with values of ≤ 50\%\textsuperscript{26}. Low hemolysis percentages may be an advantage of using bioactive products and molecules. The percentage yields little oxidation, suggesting an anti-inflammatory and antioxidant role for flavonoids, including vitexin\textsuperscript{11,27,28}. Taking into account the low RBC lysis rate, it is believed that the vitexin concentration of 500 µg/mL yielded the best result, exhibiting a low percentage of hemolysis (15\% to 17\%) in all of the blood types analyzed. Blood type A presented the best vitexin interaction with the lowest percentage of hemolysis.

Genotoxicity tests are performed to identify the ability of extracts/substances in low concentrations to interact with nucleic acids. When toxic agents interact with DNA, they often produce chromosomal aberrations, alterations of the DNA structure, which in turn may result in irreversible cell changes that lead to cell death by necrosis/apoptosis. This also can lead to damage that will be passed on to daughter cells during cell division\textsuperscript{20,29}. Cell toxicity was assessed through the presence of cellular indicators (micronuclei, binucleation, karyolysis, karyorrhexis, and macronuclei) found in the oral mucosa, as shown in Figure 3. Nuclear hyperactivity is due to exposure to harmful substances and results in large and hyper stained nuclei that can lead to cell division and the occurrence of bi-nucleation and an increased number of micronuclei. Micronuclei are broken, fragmented chromosomes that were lost during mitosis. Their frequency determines the degree of DNA exposure and consequent damage caused by exogenous agents\textsuperscript{30,31}.

Figure 2. Cytotoxic effect of vitexin (\textit{W. viscosissima}) against RBC; (C) Negative control (erythrocytes 0.5\%), (C+) Positive control (1\% Triton X-100). P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***) versus positive control.
For the groups treated with differing concentrations, vitexin induced few cell changes when compared to the positive control group, but presented results similar to those of the negative control (Table 4). Vitexin exhibited low toxicity (< 90%) at all concentrations (50 to 1000 µg/mL), but major cell changes, including macronuclei, and bi-nucleation were observed at 1000 µg/mL. Although the alterations were less than those of the positive control they demonstrate the low toxicity threshold concentrations of the test substances. The results corroborated the information described in the predictive analyses which indicated the potential role of vitexin in inducing apoptosis (considering a probability of being active at 0.737 versus a probability of being inactive at 0.012). The results indicated apoptosis inhibitory activity, with a probability of being active of 0.251 versus a probability of being inactive of 0.066.

Table 4. Vitexin genotoxic profile

| Group            | Micronucleus | Binucleation | Karyolysis | Karyorrhexis | Macronucleus | Normal        |
|------------------|--------------|--------------|------------|--------------|--------------|---------------|
| Negative control | 2.22 ± 0.11% | 1.88 ± 0.11% | 0.77 ± 0.22% | 0.55 ± 0.22% | 0.001 ± 0.00% | 94.56 ± 0.22% |
| Positive control | 4.00 ± 0.88% | 4.33 ± 0.38% | 5.44 ± 0.72% | 5.11 ± 0.67% | 4.55 ± 0.80%  | 76.56 ± 3.08% |
| Vitexin          |              |              |            |              |              |               |
| 1000 µg/mL       | 0.55 ± 0.29%*| 4.00 ± 0.33% | 1.33 ± 0.50%*| 2.77 ± 0.22%*| 4.66 ± 0.33% | 86.67 ± 0.38%*|
| 500 µg/mL        | 0.55 ± 0.22%*| 2.22 ± 0.22%*| 1.66 ± 0.33%*| 2.22 ± 0.11%*| 2.88 ± 0.11%*| 90.44 ± 0.48%*|
| 100 µg/mL        | 0.44 ± 0.11%*| 1.44 ± 0.29%*| 1.88 ± 0.40%*| 1.11 ± 0.22%*| 3.222 ± 0.29%*| 91.89 ± 0.40%*|
| 50 µg/mL         | 2.11 ± 0.29%*| 4.66 ± 0.38%*| 3.66 ± 1.64%*| 2.55 ± 0.48%*| 2.88 ± 0.29%*| 84.11 ± 0.22%*|

Values are means ± standard deviation. *p < 0.05 versus positive control

CONCLUSION

The in silico toxicological assessments suggest that vitexin may hold potential for therapeutic use, with good oral bioavailability and good absorption, denoting effective crossing of the cell membrane. The in silico toxicity prediction indicated promising protective effects against oxidative stress-induced cell damage, and also other potential biological properties such as antioxidant and pro-apoptotic activity. Evaluation of hemolytic and genotoxic effects revealed that vitexin did not increase the RBC destruction rate and was non-toxic to oral mucosa cells.

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Authors' contributions
Aleson Pereira de Sousa: conceptualization, writing, data curation, methodology, formal analysis; Maria Denise Leite Ferreira, Diêgina Araújo Fernandes and Maria de Fátima Vanderlei de Souza: carried out the phytochemical study of the molecule; Laisa Vilar Cordeiro and Abrahão Alves de Oliveira Filho: supervised the biological assay, revision and formal analysis; Hilzeth de Luna Freire Pessoa and Rita de Cássia da Silveira e Sá: conceptualization, funding acquisition, supervision, formal analysis.