

# Chemical Fingerprint and Anti-Sickling Activity of Rosmarinic Acid and Methanolic Extracts from Three Species of *Ocimum* from DR Congo

Dorothee D. Tshilanda<sup>1</sup>, Paulin K. Mutwale<sup>2</sup>, Damase V. N. Onyamboko<sup>1</sup>,  
Philippe B. Babady<sup>3</sup>, Philippe V. Tsalu<sup>1</sup>, Damien S. T. Tshibangu<sup>1</sup>, Nadege K. Ngombe<sup>2</sup>,  
M. Frederich<sup>4</sup>, Koto-te-Nyiwa Ngbolua<sup>5</sup>, Pius T. Mpiana<sup>1\*</sup>

<sup>1</sup>Department of Chemistry, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo

<sup>2</sup>Centre d'Etudes des Substances Naturelles d'Origine Végétale (CESNOV), Faculty of Pharmaceutical Sciences, University of Kinshasa, Kinshasa, Democratic Republic of the Congo

<sup>3</sup>Bila Nutraceuticals Company, Dartmouth, Canada

<sup>4</sup>Laboratory of Pharmacognosy, Department of Pharmacy, CIRM, University of Liège, CHU, Liège, Belgium

<sup>5</sup>Department of Biology, Faculty of Science, University of Kinshasa, Kinshasa, Democratic Republic of the Congo  
Email: <sup>\*</sup>ptmpiana@yahoo.fr

Received 31 October 2015; accepted 11 January 2016; published 14 January 2016

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## Abstract

The aim of this study was to characterize the polyphenolic composition by determination of chemical fingerprints of Methanolic extracts of *Ocimum canum* Sims, *Ocimum basilicum* L. and *Ocimum gratissimum* L. from Democratic Republic of Congo and to compare their antisickling activity of that of rosmarinic acid, the major compound to those of methanolic extracts. Phytochemical analysis performed by TLC and HPLC analysis, showed that rosmarinic acid is the most abundant phenolic acid in these *Ocimum* species according to the following order *O. basilicum* L., *O. gratissimum* L. and *O. canum* Sims. Methanolic extracts of these three species and pure rosmarinic acid showed significant antisickling activities with minimal concentration of normalization values of  $0.18 \pm 0.03$ ,  $0.23 \pm 0.04$ ,  $0.26 \pm 0.04$  and  $0.31 \pm 0.05$  mg/mL for rosmarinic acid, *O. basilicum* L., *O. gratissimum* L. and *O. canum* Sims methanolic extracts respectively. The antisickling activity order is the same as that of the rosmarinic acid content, indicating that this polyphenolic acid would be among the main active molecules in these extracts.

\*Corresponding author.

## Keywords

Antisickling, Chemical Fingerprint, *O. canum*, *O. basilicum*, *O. gratissimum*, Rosmarinic Acid

## 1. Introduction

Sickle cell disease (SCD) or sickle anemia is a hereditary blood disorder due to an abnormal hemoglobin, hemoglobin S (HbS). In low oxygen pressure condition, HbS aggregate into intracellular polymers, that confer the shape called sickle form to erythrocytes. This Red blood cells (RBCs) shape modification makes them fragile and less flexible; what causes many complications of sicklers [1] [2].

This chronic disease affects mainly sub-Saharan African. In Democratic Republic of the Congo (RDC) more than one and half millions of people are affected [3]-[5].

Some proposed therapeutic options seem inappropriate for low-income countries population, mainly in Africa. The World Health Organization (WHO) reported that 80% of the African population relies on herbal medicine for therapy. The extensive use of herbal medicine in Africa, has been argued to be linked to cultural and economic reasons. This is why the WHO encourages African member states to promote and integrate traditional medical practices in their health system [6].

Surveys undertaken by our research team in DRC conducted to more than 115 medicinal plants used in Congolese traditional medicine to manage sickle cell disease among which was *O. basilicum* [5] [7]-[15]. In fact, in our previous works, our research team has shown that *O. basilicum* like some of medicinal plants used in Congolese herbal medicine in the management of sickle cell disease have antisickling activity *in vitro*. This biological activity is mainly displayed by polar fractions among which methanolic one and anthocyanins, organic acids and their derivatives were among the active chemical groups [3]-[5] [12]-[20]. *O. canum* and *O. gratissimum*, two other species of *Ocimum* genus found in DRC, even if they are not cited by traditional healers in the management of sickle disease, showed also *in vitro* antisickling activity. This, confirming similar results already obtained for some species of *Justicia* and *Zanthoxylum* (*Fagara*) genera [11] [21].

*O. basilicum* L., *O. canum* Sims and *O. gratissimum* L. are aromatic herbs which are used extensively to add a distinctive aroma and flavor to food. The leaves can be used fresh or dried when they are used as spice. These aromatic herbs have been used as medicinal plants in the treatment of several sickness [22]-[25].

Generally, the aromatic plants and spices of the Lamiaceae family, among which *Ocimum* species are rich in polyphenolic compounds and a large number of them are well known for medicinal properties [26]-[28].

Recently, our research team have tested antioxidant activity of different extracts from the three species and showed that methanolic extracts exhibited the highest activity [17] [19] [20]. Furthermore, ursolic acid isolated from *O. gratissimum* [18] and butyl stearate isolated from *O. basilicum* leaves [17] showed antisickling activity *in vitro*.

The aim of this work was to analyze the chemical fingerprints of the methanolic extracts of the three quoted species of *Ocimum* from RDC, to correlate their antisickling activity for a better characterization and understanding of the three methanolic extracts activities. And, to screen the presence of rosmarinic acid (RA), a naturally occurring phenylpropanoid known for its antioxidant and anti-inflammatory activity [29]-[31] in the extracts and to test the antisickling activity of a pure RA.

## 2. Materials and Methods

### 2.1. Plant Material

Leaves of *Ocimum* species were collected in the vicinity of Mbuji-Mayi (*O. canum*) and of the University of Kinshasa (*O. basilicum* and *O. gratissimum*) in DRC on May 2013. The collected plants were identified by comparison with reference specimen available at Biology department of University of Kinshasa, DRC. Voucher specimens (*Ocimum basilicum*: 425/Devred, *Ocimum canum*: 686/A. Thieband, *Ocimum gratissimum*: 8016/R. Dechamps) were deposited at the herbarium of the same department. Leaves of plants were dried at the room temperature and finely grounded in a high speed mill (Retsch ZM 100 Model) to 0.02 inches size. The powder leaves were stored in the dark at the room temperature and used for solvent extraction.

## 2.2. Biological Material

The blood samples used for the bioassays in this study were taken from adolescent sickle cell disease patients attending the “Centre de Médecine Mixteet d’Anémie SS” (CMMASS), located in Kinshasa, DRC. A written consent for each patient was approved by the national ethic committee (N° BE117). Ethical clearance on the use of SS blood was strictly observed according to international rules [32].

In order to confirm their SS nature, the above mentioned blood samples were first characterized by hemoglobin electrophoresis on cellulose acetate gel at pH 8.5 and then stored at 4°C in a refrigerator.

## 2.3. Biological Assays

Emmel’s tests were performed as previously reported [3]-[5] [7] [10] [13]. The red blood cells digitized micrographs were treated with a computer assisted image analysis system (Motic Images 2000, version 1.3), Statistical analysis and curves were processed using Microcal Origin 8.6 package Software. All anti-sickling experiments were carried out in triplicate using a sodium citrate suspension of freshly collected blood as previously reported [3] [5] [13] [14] [16].

## 2.4. Chemicals

All solvents were of analytical grade and purchased from Merck VWR (Leuven, Belgium).

Rosmarinic acid, luteolin, vitexin were HPLC grade from Sigma Aldrich. Water was treated in a Milli-Q water ultra-purification system (Easy Pure Purification System).

## 2.5. Preparation of Samples

The samples were extracted from 1 g plant powder sonicated for 15 min in 10 mL methanol and filtered on a what man filter paper. One milligram of RA of HPLC analytical grade was dissolved in 10 mL methanol and gently shaken.

## 2.6. Thin Layer Chromatography Analysis

Analytical thin layer chromatography (TLC) of 10 µL of solution was carried out on Silica gel 60 F<sub>254</sub> plates (Merck), using ethyl acetate/formic acid/glacial acetic acid/water (100:11:11:26) and methylene Dichloride/acetone/formic acid (85:25:8.5) as eluents. Luteolin, vitexin, rosmarinic acid were used as standards. The plate was sprayed with Natural Products-PEG reagent and observed at UV-365 nm.

## 2.7. High Performance Liquid Chromatography Analysis

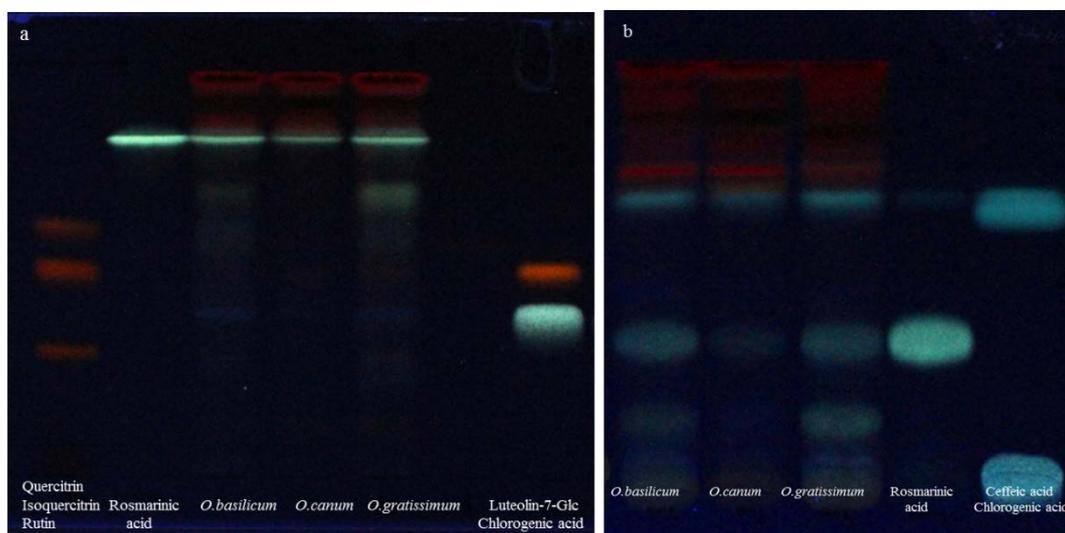
The High performance liquid chromatography (HPLC) analysis was carried out at 25°C by an “Agilent 1100” HPLC chain connected to a diode array detector (DAD). All samples and standards were filtered through a 0.45 µm pore size syringe-driven filter before 20 µL of each one were injected into the HPLC-UV/DAD system. The separation was carried out using an Hypersil ODS column (4 mm × 250 mm) with a nonlinear gradient of acetonitrile (solvent A) and 0.05% trifluoroacetic acid in ultra-pure water (solvent B) in the following composition: T0: 0% A, 100% B; T1: 3% A, 97% B; T45: 40% A, 60% B; T46: 0% A, 100% B and T60 stop. The time (T) is expressed in minutes. The compounds were eluted at a flow rate of 1 mL/min and detected with UV-DAD. The UV spectra of elution peaks were recorded in the range from 250 to 340 nm and the chromatograms were monitored at 280 and 340 nm. The identification was based on the retention time and the absorption spectra in comparison to the references and the data available in the database of the system [33].

## 3. Results and Discussion

### 3.1. Thin Layer Chromatography (TLC) Analysis

**Figure 1(a)** and **Figure 1(b)** show the TLC fingerprint of methanolic extracts of the three species of *Ocimum* firstly alone and then with phenols controls, respectively.

As above noticed, TLC analysis show that the different extracts of *Ocimum* contain polyphenols, including: Chromatogram (a): flavonoids of Kampférolkind (yellow-orange fluorescent spots) and phenolic acids (blue



**Figure 1.** (a) TLC Chromatogram of methanolic extracts from *O. basilicum*, *O. canum* and *O. gratissimum*; developed with Ethyl acetate/formic acid/glacial acetic acid/water 100:11:11:26 and visualized at 365 nm with Natural Products-PEG reagent. Flavonoids are detected as yellow-orange fluorescent spot and phenolic acids as blue fluorescent spot [34]; (b) TLC Chromatogram of methanolic extracts from *O. basilicum*, *O. canum* and *O. gratissimum* with rosmarinic acid, luteolin and vitexin as standards; developed with Methylene Dichloride/acetone/formic acid 85:25:8.5 and visualized at 365 nm with Natural Products-PEG reagent.

fluorescent spots); chromatogram (b): phenols acids RA.

The presence of RA was confirmed by HPLC fingerprint of control pure RA in superimposing this last to the fingerprint of each methanolic extract of the three *Ocimum*.

### 3.2. High Performance Liquid Chromatography (HPLC) Analysis

**Figure 2** shows the structure and the HPLC fingerprint of pure RA used as control by reason of comparison to the three HPLC fingerprints of the three methanolic extracts of three *Ocimum*.

As it can be clearly seen, the pure RA (a) appears as a single peak to the retention time equal to 32.408 minutes. As above noticed the profile of methanolic extract of *O. canum* (b) shows two intense peaks. The most intense and consequently major compound appears to the retention time equal to 58.719 minutes, followed by the one at 32.494 minutes. This last has approximately equal RA retention time. These results are near to what found by Berhow *et al.* [35] who has found a retention time of 30.07 min.

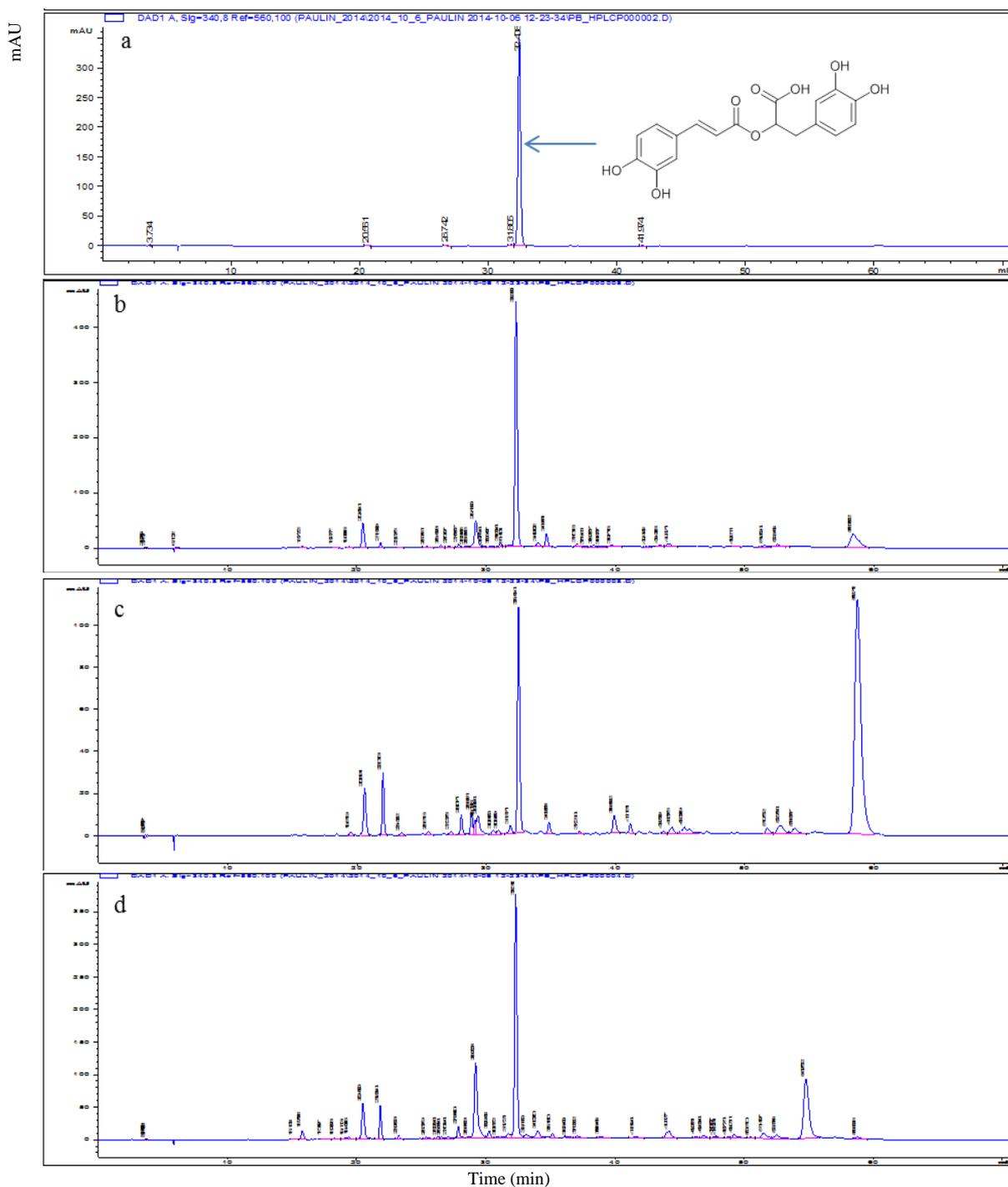
The presence of RA in the methanolic extract of *O. canum* is confirmed by the overlay of both the pure RA peak and the characterized fingerprint of methanolic extract, at the same retention time. The available data in the database used as references of the system did not allow us to identify the compound to the retention time of 58.719 minutes.

It can also be seen in the fingerprint of *O. basilicum* (b) the most intense peak to the retention time of 32.300 min. Approximately this last value is equal to the one above of RA. These results are alike to that of Vlase *et al.* [36] in methanolic extract of *O. basilicum* from Romania.

The presence of RA is demonstrated by the superimposition of the peak of the HPLC fingerprint of pure rosmarinic acid with the corresponding peak of the HPLC fingerprint of the methanolic extract of *O. basilicum*, to the identical retention time. By comparing the two intensities identified above peaks between *O. canum* and *O. basilicum*, the peak of this last (b) appears to be more intense. Thus, that result confirms that *O. basilicum* has more important amount of RA than *O. canum*.

Ultimately, it is noticed in **Figure 2** in the fingerprint of *O. gratissimum* (d) the most intense peak to the retention time of 32.310 min. This seems to be approximately to RA founded value. This result is comparable to that found by Costa *et al.* [37] in methanolic extract of *O. gratissimum* from Brazil.

As for the two first methanolic extracts, the presence of RA is shown by the overlay of the two peaks of pure RA and the one of the methanolic extract of *O. gratissimum* to the almost equal time of retention. Once more



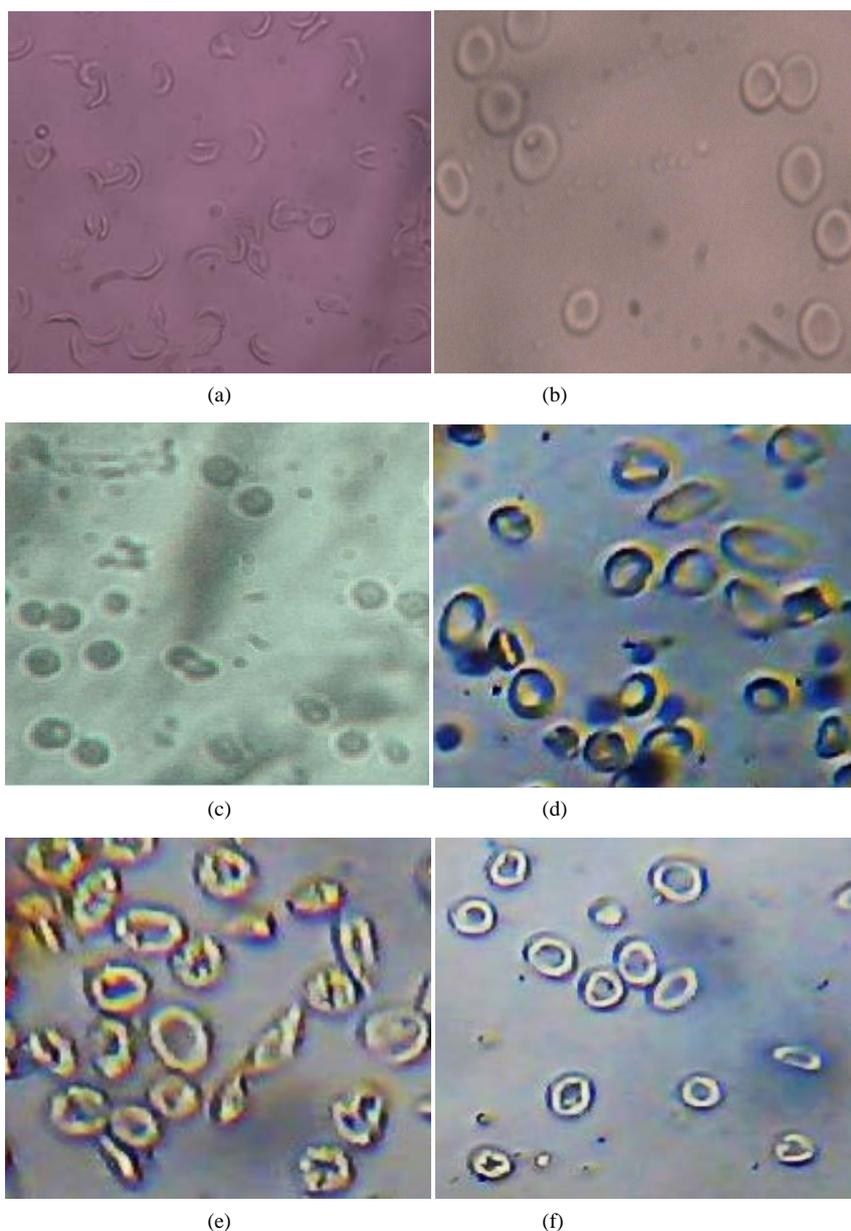
**Figure 2.** HPLC-DAD chromatograms of Rosmarinic acid (a) and methanolic extracts from *O. basilicum* (b), *O. canum* (c), *O. gratissimum* (d) using a nonlinear gradient of acetonitril (A) and 0.05% trifluoroacetic acid in ultra pure water (B): 0 min, 0:100 (A:B), 1 min, 3:97(A:B); 45 min, 40:60 (A:B); 46min, 0:100 (A:B) and 60 min, stop at 1 mL/min on Hypersil ODS column (4 mm × 250 mm) with detection at 340 nm. The elution time is given in minutes (horizontal axis). On the y axis, the height of the elution peak, expressed in arbitrary milli-unities (mAU) corresponds to the concentration of the eluted compound in the sample analysed.

again comparing the three fingerprints of the methanolic extracts of the three species according to their intensity, *O. basilicum* contains the highest amount of RA, followed by *O. gratissimum* and then *O. canum*.

### 3.3. Antisickling Activity

**Figures 3(a)-(f)** show respectively micrographies of SS blood alone in a NaCl 0.9% solution (negative control), betulinic acid (positive control) and the sickle cells blood incubated with the methanolic extract of *O. canum*, *O. gratissimum* and *O. basilicum*, and RA finally.

As it can be seen from these micrographies, the negative control (**Figure 3(a)**) contains the majority of sickle-shaped erythrocytes, confirming the Sickle cell nature of the blood. When betulinic acid, well known for its antisickling activity [17] [18] [38], is added as positive control (**Figure 3(b)**), Red blood cells show their normal circular (biconcave) shape. In the presence methanolic extracts of the three species of *Ocimum* (**Figures 3(c)-(e)**)



**Figure 3.** Morphology of erythrocytes of untreated SS blood (negative control, a); treated with 20 µg/mL of betulinic acid (positive control, b); treated with 40 µg/mL of methanolic extract of *O. canum* (c); treated with 40 µg/mL of *O. gratissimum* (d); treated with 40 µg/mL of *O. basilicum* (e); treated with 20 µg/mL of pure rosmarinic acid solution (f) [NaCl 0.9%; Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> 2%; ×500].

and RA (Figure 3(f)), the majority of erythrocytes are reversed normal-shape. This fact indicates that the methanolic extracts have an antisickling activity and show also a good normalization effect of drepanocytes in hypoxic condition. The same behavior was already observed from active extracts from some plants used in the management of sickle disease in Congolese traditional medicine, justifying their traditional use. Some molecules contained in these extracts would interact with HbS and compete with its polymerization reaction. This would prevent the sickling of red blood cells. In fact, according to Russu *et al.* [39] when investigating on molecular basis for the antisickling activity of aromatic amino acids and related compounds by NMR, phenolic acids would interact with HbS on a binding site located at or near the beta 6 position (the site containing the mutation in beta 6Glu to Val). This binding would induce conformational changes in the amino-terminal domains of the beta chains.

### 3.4. Normalization Rate

To compare the antisickling activity of different drugs, it is necessary to determine the minimal concentration of normalization (MCN) or the concentration that normalizes 50% of drepanocytes (ED50) [5] [9] [10] [15] [16].

Figure 4 gives the dose dependent antisickling activity of methanolic extracts of three *Ocimum* leaves and RA.

Figure 4 shows that the sickle cells normalization rate increases with the methanolic extracts doses until reaching the maximum threshold of which the normalization rate remains constant despite the increase of the extracts concentration. The determined minimal concentrations of normalization of each extract and RA *i.e.* the weakest concentration of extracts or RA for which the normalization rate is maximum are listed in the table.

As it can be noticed from Table 1, RA shows higher antisickling effect than all methanolic extracts. This indicates that the main secondary metabolites responsible of the antisickling effect in the three methanolic extracts have to be found as RA. This is confirmed by the fact that *O. basilicum* has highest amount of RA and exhibited

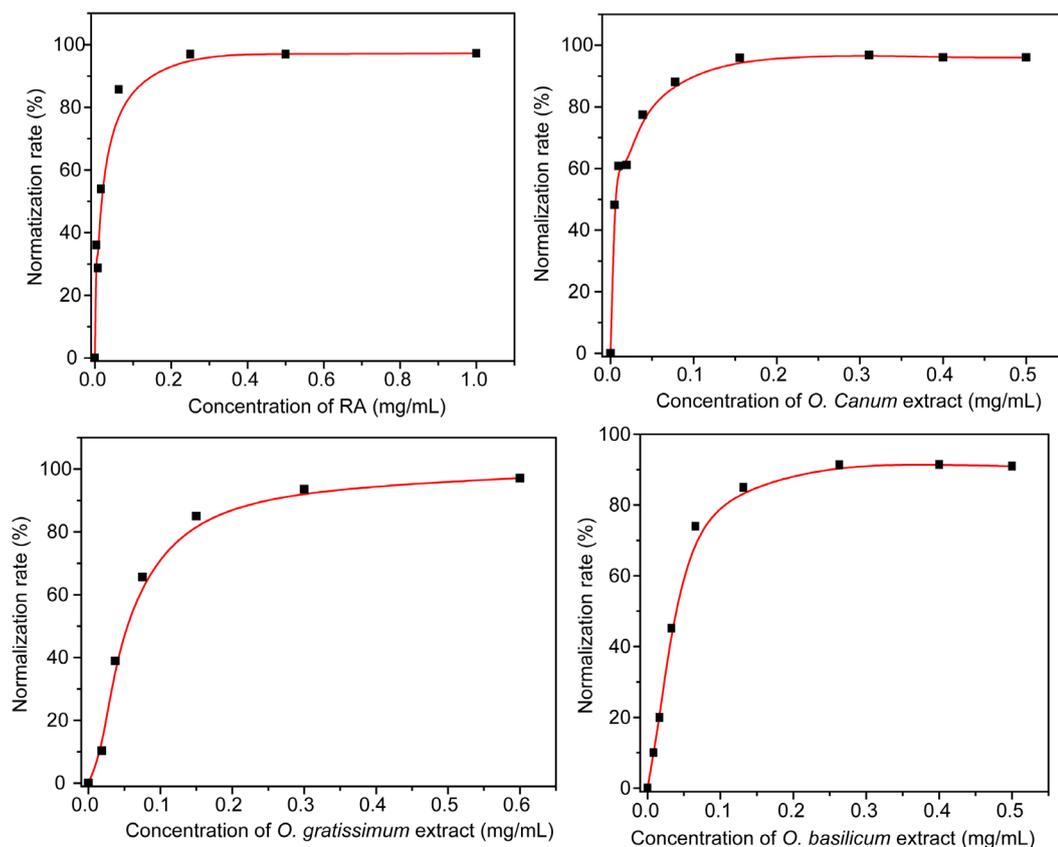


Figure 4. Evolution of the normalization rate of the drepanocytes form with the methanolic extracts of *Ocimum* and rosmarinic acid (RA).

**Table 1.** Minimal concentration of normalization (MCN) of different methanolic extracts and rosmarinic acid.

SAMPLES	MCN (mg/mL)
Rosmarinic acid	0.18 ± 0.03
<i>Ocimum basilicum</i>	0.23 ± 0.04
<i>Ocimum gratissimum</i>	0.26 ± 0.04
<i>Ocimumcanum</i>	0.31 ± 0.05

the highest antisickling activity, follow by *O. gratissimum* and then *O. canum*. In fact, most isolated antisickling active molecules are phenolic acids [18] [38]-[41]. These results indicate that rosmarinic acid is among these antisickling active organic acids. The kwon antioxidant activity of RA [29] [35] [37] may also contribute to its antisickling activity, because sickle disease is in relation with the oxidative stress [42] [43].

#### 4. Conclusion

The present study fingerprinted methanolic and tested the antisickling activity of extracts of three species of *Ocimum* from DR Congo: *O. basilicum* L., *O. gratissimum* L. and *O. canum* S. The TLC and HPLC analysis confirmed the presence of Flavonoids like kampferol derivates, phenolic acids and RA as phenol acid. This last is in high amount in the three *Ocimum* species extracts. The *O. basilicum* specie has a higher quantity of RA acid, followed by *O. gratissimum* and then *O. canum*. This order is also respected for the antisickling activity since pure RA has the highest antisickling activity and would be among the phenolic acids responsible of antisickling activity of these *Ocimum* species. RA could constitute a phyto-marker for the quality control of herbal medicines from these three plants without its isolation as pure compound.

#### Acknowledgements

One of the authors ( Prof. Pius T. Mpiana) thanks The World Academy of Sciences (TWAS) for the Research grant No. 15-156 RG/CHE/AF/AC\_G—FR3240287018 and the “Académie de Recherche et d’Enseignement supérieur (ARES) du Royaume de Belgique” for the Research grant PAH-2015 ARES/UNIKIN.

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