STUDY OF A HEPATOPROTECTIVE AND ANTIOXIDANT FRACTION FROM *ERYTHRINA SENEGALENSIS* STEM BARK EXTRACT: *IN VITRO* AND *IN VIVO*

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Summary

An hydroethanolic extract of *Erythrina senegalensis* (DC) stem bark was subjected to a bioguided fractionation by repeated chromatographies. In vitro CCl_4-induced hepatitis in rat liver slices was used for the hepatoprotective effect assessment of the obtained fractions while four model systems: 2,4-dinitrophenyl-1-picrylhydrazyl (2,4-DPPH) radical scavenging activities, ß-Carotene-Linoleic Acid Model System (ß-CLAMS), Ferric-Reducing Antioxidant Power (FRAP) assay and microsomal lipid peroxydation (MLP) were used to measure the antioxidant activity. The fraction called F_3 was found to be the most effective *in vitro* as indicated by its ability to protect rat liver slices against CCl_4 damage (protection percentage value of 92.77). The fraction also exhibited a strong antioxidant activity in ß-CLAMS, FRAP and MLP model system (respective EC_{50} values of 12.35±1.89, 10.24±0.89 and 1.47±1.29 µg/mL). The *in vivo* hepatoprotective effect of fraction F_3 was then studied against CCl_4-induced hepatitic damage in rats. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined as biochemical indices of injury. CCl_4 (0.6 ml/kg) intoxication resulted in increase levels of serum ALT (101.90±2.92 IU/L) and AST (144.48±9.86 IU/L) compared to respective normal values 23.45±3.13 and 61.41±2.27. Pre-treatment of rats with fraction F_3 (25mg/kg, orally) significantly ($P < 0.05$) reduced the serum levels of ALT (60.95±1.43 IU/L) and AST (89.31 ±3.21IU/L) respectively. Phytochemical studies of the extract revealed the presence of polyphenols and flavonoids, compounds known to be hepatoprotective and antioxidant. These results indicate that fraction F_3 of *Erythrina senegalensis* extract may be useful as potential hepatoprotective and antioxidant phytomedecine.

Key words: *Erythrina senegalensis*, hepatoprotective activity, antioxidant activity, *in vitro*, *in vivo*

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Introduction

Current research in many parts of the world focuses on the use of local medicinal plants as liver protective drug sources [1-7]. *Erythrina senegalensis* DC (Fabaceae) is a thorny shrub or small tree with bright red flowers found mainly in Sudanese Savannah regions [8]. The tree is traditionally used by the Bamun population (Western Cameroon tribe) against liver disorders [9]. Previous phytochemical studies reported the isolation from the plant of prenylated isoflavones and flavones along with their antimicrobial and antidiuretic properties and their lack of toxicity [10]. The antiinflammatory , antiplasmodial [11], toxicity and *in vitro* hepatoprotective potential effects [12, 13] of the plant extract have also been investigated. We present in this paper the bioguided isolation and the evaluation of an antioxidant and hepatoprotective activity of a fraction from *Erythrina senegalensis* ethanolic extract.

Materials and methods

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 done at the National Herbarium in Yaounde, where the voucher specimen is conserved under the reference number 35259YA.

Animals

Male wistar albino rats from the Biochemistry Department (University of Yaounde I) animal house weighing 180g- 200g were used for the assays.

Chemicals

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

Fractionation procedure

The powdered stem bark of *Erythrina senegalensis* (5 kg) was extracted with 20 L of an alcohol/water (40% v/v) mixture for 2 h under reflux. The solvent was removed under reduced pressure to yield 360 g of a dark green residue. A portion of it (355 g) was soaked 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 to give 11 fractions (A, B, C, D, E, F, G, H, I, J, K). These fractions were then tested for hepatoprotective and antioxidant activities. Active fractions were pooled 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 column chromatography and eluted gradually with hexane and hexane-ethyl acetate mixtures to yield 7 fractions (F_1, F_2, F_3, F_4, F_5, F_6, F_7). Their activities were evaluated and were found that the most active fraction was F_3 (6 g). Further separation and purification of this fraction by column chromatography over silica gel eluted with hexane-ethyl acetate (95-5) gave another 6 fractions (F_{31}, F_{32}, F_{33}, F_{34}, F_{35}, F_{36}) and their hepatoprotective and antioxidant activities were also assessed as reported in the following tables.

***In vitro* and *in vivo* hepatoprotective activity assay of fractions**

In vitro experimental design

The hepatoprotective activity of fractions was tested using carbon tetrachloride-induced hepatitis in rat liver slices by assessing lactate dehydrogenase (LDH) leakage from them by the method of Wormser and Ben [14] as modified by Njayou [12]. Liver slices were intoxicated with carbon tetrachloride (40 mM) and incubated as described previously. The *in vitro* hepatoprotective activity was calculated as:

$$\left[1 - (\text{LDHdrug} - \text{LDHcontrol} / \text{LDHCCl}_4 - \text{LDHcontol})\right] \times 100$$

Where LDH is the percentage of lactate dehydrogenase leakage from liver slices.

In vivo exprimental design

The animals were divided into seven groups of five animals each and treated as follows: Group I animals served as normal control and received maize oil (vehicule) 10 mL/kg intraperitonial (ip). Group II animals constituted the hepatotoxic group which, received 0.6 mL/kg CCl_4 suspended in maize oil 12 hours before sacrifice. Group III received silymarin (taking as reference compound) and Group IV, V, VI, VII received the most *in vitro* active fraction (250, 100, 50 and 25 mg/kg) suspended in polyvinyl pyrrolidone (PVP) 1 hour before intoxication with CCl_4 as described in Group II.

At the end of the experimental period, animals were sacrificed by cervical decapitation, blood collected and serum separated for biochemical analyses. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed in serum by the colorimetric test of Rodier and Mallein [15] as toxicity marker enzymes. The hepatoprotective activity was calculated as:

$$\left[1 - (\text{ALTdrug} - \text{ALTcontrol} / \text{ALT CCl}_4 - \text{ALTcontol})\right] \times 100$$

***In vitro* screening of antioxidant activities**

Free radical-scavenging activity

The free radical-scavenging activity of fraction was evaluated by assessing their ability to discolorate 2,4-DPPH in methanol according Brand Williams[16]. Each fraction 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 [17]. In brief, 1 mL of β-carotene (0.02 % w/v) dissolved in $CHCl_3$ was introduced in a 250 mL round-bottom flask. Linoleic acid (20 µL) and 200 mg 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 was dissolved. This test mixture was prepared fresh and used immediately. To each spectrophotometric cuvette were added 3 mL of the test mixture and 10 µL of fraction solution or water (blank). The spectrophotometric cuvettes were incubated at 50 °C for 5 min. Readings were taken at 470 nm immediately after and every 10 min for 3 hours. 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}.

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 [18]. For each fraction, four concentrations (25, 50, 100, and 200 µg/ml) were tested and the reducing power calculated for the determination of the EC_{50}

Microsomal lipid peroxydation assays (MLP)

Inhibition of lipid peroxydation was investigated using rat liver microsomes isolated by the calcium aggregation procedure as described by Garle and Fry [19]. Lipid peroxydation was non-enzymatically initiated using ascorbate as described by Ulf *et al.,* [20] and assayed for thiobarbituric acid-reactive substances (TBA-RS) according to Wills [21]. Four concentrations (1, 10, 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

In vitro fractions hepatoprotective activity

Percentages of protection of rat liver slices against CCl_4 toxicity by different fractions are presented in **Table1**.

Table 1: Effect of fractions isolated from hydroalcoolic *Erythrina senegalensis* stem bark extract on the extent of lactate dehydrogenase leakage *in vitro* after CCl4 challenge.

Extracts	LDH leakage percentages	Liver slices protection percentages
T-	27.78±01.20**	/
T+	66.69±02.54	/
Si	**34.31±03.21**	**83.21**
EH40	37.61±03.12**	74.73
A	55.92±04.25**	27.67
B	32.46±05.36**	87.97
C	36.21±02.35**	78.33
D	40.16±04.36**	68.18
E	37.97±03.90**	73.81
F	42.93±03.87**	61.06
G	46.49±01.89**	51.91
H	57.28±01.89*	24.18
I	54.29±02.58*	31.86
J	67.60±04.12	/
K	47.78±02.65**	48.59
F1	41.90±04.26**	63.71
F2	36.80±02.89**	76.81
F3	**30.59±01.98****	**92.77**
F4	34.16±02.48**	83.60
F5	39.90±06.28**	68.85
F6	42.70±00.03**	61.65
F7	41.76±04.13**	64.07
F31	32.58±03.59**	87.66
F32	33.26±02.67**	85.91
F33	34.38±03.59**	83.03
F34	36.40±04.02**	77.84
F35	36.59±02.59**	77.35
F36	41.89±02.49**	63.73

Values are mean ± DS of four observations of a triplicate experiment. Values significantly different from CCl_4-treated controls at *$p<0.05$ **$p<0.01$. T+: CCl_4-treated controls; T-: vehicule control; Si: Sylimarin (reference compound); EH_{40}: Crude extract; A, B, C, D, E, F, G, H, I, J, K, F_1, F_2, F_3, F_4, F_5, F_6, F_{31}, F_{32}, F_{33}, F_{34}, F_{35} , F_{36} are different fractions isolated from EH_{40}.

Apart from fraction **J**, the others protected rat liver slices from the toxin damage. The most important hepatoprotective activity found regards the fraction F_3.

Fractions antioxidant activities

Hydroethanolic *Erythrina senegalensis* stem bark extract fractions were otherwise tested for their antioxidant activities. Four biochemical parameters were use for this estimation: DPPH radical-scavenging activity, β-CLAMS, FRAP assays, and microsomal lipid peroxidation. The results are shown in the **Table 2**.

Table 2: Antioxidant activities of fractions obtained from 40% hydroalcolic *Erythrina senegalensis* stem bark extract

	Biochemical antioxydant parameters (EC50 µg/mL)			
Fractions	DPPH	B Clams	Frap	MLP
Vit C	15.36±1.20	01.25±1.25	08.6.10^{-4}±0.007	
Si	35.48±3.20	19.76±4.36	15.99±2.87	40.86±1.28
EH40	57.38±0.69	16.78±3.25	25.28±1.59	21.36±2.57
A	18.00±1.32	45.68±2.68	55.38±10.17	56.24±1.24
B	54.94±1.25	46.55±2.59	46.95±1.47	60.38±2.49
C	59.76±2.32	35.86±3.17	49.81±.97	13.13±3.48
D	48.69±.79	32.00±1.98	47.38±1.24	11.1±3.57
E	54.62±1.28	25.05±2.89	51.83±2.04	30.96±2.86
F	73.99±3.56	25.98±3.45	34.34±2.17	3.32±1.57
G	44.18±3.24	17.57±2.38	47.15±0.29	18.07±1.27
H	53.67±2.87	21.56±3.49	46.67±0.14	54.1±1.49
I	67.48±0.59	37.66±3.27	54.69±2.17	**b**
J	37.85±0.45	31.04±4.29	46.51±0.47	**c**
K	33.05±0.23	38.14±5.28	68.05±1.27	94.62±7.89
F1	72.78±1.25	44.4±3.14	29.39±0.12	30.45±5.67
F2	74.33±2.49	24.8±2.59	23.00±0.79	16.12±4.27
F3	33.27±3.49	12.35±1.89	10.24±0.89	10.47±1.29
F4	41.4±3.49	32.23±2.79	21.37±1.58	70.98±3.57
F5	45.57±1.25	33.89±3.58	18,98±3.478	26.57±0.49
F6	45.85±0.03	37.39±1.58	19.23±1.28	42.53±1.49
F7	56.34±2.48	42.21±3.27	28.65±3.49	36.33±2.49
F31	60.54±3.24	30.42±3.25	20.33±2.47	30.21±2.47
F32	76.02±0.49	29.32±1.89	19.71±1.57	80.63±3.49
F33	45.67±2.17	33.22±2.58	14.00±1.89	20.14±3.479
F34	69.26±1.26	37.83±1.59	20.74±0.25	30.25±2.57
F35	87.35±0.89	30.51±2.89	32.94±1.26	33.25±3.49
F36	89.59±1.25	17.35±3.48	45.77±0.49	42.35±3.49

Values are EC_{50}± SD of different fractions with sylimarin (Si) treat controls. Each EC_{50} value was obtain by testing four doses (25, 50, 100, 200 µg/ml) in a triplicate experiment. Vitc: vitamin C; **b,c**: fractions I and J exhibited no lipid inhibition activities in rat liver microsomes. Optical densities obtained with these fractions were high than in positive controls.

Compared to sylimarin and vitamin C, all fractions possess a dose dependant antioxidant activity even though the antioxidant activity was more pronounced with fraction F_3.

In vivo F_3 fraction hepatoprotective activity

Table 3: Hepatic protection of different doses from fraction F_3 and sylimarin on CCl_4-induced ALT and AST increase in rats.

Group	Protection Percentages (%)	ALT(IU/L)	AST(IU/L)
Control	/	23.450±3.12	61.41±2.47
CCl_4	/	101.90±2.92 **	144.48±9.86**
Si (100mg/kg)	19.24±2.46	86.80±1.46 *	104.25±2.19*
F3 (25mg/kg)	52.19±4.19	60.95±1.43*	89.31±3.21*
F3 (50mg/kg)	38.55±3.67	71.65±1.76*	100.56±2.40*
F3 (100mg/kg)	26.70±2.48	80.95±1.22*	114.12±2.08*
F3 (250mg/kg)	0.19±1.46	101.75±0.95	133.74±3.42

Each value represents the mean ± SD of five rats per group. *p< 0.05 significantly different values from CCl_4-group. **p<0.01 significantly different values from control-group.

As shown in Table 3, administration of CCl_4 (0.6mL/kg IP) resulted in a marked increase of ALT and AST significantly different ($p<0.01$) from the control group. Pretraitment of rats with different doses (25, 50 and 100 mg/kg) of fraction F_3 and sylimarin (100mg/kg) resulted in the significant reduction of these elevated levels not in dose-related manner. However fraction F_3 was more active ($p<0.05$) at the dose of 25 mg/kg.

Phytochemical characterisation of fraction F_3

Phytochemical studies of fraction F_3 revealed the presence of flavonoids and polyphenols among others compounds as shown in **Table 4**.

Table 4: Some phytochemical classes compounds of *E. senegalensis* hydroethanolic extract.

Classes of compounds	Fl	Po	Tr	St	An	Al	Co	Su	Ant	Ta
	+	+	-	-	+	-	-	+	+	+

Fl: flavonoids, Po: polyphenols, Tr: Triterpens, St: Sterols, An: Anthocyanins, Al: Alcaloids, Co: Coumarines, Su: Sugars, Ant: Anthranoids, Ta: Tanins. (+): presence of compound; (-): absence of compounds

Discussion

The liver slice system and CCl_4 as toxic were used to assess *in vitro* hepatoprotective effect of *Erytrhrina senegalensis* stem bark extracts and fractions obtained from it. It is generally accepted that the hepatotoxicity of CCl_4 depend on the cleavage of the carbon-chlorine bond to generate trichloromethyl free radical (**.**CCl_3); this free radical reacts rapidly with oxygen to form a trichloromethyl peroxy radical (.CCl_3O_2). This metabolite may attack membrane polyunsaturated fatty acids and causes lipid peroxidation which play a main role in the induction of liver injury [3, 22, 23,], which lead to impairment of membrane function. The consequence is the leakage of some cytosolic enzymes including LDH. In our experiments, the *in vitro* hepatoprotective power of a fraction depends on its capacity to prevent LDH leakage from a liver slices. Considering the obtained results, at the dose of 100 µg/ml all fractions significantly ($P<0.01$) inhibited LDH leakage from liver slices showing thus their hepatoprotective activities against injuries induced by CCl_4. However this parameter was more pronounced with fraction F_3 as shown by its in vitro hepatoprotective percentage (92.77).
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 [17, 18, 24 - 29]. These systems were thus used for the assessment of the antioxidant activities of fractions. The low values of respective EC_{50} allow us to suggest that all fraction were active in the model systems studied, a part from fraction I and J regarding microsomal lipid peroxydation. However, fraction F_3 shows the lowest EC_{50} values with β-CLAMS, FRAP assays, and microsomal lipid peroxidation respective values of 12.35±1.89; 10.24±0.89 and 10.47±1.29.
These results constitute evidences that fraction F_3 of *Erythrina senegalensis* possess strong antioxidant activities. Interestingly, further fractionation of fraction F_3 gave fractions F_{31}, F_{32}, F_{33}, F_{34}, F_{35} and F_{36} that showed lower activities than that of the parent fraction. This result suggest that the active compound present in fraction F_3 may act in synergy demonstrating that fraction F_3 may be useful as a mixture..
CCl_4-induced hepatic injuries are commonly used models for the screening of hepatoprotective plant extract and the extend of hepatic damage is assessed by the level of released cytosolic transaminases including ALT and AST in circulation [3, 6, 5]. When administrated prophylacticaly, fraction F_3 exhibited protection against CCl_4 induced liver injuries as manifested by the reduction of toxin-mediated rise in serum enzymes in rats (**Table 3**) The observed preventive and the *in vitro* antioxidant activities of fraction may be attributed to the presence of polyphenols and flavonoids which have been evidenced, amongst others, as phytochemical constituents of the fraction. In fact, these metabolites are known to be antioxidant and hepatoprotective [30]
Considering the overall results, this study indicates that fraction F_3 shows strong hepatoprotective and antioxidant activities. Accordingly, this mixture of compounds might be useful for the prevention of toxic-induced liver diseases and free radical-mediated diseases since antioxidant compounds have been suggested as prophylactic agent [31 - 34]. Further biochemical and phytochemical studies of this fraction are currently going on in our laboratory.

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