In Vitro Hepatoprotective and Antioxidant Activities of Diprenylated Isoflavonoids from *Erythrina Senegalensis* (Fabaceae)

Jean Hubert Donfack[a], Frederic Njayou Nico[a], Barthelemy Ngameni [b], Angele Tchana[a], Pascal Djamen Chuisseu[a], Paola Vita Finzi [c], Bonaventure Tchaleu Ngadjui[b] and Paul Fewou Moundipa[a*]

[a] *Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of Science, University of Yaounde I, P.O. Box . 812 Yaounde, Cameroon*
[b] *Laboratory of Organic Chemistry, Department of Organic Chemistry, Faculty of Science, University of Yaounde I, P.O. Box . 812 Yaounde, Cameroon*
[c] *Laboratorio di Chimica delle Sostanze Organiche Naturali, Dipartimento di Chimica Organica, Universita'degli Studi di Pavia, Via Taramelli 10, 27100 Pavia, Italy*

Summary

The 40% hydroethanolic stem bark extract (HE40) from *Erythrina senegalensis* was subjected to purification by repeated column chromatographies. Three diprenylated isoflavonoids were isolated and identified as 2,3-dihydro-2'-hydroxyosajin (**1**), osajin (**2**) and 6,8-diprenylgenistein (**3**). These compounds were tested for hepatoprotective activities against *in vitro* CCl_4-induced hepatitis in rat liver slices. The following four model systems were used to measure the antioxidant activity of these three isoflavones: 2,4-dinitrophenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, ß-Carotene-Linoleic Acid Model System (ß-CLAMS), Ferric-Reducing Antioxidant Power (FRAP) assay and microsomal lipid peroxidation. The obtained hepatoprotective percentages of compounds (**1**) (**2)** (**3**) were respectively 71.8±1.45, 67.54±3.56 and 69.41±2.56. By comparison to compound **(2)** and **(3)**, compound (**1**) showed significant antioxidant effect with EC_{50} values of 41.28±1.2, 31.27± 2.14, 19.17±1.2 and 15.99±0.49 µg/ml respectively for the radical-scavenging action, inhibition of microsomal lipid peroxidation, ß-CLAMS and FRAP assays. The hepatoprotective activity of silymarin used as reference compound was lower than the activities of isolated compounds.
The results obtained provide promising baseline information for the potential use of this crude extract as well as some of the isolated compounds for their hepatoprotective and antioxidant activities. It is also worth noting that these results validate, by *in vitro* tests, the therapeutic use of the plant in traditional medicine.

Keywords: *Erythrina senegalensis*; Fabaceae; Isoflavonoids; Antihepatotoxic, Hepatoprotective, Antioxidant.

[*]Corresponding author: Professor Paul F. MOUNDIPA, Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of Science, University of Yaounde I.
E-mail : pmoundipa@hotmail.com. Phone +237 22016043/77310383

Introduction

Erythrina senegalensis DC (Fabaceae) is a thorny shrub or small tree with bright red flowers found in Sudanese Savannah regions [1]. The stem bark of this tree is traditionally used by the Bamun population (Western Cameroon tribe) against liver disorders as a decoction [2] . Previous phytochemical studies had reported the isolation from the plant of prenylated isoflavones and flavones [3]. The antiinflammatory and antiplasmodial effects of the plant have also been investigated [4]. In addition, the isoflavone called osajin has been previously isolated from the infructences of *Maclura pomifera*, *(Moraceae)* and from *Flemingia macrophylla* and have been shown to possess cardioprotective, neuroprotective and antioxidant activities [5 6 7].

We recently reported that the 40% hydroethanolic (HE40) extract of *Erythrina senegalensis* stem bark possesses *in vitro* high antihepatotoxic potential and contains polyphenols and flavonoids (in publication). Some of these groups of compounds are reported to be antioxidant and hepatoprotective [8]. So in our ongoing search for hepatoprotective compounds from natural sources, a research to isolate, identify the active principles of the HE40 extract and to investigate the hepatoprotective and antioxidant activities of has been carried out and the results are here reported

Materials and methods

Optical rotation was recorded on a Perkin-Elmer 241 polarimeter. UV spectra were measured on a Varian Cary 1E spectrophotometer. IR spectra were recorded on a Nicolet Avatar 370 spectrophotometer in a CCl_4 solution. NMR spectra were obtained with a Varian Gemini-300 spectrometer. ESIMS were recorded on a Micromass Quattro LC mass spectrometer. Thin layer chromatography (TLC) was performed on silica gel F254 (Merck) precoated aluminium sheets and spots were visualized under UV and by spraying with molybdenum or sulphuric acid solution and heating.

Plant material

The stem barks of *Erythrina senegalensis* were collected from Foumban (West province of Cameroon) in August 2002. The botanical identification of the plant was at the National Herbarium in Yaounde, where the voucher specimen are conserved under the reference number 35259YA.

Animals

The study was carried out on male wistar albino rats (180-150g). The rat were bred in colony in the animal house of Biochemistry Department (University of Yaounde I). They were fed with a

standard pellet diet and water ad libitum. Before their use in the experiment, rats were kept in standard environment condition.

Chemicals

All reagents used in the study were of high purity and purchased from SIGMA Chemicals Co. (Dorset, UK) and Prolabo (Paris, France).

Extraction, isolation and identification of metabolites

The powdered stem bark of *Erythrina senegalensis* (5 kg) was extracted with 20 L of an ethanol/water (40% v/v) mixture for 2 h under reflux to obtain an extract similar to the folk decoction. The solvent was removed under reduced pressure to yield a dark green residue (360 g). A portion of it (355 g) was again extracted using n-hexane-ethyl acetate with a continuous gradient (from 95:5 to 60:40, and with pure ethyl acetate), followed by ethyl acetate-methanol mixtures and pure methanol as solvent and 11 fractions have been obtained. These fractions were then tested for hepatoprotective and antioxidant activities. Active fractions were pooled together according to their similarities provided by thin layer chromatography analysis to give 184 g of material. This mixture (184 g) of products was subjected to silica gel (50g) column chromatography and eluted gradually with hexane and hexane-ethyl acetate mixtures to yield 7 fractions whose activities were evaluated and the most active fraction (6 g) selected. Further separation and purification of this fraction by column chromatography over silica gel (50g) eluted with hexane-ethyl acetate (95-5) gave three compounds (**1**) (**2**) and (**3**) with respective weights of 0.678g, 0.54g and 0.68g.

The structures of the isolated compounds were established using NMR spectral analysis such as 2D experiments, COSY, HMQC and HMBC, and direct comparison with published information and with authentic specimens obtained by our research group.

Hepatoprotective activity assay of isolated compounds

The hepatoprotective activity of compounds was tested using carbon tetrachloride-induced hepatitis in rat liver slices by assessing lactate dehydrogenase (LDH) leakage from them. Liver slices were intoxicated with carbon tetrachloride (40 mM) and incubated as described previously [9]. Silymarine was use as reference hepatoprotective compound.

Screening of antioxidant activities

Free radical-scavenging activity

The free radical-scavenging activity of the compounds was evaluated by assessing their ability to discolorate 3,4-DPPH in methanol according to Brand-Williams [10]. Each compound was tested at

doses of 25, 50, 100 and 200 µg/ml. The decrease in absorbance was monitored at 517 nm and exactly 30 seconds after adding the appropriate volume of the extract or methanol to the blank. Then the percentage of discoloration was calculated for the determination of the EC_{50}.

β-Carotene-linoleic acid model system (β-CLAMS) assay

The β-CLAMS method is based on the discoloration of β-carotene by the peroxides generated during the oxidation of linoleic acid (a free radical chain reaction) at high temperature [11]. In brief, 1mL of β-carotene (0.02 % w/v) dissolved in $CHCl_3$ was introduced in a 250mL round-bottom flask. Linoleic acid (20µL) and 200mg of tween 20 were added to the mixture. $CHCl_3$ was removed using a rotary evaporator. Fifty mL of distilled water were added and the flask was shaken vigorously until all the material got dissolved. This test mixture was prepared fresh and used immediately. To each spectrophotometric cuvette was added 3 mL of the test mixture and 10µL of compound solution or water (blank). The spectrophotometric cuvettes were incubated at 50°C for 5 min. Readings were taken at 470nm immediately after and every 10 min for 3 hours. Four concentrations (25, 50, 100, and 200 µg/ml) of each compound were tested and the percentage of inhibition calculated for the determination of the EC_{50}.

Ferric-reducing antioxidant power "FRAP"

The ferric-reducing antioxidant power (FRAP) assay measures the potential of antioxidants to reduce the Fe^{3+}/2,4,6-tripyridyl-s-triazyne (TPTZ) complex present in stoichiometric excess to the blue coloured Fe^{2+} form which increases the absorption at 593 nm. This method was used as described by Benzie and Strain [12]. For each compound, four concentrations (25, 50, 100, and 200 µg/ml) were tested and the reducing power calculated for the determination of the EC_{50}.

Reference compound used for the evaluation of these three antioxidants assays (Free radical-scavenging activity, β-CLAMS and FRAP tests) was vitamin C.

Lipid peroxidation assays

Inhibition of lipid peroxidation was investigated using rat liver microsomes isolated by the calcium aggregation procedure as described by Garl and Fry [13]. Lipid peroxidation was non-enzymatically initiated using ascorbate as described by Ulf et al. [14] and assayed for thiobarbituric acid-reactive substances (TBA-RS) according to Wills [15]. Four concentrations (25, 50, 100, and 200 µg/ml) of each fraction were tested and the percentage of inhibition calculated for the determination of the EC_{50}.

Calculations and statistical analyses

LDH leakage percentages were analysed by ANOVA using the Graph Pad Prism software and $P<0.05$ was taken as significant. EC_{50} values denoted as the concentration of the sample required to scavenge 50% DPPH or to inhibit 50% of another oxidant mechanism were estimated using Graph Pad Prism 3.0.

Results and Discussion

The compounds isolated from the 40% hydroethanolic stem barks extract (**Figure 1**) belong to a secondary metabolite class named diprenylated isoflavonoids and were identified as 2,3-dihydro-2'-hydroxyosajin (**1**), osajin (**2**) and 6,8-diprenylgenistein (**3**) by comparison with literature data [16 17 18]

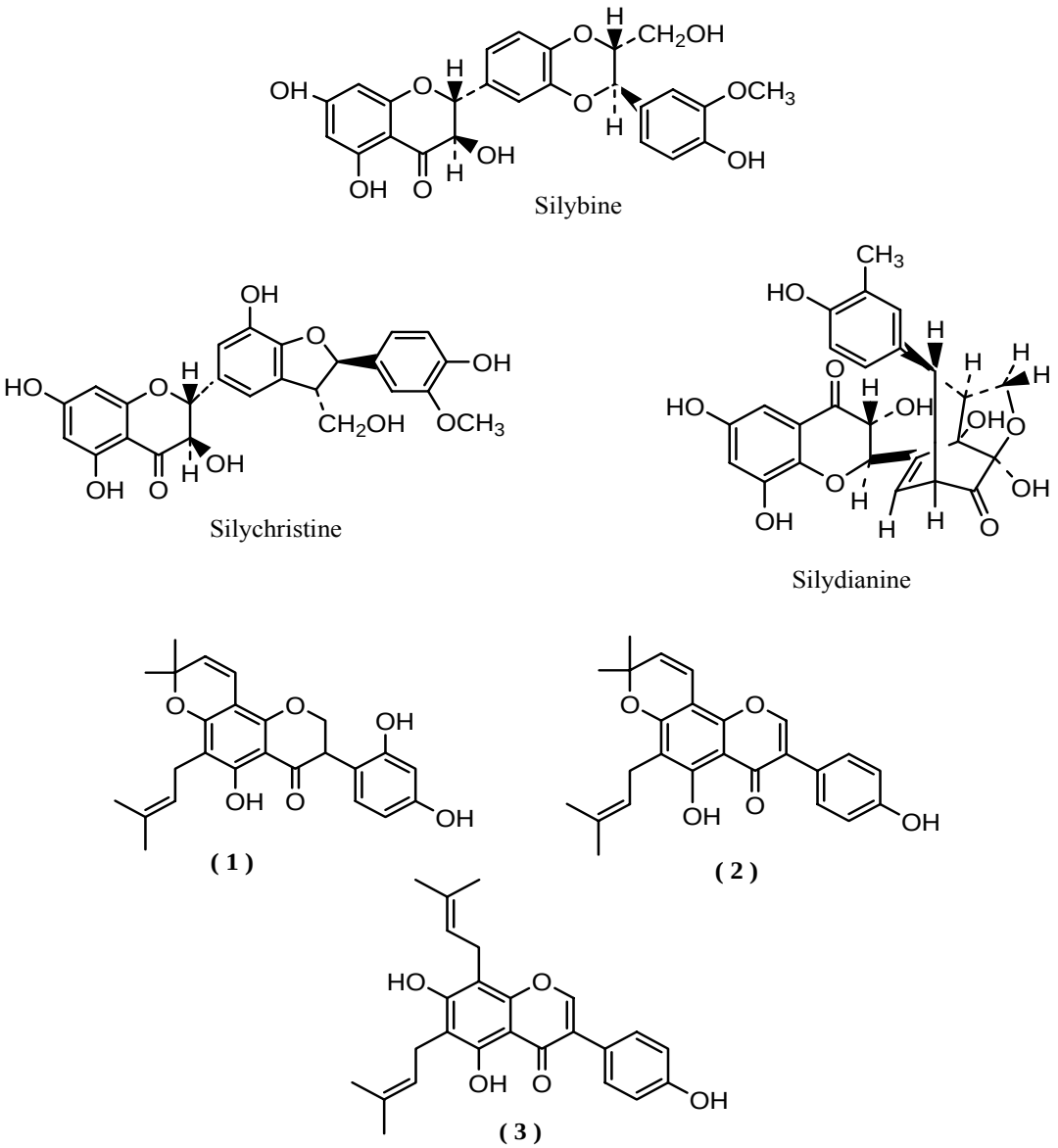

Figure 1: Structures of isolated compounds and silymarine (which is a mixing of silybine, silydianine and silychristine). (**1**): 2,3-dihydro-2'-hydroxyosajin, (**2**): osajin, (**3**): 6,8-diprenylgenistein

The liver slice system with CCl_4 as toxic agent was used to assess the *in vitro* hepatoprotective effect of compounds derived from the 40% hydroethanolic *Erythrina senegalensis* extract.

Liver slices have proved to be useful for several pharmacological and toxicological investigations since the model permits the maintenance of the normal lobular architecture and cell-cell interactions within their original matrix. Thus, this *in vitro* model may have an advantage over isolated, cultured hepatocytes due to the preservation of the tissue architecture [19 20 21 22].

It is generally accepted that the hepatotoxicity of CCl_4 depends on the cleavage of the carbon-chlorine bond to generate a trichloromethyl free radical (CCl_3). This free radical reacts rapidly with oxygen to form a trichloromethyl peroxyl radical (CCl_3O_2). This metabolite may attack the membrane polyunsaturated fatty acids and causes lipid peroxidation which plays a main role in the induction of liver injury [23 24 25], which leads to the impairment of membrane function. The consequence is the leakage of some cytosolic enzymes, including LDH. In our experiments, the hepatoprotective power of a compound depends on its capacity to prevent LDH leakage from a liver slice. At the dose of 100µg/mL, all the tested compounds were active in preventing LDH leakage from liver slices as shown by the exhibited percentages of LDH leakage (**Figure 2**).

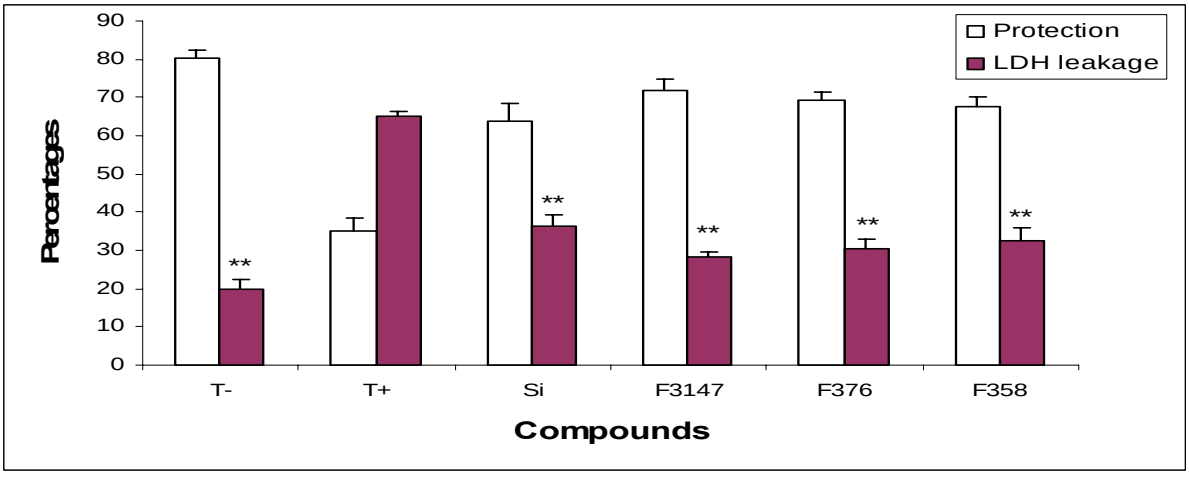

Figure 2: Effect of isolated compounds on the extent of lactate dehydrogenase leakage *in vitro* after CCl_4 challenge. Values are mean ± SD of four observations in triplicate. **comparison with CCl_4-treated control ($p<0.05$).T+: CCl_4-treated control; T-: vehicle control, Si: Silymarin (reference compound); F3147 (**1**), F358 (**2**), and F376 (**3**) (isolated compounds)

The compounds significantly ($p<0.01$) protected rat liver slices from injuries induced by CCl_4 in intoxicated and treated sets as compared to controls. Compared to silymarin (**Figure 1**) taking as reference compound with a hepatoprotection of 63.8 ± 3.89 % the hepatoprotection was pronounced with isolated compounds: 71.8±1.45, 67.54±3.56 and 69.41±2.56 % respectively for compound (**1**),

(**2**) and (**3**). The fact that antioxidant agents inhibit carbon tetrachloride-induced liver damage [26 27 28 29]prompted us to study the antioxidant effect of the isolated compounds. The DPPH radical-scavenging activity, β-CLAMS and FRAP assays, and microsomal lipid peroxidation are still frequently used by researchers for a rapid evaluation of antioxidant activity [11 12 30 31 32 33 34]. These systems were then used for the assessment of the antioxidant activities of the isolated compounds using vitamin C as reference. The results of these tests are combined in **Table 1**.

Biochemical antioxydant parameters (EC_{50} µg/mL)				
Compounds	**DPPH**	**IPL**	**β-CLAMS**	**Frap**
F3147	41.28±1,20	31.27±0.49	19.17±1.20	15.99±2.14
F376	53.00±0,89	37.48±2.45	24.95±2.54	19.17±3.1
F358	61.18±2,58	42.90±3.12	49.15±1.89	44.04±1.59
VitC	15.36±0.78	**α**	1.25±2.54	$08.6.10^{-4}$±7.10^{-5}
Correlation cœfficient	0,88	0,99	0,9	0,97

Table 1: Antioxidant activities of isolated compounds. Values are EC_{50} ± SD of two experiments in triplicate **DPPH**: radical scavenging activity; **IPL**: Lipid peroxidation assay; **FRAP**: ferric-reducing antioxidant power. **β-CLAMS** Inhibition of degradative oxidation of β-carotene, F3147 (**1**), F358 (**2**), and F376 (**3**) are isolated compounds, **α**: Vitamin C was use to initiate lipid peroxidation thus was not tested for microsomal lipid peroxidation assays.

As showing by these results, all compounds tested in the present study exhibited antioxidant activities as inhibitors of the degradative oxidation of β-carotene and rat microsomal lipid peroxidation, scavengers of free radicals and ion chelators. However, compound (**1**) seems to be more antioxidant, as indicating by its low EC_{50} values (15.99±3.1; 19.17±2.54 and 31.73±0.49) respectively for FRAP, ß-CLAMS and lipid peroxidation assays as compared to that of silymarine (86.74±1.5; 51.2±2.35; and 49.73±1.24). However, compare to Vitamine C these antioxidant parameters of compound (**1**) were very low.

Since the FRAP and ß-CLAMS assays of a compound may count as strong indicators of antioxidant potential, the latter compound might possess antioxidant activity. The best correlation was obtained between hepatoprotective and antioxidant activities of different isolated compounds as indicated in **Table 1**.

Taken together, these findings constitute evidence that isolated compounds show hepatoprotective and antioxidant activities. Accordingly, the compounds might be useful for the prevention of toxin-induced liver diseases and free radical-mediated diseases. *In vivo* studies are currently going on in our laboratory to confirm these activities.

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