Effect of *Artemisia annua* (Asteraceae) Extracts on Hemolysis in Individuals with G6PD-Deficiency

Jescica Bonarck Sohou, Casimir Jiras Kpego Ligan, Salomon Ezechiel Mahougnon Fiogbe, Thierry Comlan Marc Medehouenou, Fifame Esperance Elvire Kougnimon, Casimir Dewanou Akpovi*

Unité de Recherche sur les Maladies Non Transmissibles et le Cancer (UR-MNTC), Laboratoire de Recherche en Biologie Appliquée, École Polytechnique d’Abomey-Calavi, University of Abomey-Calavi, Cotonou, Benin

Email: *casimir.akpovi@gmail.com, casimir.akpovi@uac.bj*

### Abstract

Individuals with Glucose-6-phosphate dehydrogenase (G6PD) deficiency are susceptible to hemolytic anemia when exposed to pro-oxidant substances. This study investigates the hemolytic impact of *Artemisia annua* (*A. annua*) extracts in G6PD-deficient subjects through a mixed experimental approach. In the *in vitro* phase, red blood cells from G6PD-deficient individuals and rats induced with Dehydroepiandrosterone (DHEA) were exposed to various concentrations of *A. annua* infusion, with distilled water and physiological saline as positive and negative controls respectively. The *in vivo* study involved G6PD-deficient Wistar rats divided into three groups receiving *A. annua* infusion, quinine (positive control), and distilled water (negative control) via gavage. Blood samples were collected for biochemical and hematological analyses. Notably, at a 40% concentration of *A. annua* infusion, there was a significant increase in the hemolysis rate of G6PD-deficient red blood cells compared to controls (*p* < 0.001). Rats receiving *A. annua* exhibited elevated aspartate aminotransferase (129.25 ± 4.55 U/L vs. 80.09 ± 4.03 U/L; *p* < 0.001) and total bilirubin levels (3.50 ± 1.73 U/L vs. 0.86 ± 0.24 U/L; *p* < 0.001), decreased hemoglobin levels (10.45 ± 1.01 g/dL vs. 13.25 ± 0.20 g/dL; *p* < 0.05), and the presence of Heinz bodies in blood smears contrary to controls. Additionally, the *A. annua* infusion tested positive for saponins. These findings underscore the risk of hemolysis in G6PD-deficient individuals upon ingesting *A. annua*.

### Keywords

*Artemisia annua*, Infusion, Saponins, G6PD Deficiency, Hemolysis
1. Introduction

Throughout human history, medicinal plants have been extensively harnessed for their therapeutic advantages. Nature has consistently provided an invaluable wellspring of medicinal compounds over the ages, with a substantial portion of contemporary pharmaceuticals originating from these natural resources [1]. According to the World Health Organization (WHO), more than 80% of the population in developing nations depends on medicinal plants as their primary healthcare needs [2]. This reliance continues to expand globally, driven by the potential of these plants to offer novel medicines through their bioactive constituents, which constitute the foundation of modern medicine and therapeutic innovation [3].

However, although herbal products are often perceived as natural and therefore safe [4], it is essential not to underestimate the intrinsic toxicity of some of their constituents. Plants that are considered nontoxic can produce side effects when administered inappropriately [5]. Studies have reported the mutagenic, cytotoxic, and genotoxic effects of many plants used in both food and traditional medicine [6]. Some of these plants contain chemicals that can induce hemolytic or anti-hemolytic reactions in human erythrocytes [7]. Plant extracts can disrupt the cytoplasmic membrane of red blood cells [8], and various plants have been associated with severe adverse effects, including the induction of hemolytic anemia [9]. In light of these challenges, research on medicinal plants has emerged as a prominent field globally [10]. It becomes imperative to assess the potential risks of efficacy, toxicity, and hemolytic activity inherent in medicinal plants [9]. In drug development, the toxicity of active molecules is of crucial importance, and hemolytic activity serves as a significant starting point. Indeed, it provides vital information about the interaction between molecules and biological entities at the cellular level. The hemolytic activity of a compound is a major indicator of its overall cytotoxicity toward normal cells [11]. In general, saponins, a group of phytochemical compounds found in plants, have exhibited hemolytic activity by altering erythrocyte membranes. Specific medical conditions, such as sickle cell disease and glucose-6-phosphate dehydrogenase (G6PD) deficiency, which are highly prevalent in Benin [12], may increase the sensitivity to erythrocyte hemolysis in the presence of hemolytic factors, whether they are in the form of medications or food.

*Artemisia annua* (*A. annua*) is selected for this study due to its established use in treating malaria in Benin, a disease that remains a significant health concern in the region. The choice to investigate its hemolytic effects on G6PD-deficient individuals is driven by the need to ensure the safety of this medicinal plant for all population segments, especially those at increased risk of adverse reactions. This research aims to bridge the gap in understanding the comprehensive health implications of *A. annua* use, contributing to safer healthcare practices and informed use of medicinal plants in communities dependent on traditional medicine.
2. Methodology

2.1. Animal Model

This is an experimental study of the exposed/nonexposed type conducted on an animal model, *Rattus norvegicus* (Wistar strain rats), which were rendered deficient in G6PD. The number of rats needed for the study was determined using the “resource equation” method [13] [14]. This approach was preferred owing to the need to determine several parameters for which reference standard deviations are not known in rats. The method is based on calculating the degrees of freedom (E) needed for the analysis of variance (ANOVA) and proved to be suitable for the experimental setup of the study. The degrees of freedom were calculated using the following formula: $E = N - n$, where “N” represents the total number of animals to be used and “n” is the number of groups to be formed. The estimation resulted in a total of 18 rats. We selected 9 females and 9 males, aged between 20 and 24 weeks, with an initial weight ranging from 150 to 200 grams at the beginning of the study.

We followed international guidelines for animal research ethics, consulting external veterinary experts due to the absence of an institutional ethics committee. By applying the 3Rs (Replacement, Reduction, Refinement), we minimized animal distress and ensured human treatment, including using fewer animals and enhancing welfare. Our commitment to high standards of animal welfare reflects our dedication to ethical research practices.

2.2. Induction of G6PD Deficiency

G6PD deficiency was induced by intraperitoneal injection of dihydroepiandrosterone (DHEA) at a dose of 100 mg/kg of body weight for 35 consecutive days in 36 rats (18 females and 18 males). One week after the last injection of DHEA, a retro-orbital blood sample was collected from the rats to confirm the successful induction of G6PD deficiency. Rats that developed G6PD deficiency were used for the experiments.

2.2.1. In Vivo Study

A total of 18 rats, all with G6PD deficiency, were divided into 3 groups, with each group consisting of 6 rats, including 3 females and 3 males. A 2-week acclimatization period was observed before the start of the study. During this phase, the rats were fed a standard diet consisting of pellets and water ad libitum. Before the commencement of treatment, the rats were weighed, and a retro-orbital blood sample was collected.

The groups of rats thus formed were subjected to different treatments for 7 days. Specifically, rats in Group 1 were administered distilled water (10 ml/kg of body weight), rats in Group 2 received quinine dissolved in distilled water (10 ml/kg of body weight), and rats in Group 3 were treated with an *A. annua* extract (1000 mg/kg of body weight) in distilled water (10 ml/kg of body weight) by gavage. After these 7-day treatments, the rats were reweighed, and blood
samples were collected for posttreatment tests.

2.2.2. In Vitro Study

In vitro experiments were conducted using samples of red blood cells from both humans and rats. Blood was collected from 12 individuals, including 6 with G6PD deficiency and 6 without G6PD deficiency, as well as from 12 rats, with 6 G6PD deficient and 6 normal.

The assessment of hemolytic activity was carried out using an A. annua infusion obtained from the National Herbarium at the University of Abomey-Calavi in Benin. The infusion was prepared in physiological saline buffer at concentrations of 20%, 25%, 40%, 50%, 80%, and 100% A. annua. The collected blood samples were washed with physiological saline buffer, and the resulting red blood cell pellets were used for the study of the hemolytic effect of A. annua.

The evaluation of in vitro hemolytic activity was performed using a spectrophotometric method [15]. The washed red blood cell samples were suspended with A. annua infusion, incubated for 30 minutes at 37 °C, and then centrifuged at 1500 × g for 10 minutes. The amount of free hemoglobin in the supernatant was measured using a spectrophotometer at a wavelength of 540 nm. Distilled water was used as the positive control, and physiological saline buffer was used as the negative control. Each experiment was repeated three times for each sample tested, and the hemolysis rate was determined by the formula:

\[
\text{Hemolysis}(\%) = \left( \frac{\text{At} - \text{An}}{\text{Ap} - \text{An}} \right) \times 100, \quad \text{where } \text{At: Absorbance of the tested sample}, \text{An: Absorbance of the negative control (physiological saline buffer)}, \text{and } \text{Ap: Absorbance of the positive control (distilled water)}. 
\]

2.3. Saponin Detection

Saponin detection was performed using a foam test [16]. A suspension was prepared by mixing 1 g of A. annua powder with 100 ml of distilled water (1% suspension). The mixture was gently heated and kept at a boiling point for 30 minutes. After filtering the mixture, the resulting liquid was divided into 10 graduated 15 ml tubes and diluted with distilled water to achieve dilution levels ranging from 1/1 to 1/10 of the A. annua suspension. The tubes were then vigorously agitated for 15 minutes, and the foam height was measured immediately and again after 15 minutes. In the event of a positive reaction, foam with a minimum height of 1 cm was observed.

2.4. Heinz Body Detection

Whole blood collected in an anticoagulant tube was mixed with 0.5% brilliant cresyl blue in 9 g/L NaCl at a blood-to-solution volume ratio of 1:4. The mixture was thoroughly suspended and left to stand for 10 minutes at room temperature. Smears were prepared from the suspension, allowed to dry, and then examined under a microscope. Heinz bodies appear as intensely violet-colored intracellular inclusions within erythrocytes.
2.5. Statistical Analyses

Raw data from the experiments were recorded using Microsoft Excel 2016. Statistical analyses and graphs were generated using SigmaPlot 14 software. The results are presented as the mean ± standard error of the mean (SEM). To compare the results of the in vitro tests, we used Student’s t test (parametric test) and the nonparametric alternative, the Mann-Whitney test. The exposed groups (B, C, and D) were compared to Group A, which represents the control group (unexposed). The significance level was set at 0.05.

3. Results

3.1. Detection of Saponins in Artemisia annua infusion

The foam was persistent to a height of over 1 cm after 15 minutes from tube No. 3 containing A. annua infusion at a rate of 3/1000 and beyond (Figure 1). This result indicates the presence of saponins in the studied A. annua extract.

3.2. Effect of Artemisia annua on in vitro Hemolysis

The hemolysis rates of erythrocytes from G6PD-deficient patients and controls did not show significant variation over a range of A. annua concentrations from 0 to 25%. However, a significant increase in the hemolysis rate was observed starting at an A. annua concentration of 40% in G6PD-deficient patients (p < 0.001) compared to the unchanged baseline level in the control group (Figure 2(a)). The substantially reduced G6PD enzymatic activity in deficient subjects (p < 0.0005) compared to nondeficient control subjects confirms G6PD deficiency in the subjects under consideration (Figure 2(b)).

In G6PD-deficient rats, the hemolysis rate increased progressively starting from an A. annua concentration of 40% to a maximum rate of 2% at a concentration of 100% A. annua (Figure 3(a)). The G6PD enzymatic activity rate was significantly lower in the G6PD-deficient rats (p < 0.01) than in the normal rats (Figure 3(b)).

Figure 1. Assessment of the presence of saponins in Artemisia annua infusion. Foam thickness generated by the A. annua powder suspension was rigorously evaluated in a sequence of ten tubes obtained through a stepwise dilution from the most dilute (T1) to the most concentrated (T10). Measurements were conducted with the aid of a graduated ruler to ensure accuracy. The results of these assessments are visually represented in a histogram, illustrating the relationship between dilution concentration and foam thickness. A foam thickness measured 15 minutes post-agitation, greater than 1 cm indicates the presence of saponin.
Figure 2. Effect of Artemisia annua infusion on in vitro hemolysis in human. The hemolysis rate was determined in vitro using erythrocytes from G6PD-deficient (Def-G6PD) and nondeficient control subjects. Physiological saline and distilled water were used as the negative and positive controls (Ctrl). Comparisons were made between the hemolysis rate of deficient subjects and that of the control group exposed to different concentrations of A. annua (Figure 2(a)). The G6PD enzymatic activity in deficient and control subjects is presented (Figure 2(b)). The results are presented as the mean ± SEM from 6 independent experiments (***p < 0.0005; ++p < 0.001).

Figure 3. Effect of Artemisia annua infusion on in vitro hemolysis in Wistar rat. The hemolysis rate was determined in vitro using erythrocytes from rats rendered G6PD-deficient (Def-G6PD) and nondeficient control rats (Ctrl). Comparisons were made between the hemolysis rate in G6PD-deficient rats and the control group at various concentrations of A. annua (Figure 3(a)). The G6PD enzymatic activity in both rat groups is indicated (Figure 3(b)). The results are presented as the mean ± SEM of 6 independent experiments (*p < 0.05; +p < 0.01).

3.3. Effect of Artemisia annua in G6PD-Deficient Wistar Rat

3.3.1. Effect on Weight
Rat weight showed a significant decrease (p < 0.001) after G6PD deficiency induction compared to normal rats. Treatment with quinine (p < 0.01) and A. annua (p < 0.01) further resulted in a complementary weight reduction compared to the G6PD-deficient rats without any other treatment (Figure 4).

3.3.2. Effect on G6PD Enzyme Activity
The enzymatic activity of G6PD significantly decreased (p < 0.005) compared to the activity before induction (Figure 5). Treatment with quinine or A. annua did not affect the G6PD enzymatic activity levels (Figure 5).
Figure 4. Effect of *Artemisia annua* on Wistar rat weight. G6PD-deficient rats were divided into three groups: Group A received only water, Group B was treated with quinine (G6PD Def. + Qne), and Group C was treated with *A. annua* (G6PD Def. + A. ann). The rat weights were measured before and after treatment in each group. Comparisons were made within each group between the rats before induction (Ctrl) and after induction (G6PD Ind.), as well as between Groups B and C after treatment (+p < 0.01; ++p < 0.001).

Figure 5. Effect of *Artemisia annua* on G6PD Enzymatic Activity. G6PD-deficient rats were divided into three groups: Group A received only water, Group B was treated with quinine (G6PD Def + Qne), and Group C was treated with *A. annua* (G6PD Def + A. ann). G6PD enzymatic activity was measured before and after treatment in each group. The results were compared to the control group and between the treated groups (**p < 0.005).

3.3.3. Effect on Hemolysis Biomarkers

The administration of quinine to G6PD-deficient rats resulted in a significant increase in AST levels (p < 0.001), total bilirubin (p < 0.01), and direct bilirubin (p < 0.05), along with a reduction in hemoglobin levels (p < 0.05), compared to the nondeficient control group (*Table 1*). Conversely, treatment with *A. annua* in G6PD-deficient rats led to a significant increase in AST levels (p < 0.001) and total bilirubin (p < 0.005), but not direct bilirubin, while causing a significant decrease in hemoglobin levels (p < 0.05) compared to the nondeficient control group (*Table 1*).
Table 1. Effect of *Artemisia annua* on intravascular hemolysis biochemical markers.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Distilled water</th>
<th>Quinine</th>
<th>A. annua</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/L)</td>
<td>80.09 ± 4.03</td>
<td>87.33 ± 4.63</td>
<td>133.00 ± 4.10**</td>
<td>129.25 ± 4.55**</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>27.63 ± 3.38</td>
<td>41.66 ± 3.18</td>
<td>39.75 ± 7.77</td>
<td>37.25 ± 7.04</td>
</tr>
<tr>
<td>Total Bili. (µmol/L)</td>
<td>0.86 ± 0.24</td>
<td>0.60 ± 0.17</td>
<td>2.85 ± 0.65*</td>
<td>3.50 ± 0.73**</td>
</tr>
<tr>
<td>Direct Bili. (µmol/L)</td>
<td>0.79 ± 0.15</td>
<td>1.23 ± 0.06</td>
<td>3.82 ± 1.14*</td>
<td>0.85 ± 0.24</td>
</tr>
<tr>
<td>Calcium (mg/L)</td>
<td>10.21 ± 0.20</td>
<td>9.94 ± 0.48</td>
<td>11.06 ± 1.73</td>
<td>13.45 ± 1.57</td>
</tr>
<tr>
<td>Magnesium (mg/L)</td>
<td>20.42 ± 0.84</td>
<td>20.90 ± 0.43</td>
<td>23.37 ± 3.76</td>
<td>23.12 ± 1.69</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.25 ± 0.20</td>
<td>14.66 ± 0.29</td>
<td>11.70 ± 0.34*</td>
<td>10.45 ± 1.01*</td>
</tr>
</tbody>
</table>

Initial assessment of biological parameters (control) was conducted, followed by evaluation in rats with G6PD-induced deficiency after exposure to distilled water, quinine, and *A. annua* treatment. The results are expressed as the means ± SEM. The average parameter values were subjected to comparison with the control group utilizing ANOVA (*p < 0.05; **p < 0.005; + p < 0.01; ++ p < 0.001: Assay vs. Control; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; Bili.: Bilirubin).
4. Discussion

The results presented in this study highlight the significant impact of *A. annua* on hemolysis in individuals with G6PD deficiency. This observation is of great importance, as it confirms that the use of *A. annua* can lead to potentially severe complications in individuals with this enzymatic deficiency.

*A. annua* is widely used in traditional and alternative medicine to treat various conditions [17] [18] [19], including malaria [20] [21]. However, patients with G6PD deficiency are more sensitive to hemolysis induced by certain oxidizing agents [22] [23] [24] [25], such as compounds present in *A. annua* [26] [27]. Saponins are common in many plants and play a significant role in both human and animal diets [28] [29]. Our results show the presence of saponin in *A. annua*, as reported previously [26]. Saponins are effective at very low doses and exert their hemolytic properties by creating pores in erythrocyte membranes [30]. This property contributes to the plant’s antimalarial effect. Our work confirms that the infusion of *A. annua* can trigger a significant increase in hemolysis rates in G6PD-deficient patients, particularly at high concentrations of the plant infusion. This is particularly crucial given the significant increase in hemolysis rates observed in our study, especially at higher concentrations of *A. annua* infusion. Such findings echo the intricate balance between the therapeutic benefits and potential risks of herbal medicines, emphasizing the need for personalized medical approaches.

G6PD plays a crucial role in protecting red blood cells against oxidation. When this enzyme is deficient, red blood cells become more susceptible to oxidation and rupture, leading to hemolysis [31]. The impact of *A. annua* on G6PD activity confirms that this plant can exacerbate the fragility of red blood cells in individuals with this deficiency. Furthermore, the significant decrease in the weight of G6PD-deficient rats exposed to *A. annua* is concerning. This suggests that exposure to plant extracts can have a detrimental effect on the health and growth of these individuals. This finding reinforces the idea that *A. annua* may have adverse consequences for G6PD-deficient individuals beyond hemolysis. Serum biochemical markers in rats reveal concerning results, with a significant increase in AST levels and total bilirubin in G6PD-deficient rats exposed to *A. annua* as previously reported by team [32]. These data clearly indicate liver disruption and an increase in bilirubin, which are typical signs of hemolysis and liver dysfunction. These results once again show that *A. annua* can have a negative impact on the health of G6PD-deficient individuals. Finally, the presence of Heinz bodies in the red blood cells of rats exposed to *A. annua* is visual evidence of hemolysis [33]. Heinz bodies are abnormal inclusions in red blood cells that are typically associated with excessive oxidation [34]. Their presence in rats exposed to *A. annua* confirms that this plant causes increased oxidation of red blood cells, leading to their destruction.

While this study underscores the potential risks associated with *A. annua* in G6PD-deficient individuals, it is crucial to emphasize that further research is...
imperative to determine the clinical significance of these findings. The study primarily focused on the impact of *A. annua*, neglecting a comprehensive exploration of other variables and potential interactions that might contribute to hemolysis. Focusing solely on G6PD deficiency, this study missed the complexity of individuals who often contend with multiple health conditions or genetic variations that could significantly influence the outcomes. Conducted in animals, this study leaves unanswered questions about the extent to which these findings can be translated to humans, particularly in the context of clinical applications. This highlights the need for a broader understanding of the potential risks and benefits of *A. annua* in various clinical scenarios.

In summary, our study underscores the clinical importance of recognizing the risks associated with *A. annua* for individuals with G6PD deficiency, emphasizing the need for healthcare providers to exercise increased vigilance and adopt preventive strategies to protect vulnerable groups. This research encourages further exploration into the varied clinical effects of *A. annua* across different genetic profiles, with the goal of enhancing personalized medicine approaches to ensure the safety and well-being of patients.

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Author Contributions

Conceptualization: JBS, CDA; methodology: JBS, SEMF, CDA; formal analysis, data interpretation and investigation: CJKL, TCMM, CDA; writing-original draft: CJKL, CDA; writing-review and editing: CJKL, TCMM, EFEK; study supervision: CDA; infrastructure and administration: SEMF, EFEK, CDA. All authors have read and approved the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References


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