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Inhibition Of Platelet Aggregation By Anthocyanins Purified From Roselle (*Hibiscus Sabdariffa* L.) Callus.

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ABSTRACT

In the present study, the anthocyanins of Roselle were subjected into the evaluation of their antiplatelet aggregation. Among the tested compound, delphinidin-3-O sambubioside, cyanidin 3-O-sambubioside, delphinidin 3-O-glucoside and cyanidin 3-O-glucoside exhibited strong inhibitory activity against platelet aggregation induced by collagen than acetylsalicylic acid (aspirin) antiplatelet reference. According to the results in the present research, anthocyanin extracted from callus of Roselle may have a protective effect against atherogenesis and cardiovascular system.

Keywords: Platelet aggregation ; Anthocyanin; Roselle ; Atherogenesis; Cardiovascular.

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INTRODUCTION

The antioxidant properties of Roselle are documented in the literature and Roselle flowers are ranked to have one of the highest antioxidant activities among many other vegetables [1; 2]. Roselle anthocyanins have been shown to have free radical-scavenging properties against superoxide radical (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($1O_2$), and they can also inhibit lipid peroxidation, as well as protein and lipid oxidation in liposomes [3]. Other phytochemicals found in Roselle (flavonols, flavanols, benzoic and cinnanmic acid derivatives) have attracted a great deal of attention because of their biological activity [4-8].

Several studies showed the presence of four anthocyanins namely cyanidin 3-O-glucoside, delphinidin 3-O-glucoside, delphinidin 3-O-glucoside, cyanidin 3-O-sambubioside and delphinidin 3-O-sambubioside in the flowers of Roselle [9-10]. Moreover, Production of anthocyanin in plant cell and tissue cultures of Roselle has been reported [11;12]. and anthocyanin-producing by callus allowed for the first time the synthesis of two news anthocyanin which are malvidin 3-O-glucoside and petunidin 3-O-glucoside [12]. Roselle calyx extract is a good source of anthocyanins.[13] But, they have some restrictions related to their seasonality, large plantation requirements, extended period of time to produce the source, storage conditions of flowers and the excessive use of flowers as they fruiting conditions i.e. the problem of the existence of the species, etc. [13-15]. Also, Roselle anthocyanins extracted from callus has been shown to have free radical-scavenging properties and they can also inhibit lipid peroxidation [16]. Because anthocyanins are widely consumed, finding out additional biological activities like anti-platelet aggregation effect related to these compounds would be of great interest.[17] Thus anthocyanins extracted from Roselle callus may have a protective effect against atherogenesis through their antioxidant and anti-platelet aggregation capacity.

The aim of this study was to evaluate the anti-thrombosis action of anthocyanins extracted from callus Roselle by determining their anti-platelet activity.

MATERIALS AND METHODS

Plant material

The callus lines of Roselle, highly rich in anthocyanins (red callus), were used as plant material. They were obtained according to the method described by Abeda *et al*. [12].

Biological material

The biological material used in this study is mainly composed of platelet rich plasma (PRP) and platelet poor plasma (PPP). These plasmas were obtained from blood of healthy donors. The blood comes from the Blood Center.

Extraction of anthocyanins and sample preparation

Approximately 50 mg of freeze-dried callus was mixed in 5 mL of methanol acidified with trifluoroacetic acid 0.1% (v/v) and anthocyanins were extracted overnight at 4°C with a blender. The supernatant was concentrated by evaporation of solvent using the SpeedVac Automatic evaporation system (Savant System, Holbrook, NY). Freeze-dried extract was then dissolved in a water/methanol mixture and filtered through a Millipore membrane with 0.45 µm porosity. The filtrate was twice diluted with purified distilled water.

Anthocyanin determination by HPLC

The high performance liquid chromatography (HPLC) analysis was conducted using the method described by Drust and Wrolstad [18]. Callus were harvested and freeze-dried. Approximately, 10 mg of dry biomass were extracted by 5 mL methanol during overnight at 4°C with a blender. Samples were centrifuged at 3000 rpm for 10 min, supernatant was collected and filtered through a Millipore membrane (0.45 µm). The filtrate was twice diluted with purified distilled water. The analyses were performed on a HPLC (Agilent), model-LC 1100 series, equipped with a degasser, an autosampler automatic injector, a high pressure pump

and a UV/Visible detector at multiple wavelengths wave, and running on Windows XP Workstation. HPLC experiments were conducted using a reversed-phase C18 column (Prontosil, 250 x 4.0 mm, 5 µm, Bischoff). The mobile phase used was a binary gradient eluent (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile). Acetonitrile used was of HPLC grade (Sigma/Aldrich) and was degassed in an ultrasonic bath before using. The water was distilled using a Milli-Q system (Millipore). The elution program was 5-20% B (0-5 min), 20-35% B (5-10 min), 35-100% B (10-25 min) and 100% B (25-40 min) with a flow rate of 0.8 mL/min. The chromatograms were monitored at 521 nm. The anthocyanins identification and peak assignments are based on their retention times, UV-VIS spectra comparing with standards and published data. The anthocyanin quantification was performed using cyanidin 3-O-galactoside.

Obtainment of platelet poor plasma (PPP) and platelet rich plasma (PRP)

The blood was kept on sodium citrate (3.8%) in a proportion of nine volumes of blood to one volume of citrate. Approximately 3 mL of blood was placed into tubes and then centrifuged at 3000 rpm for 20 min at 20 °C. The plasma supernatant, rich of platelet was collected and stored at room temperature in polystyrene tubes. Platelets were counted using an apparatus "Coulter counter" ® ZF model and platelet concentration was adjusted between 300 and 400 x 10^3/mm^3 by dilution with platelet-poor plasma (PPP).

PPP was obtained by centrifuging the remaining blood at high speed 10000 rpm for 20 min at 20 °C. The supernatant which constitutes the PPP was used to calibrate the aggregometer and the adjustment of the concentration of PRP.

Platelet Aggregation

Platelets in homogeneous suspension in the plasma diffract a light beam of specific wavelength. The phenomenon can be evaluated by measuring the intensity of transmitted light. When grouping platelet aggregates in the medium cleared and the light transmission increases. It is therefore possible to follow spectrophotometrically platelet aggregation or disaggregation on time. Platelet aggregation was monitored at 37°C on a aggregometer "Bio DATA Corporation" ® model PAP4 (rotation speed of the magnetic stirrer: 1100 rpm). The limit values of the recording of scale (0 to 100 % light transmission) were obtained with the device with PRP and PPP, respectively. A concentration range (0 to 250 µg/mL) was made from a stock solution of 1 mg/mL of each purified anthocyanin. Approximately 225 µL of PRP was placed in a tank at 37°C with magnetic stirring. Then the Tris HCl buffer (0.05 M) and anthocyanin to be studied, dissolved in dimethylsulfoxide (0.5% DMSO) were added (sample). A mixture without anthocyanin was used as control. The whole was incubated for 3 to 5 min then the inducer of aggregation was finally added. 10 µL of a collagen solution to 1 µg/mL was used as an aggregation inducer and acetylsalicylic acid in the same concentration as anthocyanin, as a reference anti-aggregation agent. The percentage inhibition of platelet aggregation (PIPA), measured after 6 min, was calculated from the relationship:

$$PIPA = \frac{(1 - \text{aggregation of sample})}{\text{Aggregation of control}} \times 100$$

For each test compound, the inhibitory concentration 50 (IC50) was defined as the substance concentration can halve the maximum aggregation caused by the single inductor. This value was determined graphically on a curve expressing the percentage inhibition versus concentration. The ordinate having a value of 50% inhibition on each curve has abscissa IC50.

Statistical analysis

Statistical analysis was performed with Statistica 7.5 software. A variance ANOVA (ANOVA 1) was carried from the arc sine transformed data to test for differences between the percentages of inhibition of the test substances. When a significant difference was found between the means, the test least significant difference (LSD) is conducted to determine the average, which differs significantly from the others. The significance of the test was determined by comparing the probability (P) associated with the test statistic to the theoretical value $\alpha = 0.05$. Thus, when $P \geq 0.05$ is deduced that there is no difference between the means. On the other hand when $P < 0.05$, there is a significant difference.

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RESULTS

Characterization of anthocyanins

Anthocyanins were identified by their retention times, which were compared the standards being their characteristic wavelengths. The chromatographic of callus anthocyanins monitored at 521 nm is given in figure 1.

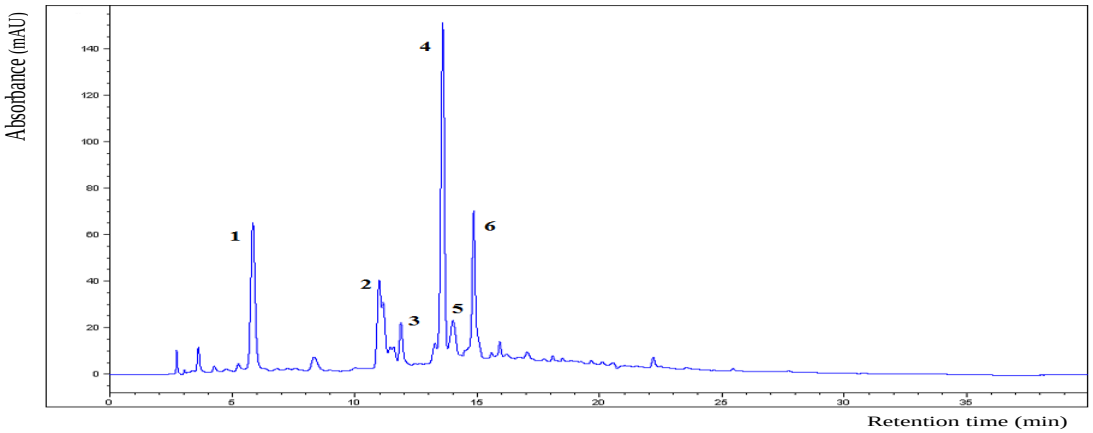

Figure 1. HPLC chromatogram of anthocyanins from callus extracts of Roselle monitored at 521nm.

Peaks were identified by comparison with reference standards when available or by HNMR data (retention time). 1. malvidin-3-O-glucoside (5.650 min); 2. delphinidin-3-O-glucoside (10.812 min); 3. petunidin-3-O-glucoside (11.704 min); 4. cyanidin-3-O-sambubioside (13.561 min); 5. cyanidin-3-O-glucoside (13.978 min); 6. delphinidin-3-O-sambubioside (14.866 min).

Six anthocyanins were detected in callus of Roselle. In this chromatogram the peaks 1, 2, 3 and 5 were identified as malvidin-3-O-glucosides (1), delphinidin-3-O-glucosides (2), petunidin-3-O-glucosides (3) and cyanidin-3-O-glucoside (5); the peaks 4 and 6 were identified as cyanidin -3-O-sambubioside (4) and delphinidin-3-O-sambubioside (6). Their chemical structures are showed in figure 2. In Roselle callus there are only malvidin (Mv-), delphinidin (Dp-), petunidin (Pt-) and cyanidin (Cy)-3-O-glucosides, along with the corresponding sambubioside derivatives of Cy- and Dp-.

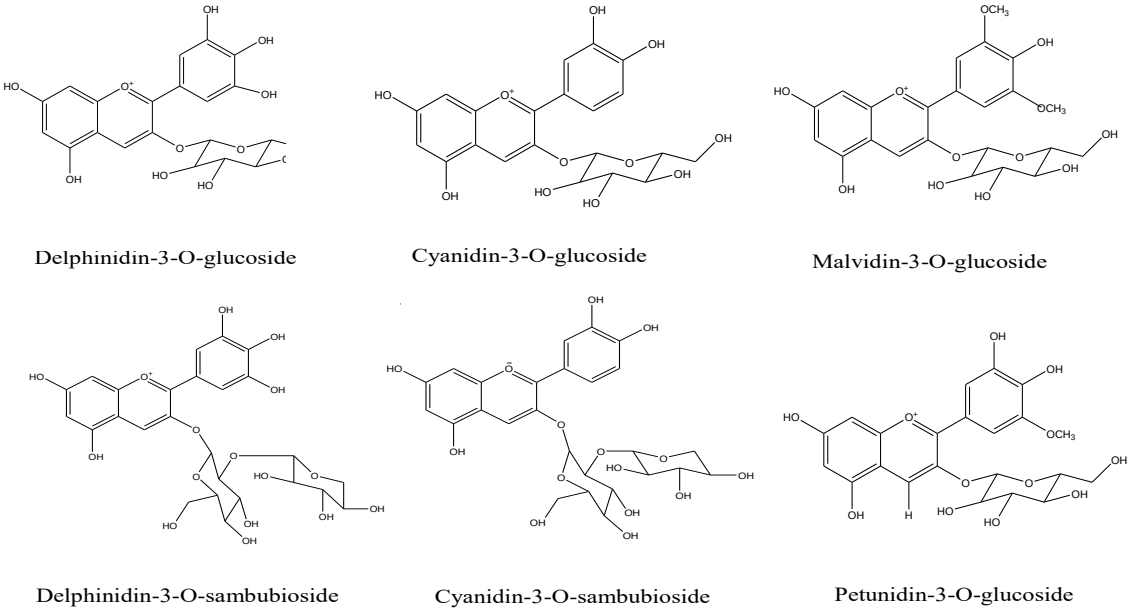

Figure 2: Structure of different anthocyanins detected in callus extracts of Roselle

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In addition, several studies showed the presence of four anthocyanins namely cyanidin-3-O-glucoside, delphinidin-3-O-glucoside, delphinidin-3-O-glucoside, cyanidin-3-O-sambubioside and delphinidin-3-O-sambubioside in the flowers of Roselle [8;19]. Quantitatively, cyanidin-3-O-sambubioside (53.93 mg/g DM) was predominant in the investigated callus. Delphinidin-3-O-sambubioside (17.07 mg/g DM), malvidin-3-O-glucoside (15.19 mg/g DM) and delphinidin-3-O-glucoside (10.04 mg/g DM) were intermediate anthocyanins followed by cyanidin-3-O-glucoside (5.01 mg/g DM) and petunidin-3-O-glucoside (3.89 mg/g DM) which were minor anthocyanins. The quantification of each is showed in table 1.

Table 1. Distribution of individual anthocyanins detected in callus extracts of Roselle.

N° peak	Name of anthocyanin	Content (mg/g DM)	Percentage (%)
1	Malvidin-3-O-glucoside	15.19 ± 0.04^{a}	14.45 ± 0.08^{a}
2	Delphinidin-3-O-glucoside	10.04 ± 0.05^{b}	09.55 ± 0.10^{b}
3	Petunidin-3-O-glucoside	03.89 ± 0.01^{c}	03.70 ± 0.03^{c}
4	Cyanidin-3-O-sambubioside	53.93 ± 0.05^{d}	51.30 ± 0.03^{c}
5	Cyanidin-3-O-glucoside	05.01 ± 0.02^{c}	04.76 ± 0.09^{c}
6	Delphinidin-3-O-sambubioside	17.07 ± 0.08^{a}	16.24 ± 0.08^{e}
Sum of monoglucoside		34.13 ± 0.08^{e}	32.46 ± 0.08^{e}
Sum of diglucoside		71.00 ± 0.08^{f}	67.54 ± 0.08^{f}
Sum of methoxylated forms		19.08 ± 0.08^{a}	18.15 ± 0.08^{a}
Sum of non methoxylated forms		86.05 ± 0.08^{g}	81.85 ± 0.08^{f}
Sum of purified anthocyanins		100.13 ± 0.08^{h}	100.00 ± 0.08^{h}

Analysis performed in triplicate ; values are means of triplicate determination (n = 3) ± standard deviation (SD); DM, dried matter; Peaks were identified by comparison with reference standards when available or by 1H-NMR data; in a column, values followed of a same letter are not statistically different (Newman-Keuls test at 5%).

The calli of Roselle has a percentage of methoxylated (Mv-3gluc and Pt-3gluc, sum 18.15 %) lower than non-methoxylated (Cy-3gluc, Dp-3gluc, Cy-3sam and Dp-3sam, sum 81.85 %) anthocyanin forms. With regard to glycosylation, the distribution of diglucoside anthocyanins form, mean 67.54 % (71.00 mg/g DM) were the most abundant fraction in callus followed by monoglucoside anthocyanins was 32.46 % (34.13 mg/g DM). The findings showed that Cy-3sam (51.30 %) was the most abundant anthocyanin in callus of Roselle. By contrast, Pt-3gluc was the least abundant anthocyanin pigment, as demonstrated. Another point worth mentioning is that the order of abundances based on percentage value of distribution for each anthocyanin was the following: Cy-3sam > Dp-3sam > Mv-3gluc > Dp-3gluc > Cy-3gluc > Pt-3gluc. Callogenesis has made it possible to induce malvidin-3-O-glucoside and petunidin-3-O-glucoside compared to the extract of Roselle's calyces [19-21].

Antiplatelet activity

The curves of inhibition percentages show that anthocyanins do not have the same antiplatelet powers. Complete inhibition of aggregation induced by collagen is caused by 75 µg/mL of cyanidin-3-O-sambubioside, 100 µg/mL of delphinidin-3-O-sambubioside, 125 µg/mL of delphinidin-3-O-glucoside and 150 µg/mL of cyanidin-3-O-glucoside. In determining the concentrations required for 50 % inhibition (IC 50), shows that delphinidin 3-O-sambubioside (34 µg/mL), cyanidin-3-O-sambubioside (27 µg/mL), delphinidin-3-O-glucoside (44 µg/mL) and cyanidin-3-O-glucoside (58 µg/mL) have antiplatelet power higher than the control acetylsalicyl acid (68 mg/mL). The antiplatelet power of acetylsalicyl acid (aspirin) is superior to that of malvidin-3-O-glucoside (146 µg/mL) and the petunidin-3-O-glucoside (194 µg/mL) (Figure 3). The most active compounds in this trial are delphinidin-3-O-sambubioside (34 µg/mL) and cyanidin-3-O-sambubioside (27 µg/mL), which are twice as active as aspirin (68 µg/mL) (Table 2).

The test substances can be grouped into three categories. These are the most active substances such as delphinidin-3-O-sambubioside, cyanidin-3-O-sambubioside and delphinidin-3-O-glucoside, moderately active substances such as cyanidin-3-O-glucoside and reference substance (aspirin) and least active substances such as malvidin-3-O-glucoside and petunidin-3-O-glucoside.

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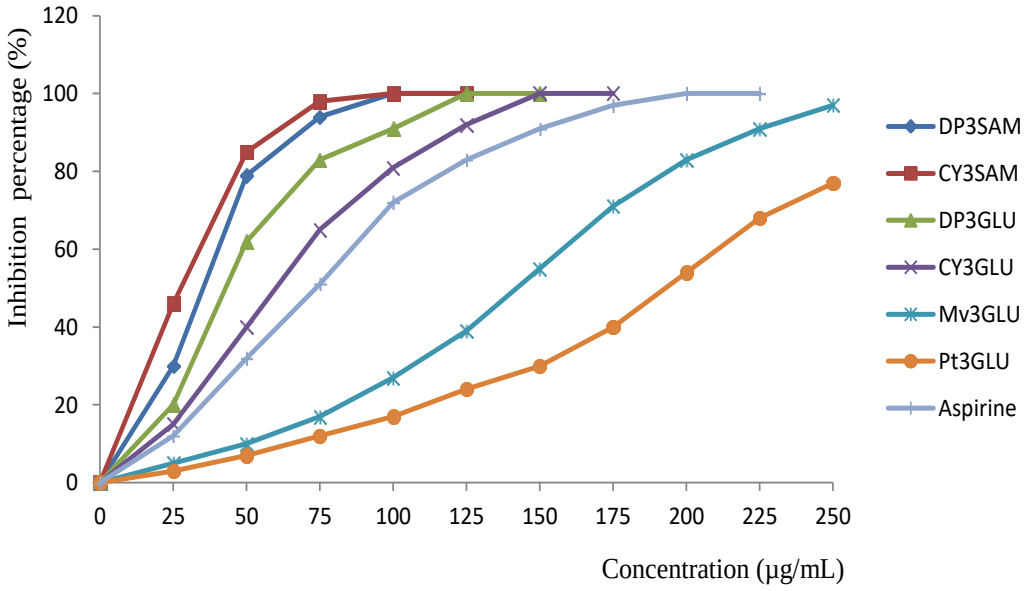

Figure 3. Effect of Roselle anthocyanins of on the inhibition of human platelet aggregation effected by collagen.

DP3SAM : Delphinidin-3-O-sambubioside ; CY3SAM : Cyanidin-3-O-sambubioside ; DP3GLU : Delphinidin-3-O-glucoside ; Mv3GLU : Malvidin-3-O-glucoside ; Pt3GLU : Petunidin-3-O-glucoside

Table 2. Inhibitory Concentration 50 obtained with anthocyanins of Guinea sorrel on antiplatelet activity.

Compounds		**IC_{50} (µg/mL)**
Delphinidin-3-0-sambubioside		34 ± 2.12 b
Cyanidin 3-O-sambubioside		27 ± 1.43 a
Delphinidin-3-O-glucoside		44 ± 2.65 c
Cyanidin-3-O-glucoside		58 ± 2.83 d
Malvidin-3-0-glucoside		146 ± 3.27 f
Pétunidin-3-O-glucoside		194 ± 3.48 g
Reference	Acetylsalicylic acid	68 ± 2.71 e

IC50: Inhibitory Concentration 50% of the tested product (anthocyanin). acéthylsalicyl Acid: Aspirin.Values are the average of three replicates. Values with the same letter in column are not significantly different according to the test of Newman-Keuls 5%.

DISCUSSION

Among the studied anthocyanins, delphinidin-3-O-sambubioside, cyanidin-3-O-sambubioside, delphinidin-3-O-glucoside and Cyanidin-3-O-glucoside showed a high anti-platelet aggregation activity in comparison with malvidin-3-O-glucoside, petunidin-3-O-glucoside and aspirin. The diglucosides such as delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside are the most active. During the metabolism, there would be a glycosylation under the action of 3-O-glucosyltarnsferase to transform Mv-, Dp-, Pt- and Cy- in their monoglucoside form [22] under the action of 3-O-diglucosyltranferase, Cy- and Dp- mainly to give diglucoside form (sambubioside) [23]. The distribution of the anthocyanins in Roselle can depend on the culture technique: Indeed, the number of anthocyanins extracted in callus was significantly greater than that of calyces [8]. Callogenesis has made it possible to induce malvidin-3-O-glucoside and petunidin-3-O-glucoside compared to the extract of Roselle's calyces [20]. Thus, the induction of the highly anthocyanins callus producing lines would increase the dietetic value of the Roselle. It is obvious that the content and the quality of anthocyanins in callus were different from that of explants which are directly taken plants [8; 24].

Platelet aggregation inhibiting activity would be based on the molecular structure. These results are in agreement with those of Varache-Lembège *et al*. [24] who worked on the action of molecular models on the inhibitory effect of human platelet aggregation. All glycosylated similar regardless of configuration, have led to

a decrease in activity. This phenomenon, already observed on various oxygenated derivatives of stilbene, may be due to the hydrophilic nature of the β-D-glucopyranosyl structure. However, Bertelli *et al*.[25] obtained results contrary to ours. This difference may be related to experimental conditions. It has been demonstrated that the IC50 of the known inhibitors vary depending primarily on the preincubation time used [24]. According Petroni *et al*. [26], the composition and quantity of anthocyanin in the extracts affect the degree of inhibition of platelet aggregation. The importance of the *in vivo* results presented here, as regards the inhibition of platelet aggregation by the anthocyanin extracts of Guinea sorrel is not known. The use of extracts of Guinea sorrel as a dietary supplement may be useful in particular as regards the effects on the cardiovascular system. Indeed the role of platelets is becoming better defined in diseases of the coronary artery and platelet aggregation.

CONCLUSION

Molecules such as delphinidin-3-O-sambubioside, cyanidin-3-O-sambubioside delphinidin-3-O-glucoside and cyanidin-3-O-glucoside exhibited strong inhibitory activity against platelet aggregation induced by collagen than acetylsalicylic acid antiplatelet reference. The use of extracts of Guinea sorrel as a dietary supplement may be useful in particular as regards the effects on the cardiovascular system. Indeed the role of platelets is becoming better defined in diseases of the coronary artery and platelet aggregation.

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