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Quantitative Determination of Oxalic and Phytic Acids of *Lippia Multiflora* Moldenke (Verbenaceae) Leaves and *In Silico* Study of Their Interaction with Haemoglobin S and 2,3-DPG-Mutase

Colette Masengo Ashande
Department of Environment, Faculty of Science, University of Gbado-Lite, Gbado-Lite, Democratic Republic of the Congo; Department of Biology, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
Email: masengo@unikin.ac.cd

Koto-Te-Nyiwa Jean-Paul Ngbolua*
Department of Environment, Faculty of Science, University of Gbado-Lite, Gbado-Lite, Democratic Republic of the Congo; Department of Biology, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo; National Scientific Council, Ministry of Scientific Research and Technological Innovation, Democratic Republic of the Congo
Email: jpngbolua@unikin.ac.cd

Néhémie Kwevi Masevo
Department of Biology, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
Please provide the Email.

Jason Kilembe Thambwe
Department of Chemistry, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
Email: jasonkilembe3@gmail.com

Oscar Shetonde Mihigo
Department of Chemistry, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
Email: shetonde.mihigo@unikin.ac.cd

Odette Kabena Ngandu
Department of Biology, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
Email: odette.kabena@unikin.ac.cd

Dorothée Tshilanda Dinangayi
Department of Chemistry, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
Email: dorothéé.tshilanda@unikin.ac.cd

Damien Tshibangu Sha-Tshibey
National Scientific Council, Ministry of Scientific Research and Technological Innovation, Democratic Republic of the Congo; Department of Chemistry, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
Email: damien.tshibangu@unikin.ac.cd

Guy Ilumbe Bayeli
Department of Biology, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
Email: guy_ilumbe@yahoo.com

Pius Mpiana Tshimankinda
National Scientific Council, Ministry of Scientific Research and Technological Innovation, Democratic Republic of the Congo; Department of Chemistry, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo
Email: pt.mpiana@unikin.ac.cd

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Abstract

The aim of this study is to measure oxalic and phytic acids from the leaves of *Lippia multiflora* and to evaluate their anti-sickle cell activity *in silico*. The histological characterization of the leaves of *L. multiflora* was performed by optical micrography. Theseanti-nutritional factors oxalic acid and phytic acid were determined by the standard method. Discovery Studio, ChemDraw and Autodock Vina software were used in molecular modeling. The pharmacokinetic and toxicological profile was evaluated using the bioinformatics descriptor SWISSADME. Histological analysis shows that fibres, sclerites and suber fragments are characteristic tissues of *L. multiflora* leaves. These leaves contain oxalic acid (insoluble oxalates: 51.457±27.653 mg/100 g DM; total oxalates: 59.483±0.704 mg/100 g MS; calcium oxalates: 0.216±0.0021 mg/100 g MS) and phytic acid (18.400±5.062 mg/100 g MS). Oxalic acid interacts more effectively with 2,3-diphospgoglycerate mutase (ΔG=-5.30±0.03 kcal/mol) while phytic acid interacts more strongly with deoxyhemoglobin S (ΔG=-9.30±0.01 kcal/mol) *in silico*. Phytic acid interacts with deoxyhemoglobin S by two hydrogen bonds (Arginine 31 and Lysine 120) while oxalic acid forms five hydrogen bonds with 2,3-DPG mutase (Arginine 10, Asparagine 17; Arginine 62; Histidine 188 and Glycine 189). The pharmacopharmacokinetic profile shows that these compounds do not inhibit cytochrome P450 complex enzymes and are not toxic. Both compounds represent added value for *L. multiflora* in the symptomatic treatment of sickle cell disease. In addition to attenuating hypersideremia, they can inhibit erythrocyte sickle formation via interaction with both protein receptors.

Keywords: Sickle cell disease; *Lippia multiflora;* Antinutritional factors; Molecular docking; ADMET.

1. Introduction

Sickle cell anemia (or SS anemia) is hemoglobinopathy caused by the presence in the blood of erythrocytes containing hemoglobin S [1, 2]. Epidemiologically, the number of people with sickle cell disease is very high, making it a major public health problem worldwide [3]. In Africa, it is the first genetic disease by the number of patients [4]. In the Democratic Republic of Congo (DRC), 2% of the population is sick and the majority of these patients die before the age of five when they are not medically treated; and those who survive have damage to some vital organs, significantly reducing their life expectancy [5].

It has been reported that in the DRC, 12% of hospitalized children are sickle cell patients and the annual cost of treatment is more than USD 1,000 per patient, a cost that is difficult to bear for the majority of the population whose average income is less than USD 2 per day and who, for primary health care needs, is mainly turning to traditional medicine based on food medicinal plants [5-7]. Indeed, the plant biodiversity of the DRC represents an alternative solution to the usual anti-sickle cell drugs [3, 8-10]. The natural substances extracted from these plants can be used to formulate phytomedicines for better management of this disease in poor environments.

Phytic acid is a chemical constituent of most vegetables found as phytin (a calcium and magnesium salt) [11]s. Although it is not toxic, its excess in a diet can negatively influence the mineral balance by the formation of insoluble complexes with divalent minerals (especially iron) at physiological pH thus reducing their bioavailability [12]. Also, phytic acid forms ionic-like interactions with proteins that can lead to a decrease in their solubility [13]. Like phytic acid, oxalic acid affects the availability of minerals in the ration, including iron, sodium, potassium or magnesium, and mainly calcium. Indeed, because of its affinity for these minerals, oxalic acid forms complexes with them by chelation, thus interfering with their assimilation and consequently causing deficiencies [14, 15];.

However, the interaction of these compounds called "antinutritional factors" with hemoglobin S and 2,3-DPG-mutase, two proteins involved in the pathophysiology of sickle cell disease, can on the contrary prevent sickle cell crises (inhibition of the formation of tactoids and 2,3-diphosphoglycerate) and make these compounds or better plants that contain them anti-sickle cell drug candidates of interest because of the very high concentration of hemoglobin S in sickle cell patients. Based on the results of the previous quantitative ethnobotanical study [16], a phytochemical study was performed on oxalic acid and phytic acid from the leaves of *Lippia multiflora* Moldenke and *in silico* to evaluate their effects on the polymerization of haemoglobin S and the Rappoport-Luebering shunt.

In this study, we formulated the following hypothesis: Two antinutritional factors (oxalic acid and phytic acid) contained in the anti-sickle cell plant *Lippia multiflora* Moldenke [17] would act either individually or synergistically, to form a thermodynamically stable complex with hemoglobin S and 2,3-diphosphoglycerate mutase. Thus, the present work aims to assay oxalates and phytates in L. *multiflora* leaves, to validate their anti-sickle cell activity *in silico* and to predict their pharmacokinetic profile. The specific objectives of this work are: To determine the oxalic and phytic acid content of *L. multiflora* Moldenke; To assess the degree of spontaneity of the reaction between these two chemical compounds (ligands) and the selected receptors (haemoglobin S and 2,3-DPG mutase);

To predict the physicochemical and pharmacokinetic properties of these two compounds using standard bioinformatics descriptors.

The interest of the present work is obvious; because if the hypothesis formulated is validated, it would open up new perspectives for scientific research that could represent the natural alternative anti-sickle cell solution sought thanks to the paradigm shift in the understanding of antinutritional factors and the management of sickle cell disease.

2. Materials and Methods

2.1. Plant Material

In this study the plant material consists of *L. multiflora*, a medicinal plant widely used in traditional Congolese medicine to treat various ailments.

2.2. Study Framework and Purchase Site

This study was carried out in the city-province of Kinshasa, capital of the Democratic Republic of the Congo (4° 18' and 4° 25' south latitude, 15° 18' and 4° 22' east longitude, average altitude: 300 m above sea level) (Fig.1).

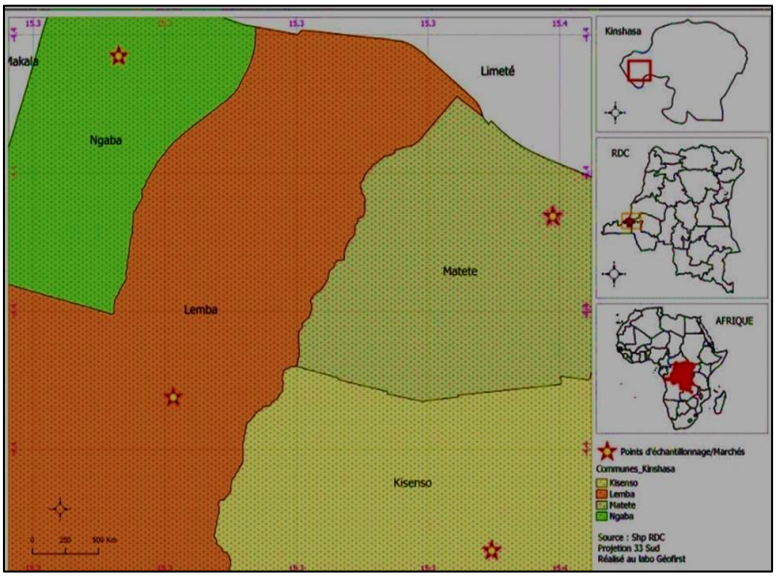

Figure-1. Geographical location of the city of Kinshasa

These samples were purchased at the Matete market in Kinshasa and the chemical analyses were carried out at the Laboratory of Ethnobiology and Medical Phytochemistry (EPHYMED Laboratory), Faculty of Science and Technology (University of Kinshasa.

2.3. Obtaining *Lippia multiflora* Moldenke Powder

The dry leaves detached from the stems are ground using an electric mill brand Blinder Burtterfly B-592, then distributed into hermetically sealed plastic vials and placed in a dry place, protected from light and moisture.

2.4. Micrograph of the Powder of the Leaves of *Lippia Multiflora* Moldenke

Observations of the powder were made using the lactic acid reagent. Two drops of lactic acid deposited on the blade were mixed with a small amount of powder, and then the mixture is covered with a covering lamella. The microscopic preparation thus obtained was heated to boiling. The microscopic observations were made with the OLYMPUS Model CH10BIMF microscope and the photos were taken with the Samsung A50 Smart Phone [3]. The purpose of this analysis was to give the histological characteristics of the leaves of L. multiflora for their characterization and the prevention of falsification.

2.5. Determination of Anti-Nutritional Factors

Both antinutrients were determined by manganimetry using standard solutions of potassium permanganate ($KMnO_4$). The oxalates were first extracted in an aqueous medium or in 2N sulfuric acid after neutralization with 0.5N sodium hydroxide (NaOH). The total oxalates obtained were precipitated with a solution of calcium chloride ($CaCl_2$) 1M in excess. The precipitate is dissolved in H_2SO_4 2N and titrated with $KMnO_4$ 0.005N. Calibration of the KMnO4 solution was performed with a range of concentrations of oxalic acid solution (C 2 O $_4$H 2, 2H_2 O) 0.001N. The content of different oxalates is determined as described above (14). Phytic acid was extracted by neutralizing the filtrate (HCl 0.5N) with soda (NaOH) 0.5N. It was then precipitated by a solution of $FeCl_3$. The precipitate (ferric phytate) was washed twice with 0,1N HCl and was then dissolved in 0,6N NaOH. The ferric hydroxide obtained was first dissociated by 6N HCl into ferric ion which is reduced to ferrous ions in the presence of zinc. The calibration of the KMnO 4 solution and the phytate content of different samples are determined as previously described [18, 19]. What kind of results are expected: What are the norms or interval thresholds of these antinutrients in leaves (foods)?

2.6. Study of the Interaction of Receptors with Ligands *in Silico*

The molecular docking was carried out in several stages as follows:

2.6.1. Preparation of Biological Receptors

Haemoglobin S (PDB ID: 2HBS) and 2,3-DPG mutase (PDB ID: 2HHJ) were used as receptors (Figure 2). Their crystallographic (3D) structures come from the "Protein Data Bank". The visualization and preparation of these proteins was done with Discovery Studio 2020 software as previously described in our previous work [20-22].

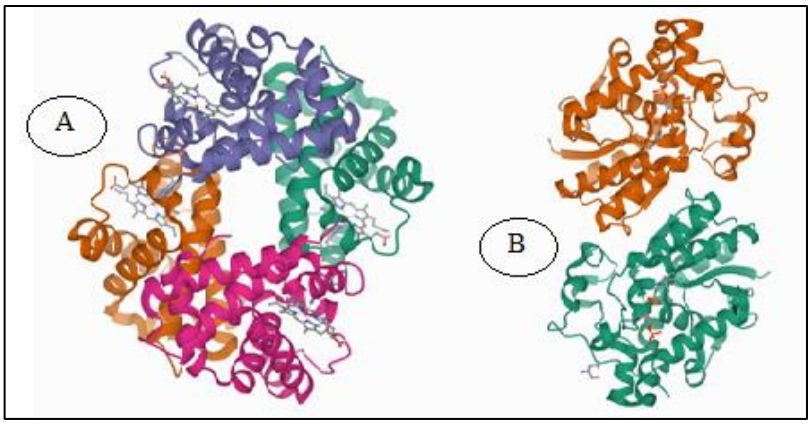

Figure-2. High-resolution crystal structure of two receptors: (a) Deoxyhemoglobin S (resolution: 2.05 Å); (b) Human bisphosphoglycerate mutase complexed with 2,3-bisphosphoglycerate (Resolution: 1.50 Å) [23, 24]

2.6.2. Preparation of Ligands

The structure of the molecules selected for molecular docking was built with ChemDraw 2016 software. The optimization of the geometry of these ligands was carried out by the force field method UFF1, which is based on a semi-empirical approach and the minimization of potential energy [25].

2.6.3. Molecular Docking

After validation of the protocol, a virtual screening was performed by rigid molecular docking for the two protein receptors in the active site using Autodock Vina software. Throughout virtual screening, the ligands were flexible and both receptors were kept rigid. The best conformation of compounds with a binding energy closer to that of the reference molecule (ursolic acid) was selected for further analysis [26].

2.6.4. Visualization

Analysis of weak molecular interactions of receptor-ligand complex structure was performed using Discovery Studio 2020 software to identify binding types [26].

2.7. Prediction of Physico-Chemical Properties and the ADMET Profile

ADMET physico-chemical, pharmacokinetic and toxicological properties (absorption, distribution, metabolism, excretion and toxicity) were evaluated with the bioinformatics descriptor SWISSADME [20-22].

2.8. Statistical Data Analysis and Microscopic Image Processing

The mean (and standard deviation), the Student test (one sample) and the Kolmogorov-Smirnov test were evaluated using Microsoft Excel version 2010, Origin version 8Pro and IBM SPSS version 20, respectively. Micrographic images were processed using Motic Images software version 1.3.

3. Results and Discussion

Figure 3 shows the histological elements of the leaves of *Lippia multiflora* Moldenke.

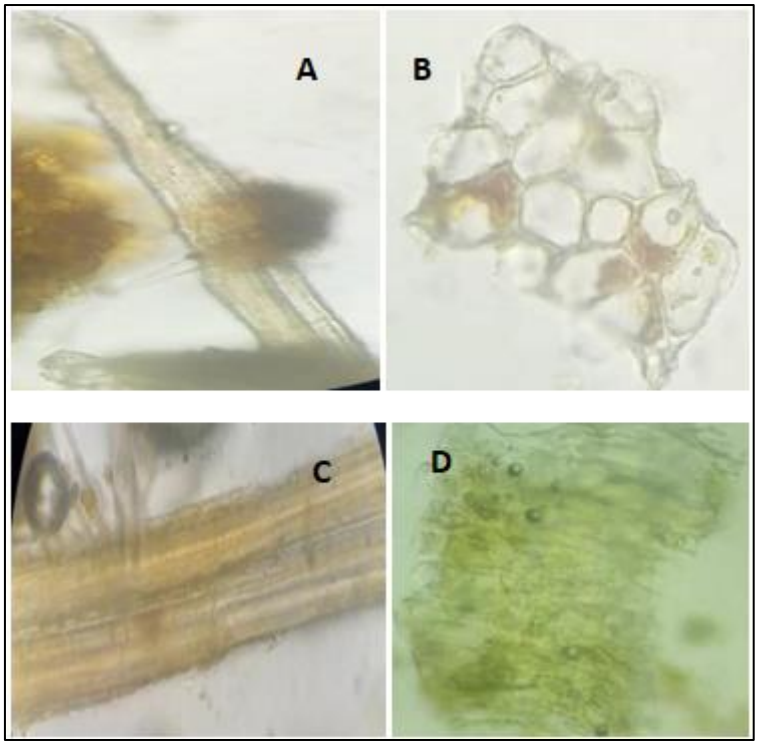

Figure-3. Histological characteristics of the leaves of *L. multiflora*: Fibers (A, C); Slerites (B); Suber fragments (D)

It appears from this figure that the leaves of *L. multiflora* contain the fibers, sclerites and fragments of suber which are histological elements characteristic of the leaves of *L. multiflora*. These results constitute a database for its characterization. To our knowledge, no histological elements of this species have been identified in previous studies. Knowledge of the microscopic characteristics of this plant is therefore one of the most valuable tools for assessing the quality and identity of plant matter.

Table 1 gives the values of some antinutritional factors measured in the leaves of *Lippia multiflora*.

Table-1. Values of different antinutrients present in L. multiflora

Sample	**Anti-nutritional factors (mg/100 g Dry matter)**			
	Insoluble oxalates	**Total oxalates**	**Calcium oxalates**	**Phytates**
A	47,45	59,55	0,219	20,6
B	87,15	60,31	0,216	11,4
C	19,77	58,59	0,214	23,2
Average	51,457	59,483	0,216	18,4
Standard deviation (Pearson)	27,653	0,704	0,002	5,062
CV (%)	53,74	1,18	0,97	27,51
p-value (one sample)	0,119*	6.998E-6**	4.51061E-5**	0,035**
Kolmogorov-S	0.214#	0.197#	0.219#	0.305#

Legend: *p>0.05 (No difference); **p<0.05 (Significant difference); #p>0.05 (Normal distribution)

It is apparent from this table that the leaves of *Lippia multiflora* Moldenke contain oxalic acid (insoluble oxalates: 51.457±27.653 mg/100 g DM; total oxalates: 59.483±0.704 mg/100 g MS; calcium oxalates: 0.216±0.002 mg/100 g MS) and phytic acid (18.400±5.062 mg/100 g MS). It should be noted, however, that these values are less than 1000 mg/100 g (reasonable threshold). Thus, although these antinutritional factors present in L. *multiflora* leaves are known for their properties to cause mineral deficiencies, they are nevertheless essential in certain diseases such as sickle cell disease characterized by iron overload (hemolytic disease) [10].

Table 2 gives the values of the interaction energy between the two ligands and the two selected receptors.

Table-2. Values of the interaction energy between ligands and selected receptors

Compounds	**ΔG (kcal/mol)**	
	2HBS (deso-oxyhemoglobin)	**2HHJ (2,3-DPG mutase)**
Ursolic acid (reference)	-6.3±0.01	-7.4±0.02
Oxalic acid	-3.8±0.02	-5.3±0.03
Phytic acid	-9.3±0.01	-5.0±0.01

It appears from this table that both ligands (oxalic acid and phytic acid) form a thermodynamically stable complex (ΔG<0) with the selected receptors. It should be noted, however, that oxalic acid interacts more effectively with 2,3-diphospgoglycerate mutase while phytic acid interacts more strongly with deoxyhemoglobin S *in silico*.

Figures 4a and 4b show the 2D and 3D structures of different thermodynamically stable complexes formed by receptors and ligands.

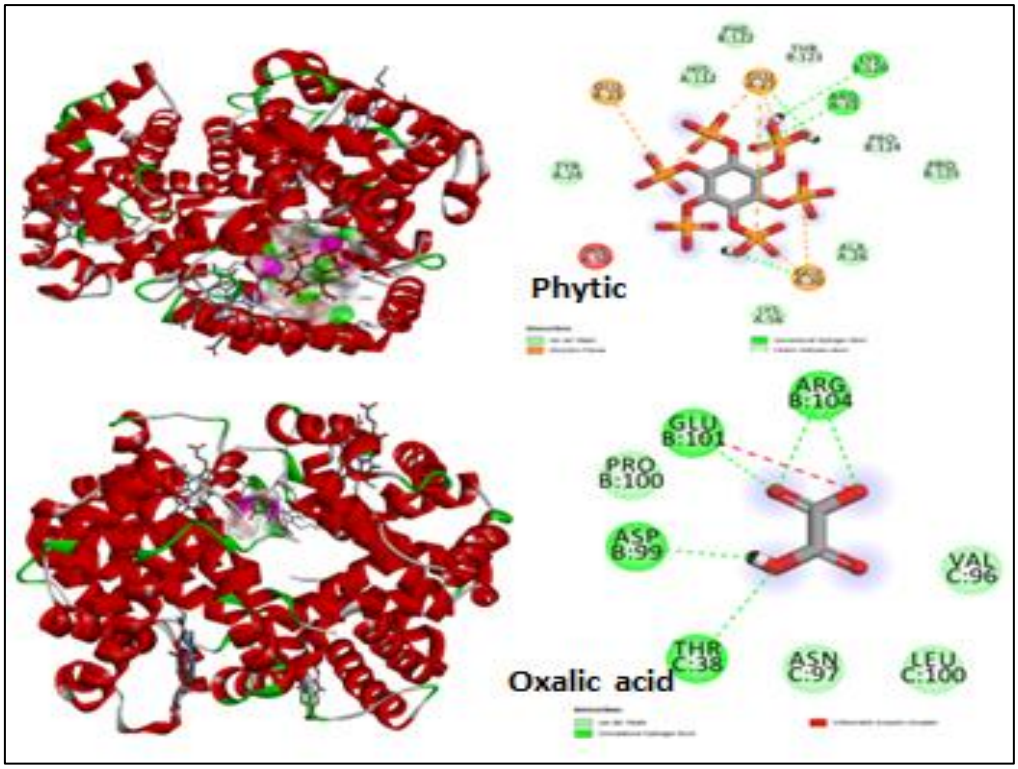

Figure-4a. 3D and 2D structures of hemoglobin S complexes with phytic acid and oxalic acid

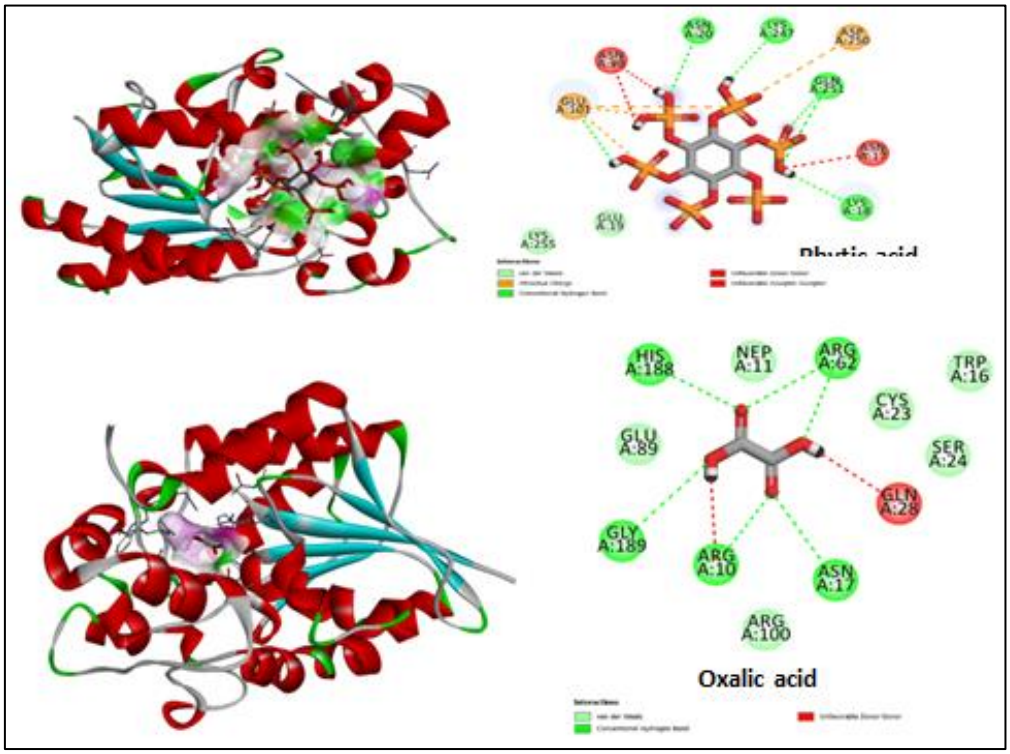

Figure-4b. 3D and 2D structures of 2,3-diphosphoglycerate mutase complexes with phytic acid and oxalic acid

Phytic acid interacts with deoxyhemoglobin S by two hydrogen bonds at arginine 31 and lysine 120 while oxalic acid forms four bonds with the same receptor at threonine 38, aspartic acid 99, glutamine 101 and arginine 104 as shown in Figure 4a.

The simultaneous presence of these two compounds in the leaves of *Lippia multiflora* is a therapeutic anti-sickle cell advantage. Indeed, in addition to other bioactive secondary metabolites present, these principles can act on several molecular targets or metabolic pathways to mitigate or suppress the pathophysiological effects of this hemoglobinopathy. As for the second receptor (2,3-DPG mutase), the present study showed that phytic acid forms four hydrogen bonds at the active site of the enzyme (lysine 18; asparagine 20; lysine 247 and glutamine 257) while oxalic acid forms five (arginine 10, asparagine 17; arginine 62; histidine 188 and glycine 189) as shown in Figure 4b.

It is well established that the docking score or thermodynamic parameter (ΔG) alone is not sufficient to assess the therapeutic potential (biological activity) of a ligand or pharmacophore. The nature of interactions between the latter and the active site of the receptor is also an important indication of bioactivity. To this end, the involvement of residues of certain so-called "key" amino acids in hydrogen bonds and weak molecular interactions such as Van der Waal or hydrophobic is also decisive. The present study shows that both chemical compounds (oxalic acid and phytic acid) are likely to inhibit the polymerization of hemoglobin S into tactoids and/or the formation of 2,3-diphosphoglycerate, a compound that decreases the affinity of hemoglobin S for oxygen. Note that the 2,3 DPG cycle or Rappoport-Luebering glycolytic shunt is grafted onto the anaerobic pathway and whose function is increased in sickle cell disease [4]. For this purpose, *Lippia multiflora* Moldenke which contains these chemical compounds can serve as an alternative to the management of this disease.

Phytic acid is a compound with antioxidant properties and can chelate iron and thus inhibit oxidative reactions catalyzed by this chemical element. This results in the inhibition of the formation of oxygen free radicals and the protection of biological macromolecules such as DNA [27, 28]. Under these conditions, plants that contain this acid can have a positive effect on excess free iron (hypersideremia) that is noted in certain pathologies such as sickle cell disease. In addition, phytic acid has been reported to reduce age-related aortic calcification, suggesting its involvement in protecting arteries from hardening [29]. It also reduces inflammation and inhibits platelet aggregation [30]. The role of phytic acid as an antioxidant is due to its ability to complex iron and remove it from circulation to prevent the production of hydroxyl radicals induced by this mineral as well as the suppression of membrane lipid peroxidation [11, 12]. Also, this compound can bind directly to certain enzymes and inhibit their biological activity [31].

In view of all these biological properties mentioned above, phytic acid is a potential anti-sickle cell drug candidate to explore. Indeed, the pharmacopharmacopharmacokinetic profile shows that this compound does not inhibit cytochrome P450 complex enzymes and is not virtually toxic. However, it is inhibitory of P-glycoprotein and its gastrointestinal absorption is low (Table 3). Figure 5 shows the physicochemical properties of oxalic and phytic acids

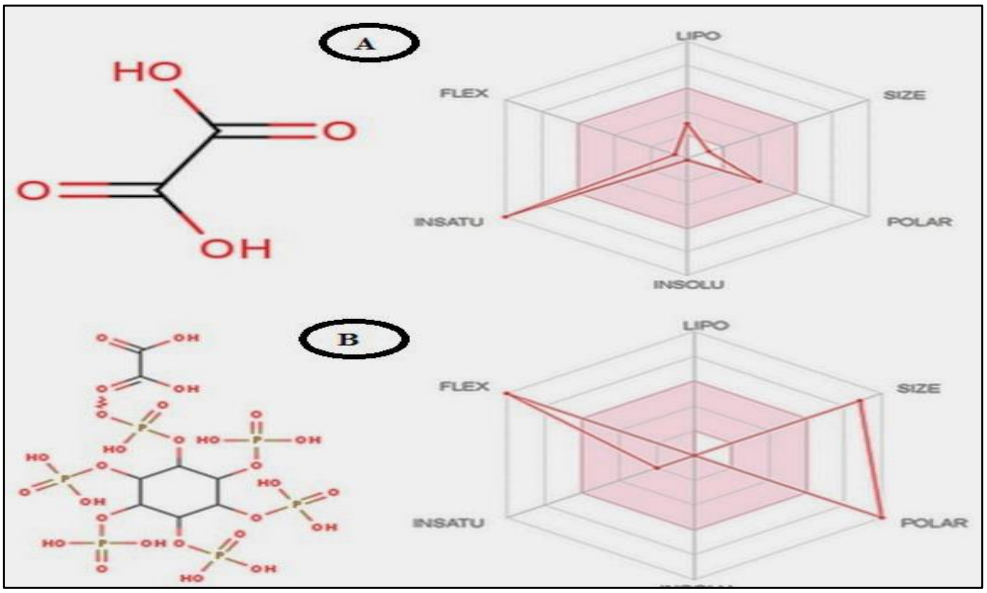

Figure-5. Chemical properties of: (a) Oxalic acid; (b) Phytic acid

(Legend: LIPO: *Liphilicity (hydrophobicity, SIZE: Molecular weight, POLAR: Polarity ar topological polar surface area, INSOLU: Insolubility in water (logs), INSATU: Unsaturation, flex = flexibility).*

The bioavailability (radar) diagram shows that phytic acid has a higher molecular weight, polarity and flexibility while oxalic acid has higher lipophilicity (hydrophobicity) and unsaturation than phytic acid. The latter is more soluble than oxalic acid (Log S: 3.34 versus -0.17), (Table 3).

Table-3. Physico-chemical properties and pharmacokinetics of oxalic and phytic acids

PROPERTIES	OXALIC ACID	PHYTIC ACID
PHYSICOCHMIC PROPERTIES		
Molecular weight	90.03 g/mol	660.04 g/mol
Number of heavy atoms	6	36
Number of aromatic heavy atoms	0	0
Fraction Csp3	0.00	1.00
Number of rotary links	1	12
Number of H-link acceptors	4	24
Number of liaison donors H	2	12
Molar refractivity	15.27	101.27
TPSA	74.60 Å²	459.42 Å²

LIPOPHILICITY		
Log $P_{o/w}$ (iLOGP)	-0.35	-4.78
Log $P_{o/w}$ (XLOGP3)	-0.25	-10.28
Log $P_{o/w}$ (WLOGP)	-0.84	-3.13
Log $P_{o/w}$ (MLOGP)	-1.51	-7.36
Log $P_{o/w}$ (SILICOS-IT)	-0.97	-8.29
Consensus Log $P_{o/w}$	-0.79	-6.77
SOLUBILITY IN WATER		
Log *S* (ESOL)	-0.17	3.34
Solubility	6.02e+01 mg/ml; 6.69e-01 mol/l	1.43e+06 mg/ml; 2.17e+03 mol/l
Class	Highly soluble	Highly soluble
Log *S* (Ali)	-0.86	1.47
Solubility	1.25e+01 mg/ml; 1.39e-01 mol/l	1.94e+04 mg/ml; 2.94e+01 mol/l
Class	Highly soluble	Highly soluble
Log *S* (SILICOS-IT)	1.47	8.57
Solubility	2.66e+03 mg/ml; 2.95e+01 mol/l	2.45e+11 mg/ml; 3.71e+08 mol/l
Class	Soluble	Soluble
PHARMACOKINETIC		
Absorption GI	High	Weak
Permeable to the BBB	No	No
P-gp substrate	No	Yes
CYP1A2 inhibitor	No	No
CYP2C19 inhibitor	No	No
CYP2C9 inhibitor	No	No
CYP2D6 inhibitor	No	No
CYP3A4 inhibitor	No	No
Log K_p (skin permeability)	-7.03 cm/s	-17.63 cm/s
MEDICINAL PROPERTY		
Lipinski	Yes; 0 violation	No; 3 violations: MW>500, NorO>10, NHorOH>5
Ghose	No; 4 violations: MW<160, WLOGP<-0.4, MR<40, #atoms<20	No; 2 violations: MW>480, WLOGP<-0.4
Veber	Yes	No; 2 violations: Rotors>10, TPSA>140
Egan	Yes	No; 1 violation: TPSA>131.6
Muegge	No; 2 violations: MW<200, #C<5	No; 5 violations: MW>600, XLOGP3<-2, TPSA>150, H-acc>10, H-don>5
Bioavailability score	0.85	0.11
MEDICINAL CHEMISTRY		
PAIN	0 alert	0 alert
Brenk	1 alert: diketo_group	1 alert: phosphor
Leadlikeness	No; 1 violation: MW<250	No; 2 violations: MW>350, Rotors>7
Synthetic accessibility	1.00	5.86
TOXICITY		
Test AMES	No	No
Max. tolerated dose (human: log mg/kg/day)	1.222	0.435
hERG I inhibitor	No	No
hERG II inhibitor	No	No
Acute oral toxicity in rats (TG_{50}: mol/kg)	1.657	2.482
Chronic oral toxicity in rats (LOAEL: log mg/kg_bw/day)	2.911	7.103
Hepatotoxicity	No	No
Skin sensitization	No	No
Toxicity to *T. pyriformis* (log μg/L)	0.086	0.285
Minnow toxicity (log mM)	2.958	22.549

4. Conclusion and Suggestions

The aim of the present study was to assay oxalates and phytates in *L. multiflora* leaves, validate their anti-sickle cell activity *in silico* and predict their pharmacokinetic profile.

The study found that:

- *Lippia multiflora* contains oxalic and phytic acids, two compounds known as antinutritional factors;
- These two compounds are endowed with anti-sickle cell properties *in silico*;
- They are neither metabolized at the hepatic level by the cytochrome P450 enzyme complex, nor mutagenic, hepatotoxic, nor cardiotoxic;
- Phytic acid is an inhibitor of glycoprotein P responsible for the efflux of xenobiotic substances.

It is therefore desirable to implement a formulation based on *Lippia multiflora* for asymptomatic treatment of sickle cell disease.

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