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Separation of biflavonoids from *Rheedia gardneriana* using chitin-Fe complex as stationary phase

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Immobilized metal affinity chromatography (IMAC) is a separation method that is based on the affinity of a determined organic compound (ligand) present in the sample to be separated and a metal-ion immobilized on a specific support [1]. The selectivity of IMAC is very high and it can be adjusted by changing the metal-ion and the properties of the compound to be separated [2]. IMAC-Cu(II) has applications in the separation of proteins, such as alpha-lactalbumin [3], lysosime [4, 5], myoglobin and cytocrome C [5]. It was also used in the separation and purification of rainbow trout pituitary gonadotropins [6]. IMAC-Fe(III) also showed wide application in the separation of phosphorylated macromolecules [2].

We have previously prepared an alternative support for IMAC, chitin modified with iron (CH-Fe), and evaluated its applicability as a support for TLC and CC for the separation of phenolic compounds [7].

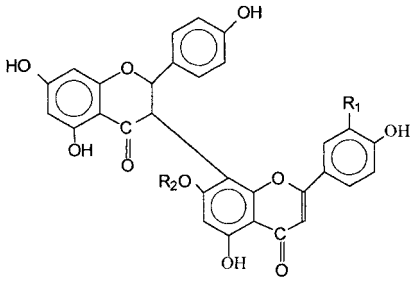
In our program to determine the presence of active principles from Brazilian plants, we have verified that *Rheedia gardneriana* leaves contain several biflavonoids with analgesic effects [8]. Although these compounds are structurally related, it is difficult to separate them by CC using silica-gel. Thus, based on the good results obtained previously with CH-Fe as a stationary phase, this study was developed to evaluate the applicability of this support in the separation of the biflavonoids from *R. gardneriana.*

Flavonoids are a widely distributed group of polyphenolic compounds with health-related properties. These properties have been found to include anticancer, antiviral, anti-inflammatory activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation [9].

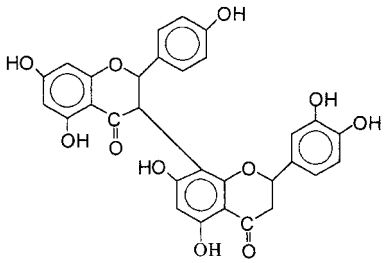
The single most important development in phenolic analysis is the application of HPLC for separation and detection. In particular, the introduction of reversed-phase silica columns and their elution with aqueous acidic method solvents has provided an almost ideal system for resolving the complex mixtures of components that are encountered in many plants.

The biflavonoids isolated from *R. gardneriana* leaves are the main chemical constituents responsible for the analgesic properties of this plant [8]. Since their molecular structures are very similar, the chromatographic separation using silica gel as stationary phase proved to be very laborious resulting in a small amount of biflavonoids. Such observation led us to determine other possible chromatographic supports, which could be used as an alternative method for this purpose.

The yields of chromatographic separation of the samples are reported in the Table. The use of CH-Fe in CC permitted the separation of four biflavonoids identified as volkensiflavone (**1**), GB-2a (**2**), fukugetin (**3**) and fukugeside (**4**). The order of elution of biflavonoids in chitin was the same as on silica gel, i.e., volkensiflavone, GB-2a, fukugetin and fukugeside [8].



(1) volkensiflavone $R_1 = R_2 = H$
(3) fukugetin R_1=OH, R_2 = H
(4) fukugeside R_1 = OH, R_2 = β Dgluc



(2) GB-2a

Table: Efficiency of chromatographic separation (150 mg of ethyl acetate fraction) from leaves of *Rheedia gardneriana*

Separated biflavonoid	Quantity of separated compound (mg)	% with regard to initial sample	% Biflavonoids
Volkensiflavone	5	3.4	1.5 [8]
GB-2a	5	3.4	3.7 [8]
Fukugeside	47	31.4	28 [11]
Fukugetin	18	12.0	– [11]

The yield of the separated biflavonoids using CH-Fe as stationary phase was higher than those achieved with silica gel [8]. This may be explained by the interaction process adsorbent – organic compound – solvent. In IMAC the separation of biflavonoids probably involves the interaction between the iron adsorbed on the polymer surface and the phenolic OH of the organic compound. The metal ion iron has strong affinity for phenolic oxygens, therefore, the more phenolic OH in the organic compound will improve the interaction with the adsorbent [7]. Our results extend previous studies with CH-Fe, suggesting its efficiency in separating different phenolic compounds such as flavonoids, biflavonoids and gallic acid derivates, among others.

Experimental

1. Preparation of the extract

Rheedia gardneriana Pl. was collected in the town of Blumenau, Santa Catarina, in the South of Brazil, in December 1997. The plant was identified by Prof. Marcos Sobral (Department of Botany, Federal University of Rio Grande do Sul (URGS), Porto Alegre). Vouchers were deposited in the Dr. Roberto Miguel Klein Herbarium (Department of Nature Science, FURB, Blumenau, SC) under numbers 534 to 540.

Air dried leaves of *R. gardneriana* (545 g) were powdered and macerated with 95% methanol (8 l) at room temperature, for approximately ten days.

After solvent removal under reduced pressure the extract was successively partitioned with hexane, $CHCl_3$, ethyl acetate and butanol to obtain the respective fractions [10].
Since the ethyl acetate fraction exhibited the highest concentration of biflavonoids, it was chosen for further study.

2. Column chromatography

The chromatographic support (CH-Fe) was prepared and characterized according to the literature [7].
The ethyl acetate fraction (150 mg) which contained the biflavonoids, was chromatographed on a chromatographic column (CC) (2.0×30 cm) using 3.5 g of CH-Fe eluted with a $CHCl_3$MeOH gradient and fractions of 5 ml were collected. After being monitored by TLC using silica gel 60 plates eluted with $CHCl_3$MeOH (70 : 30), the similar fractions, which showed positive reaction with $FeCl_3$ (2% in ethanol) solution, were combined and the compounds were identified on the basis of spectral data and Co-TLC with authentic samples previously isolated [8].

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Resorcylidene aminoguanidine improves the pathologically reduced fluidity of erythrocyte membranes in Diabetes mellitus

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Several studies in diabetic patients have reported reduced membrane lipid fluidity of red blood cells of diabetic patients as compared with normal individuals [1–3]. The rigidification of diabetic erythrocyte membranes seems to result from an enhanced cholesterol to phospholipid molar ratio and/or from an increased non-enzymatic glycation of membrane proteins and subsequent formation of advanced glycation end products [4, 5]. The pathologically reduced membrane fluidity occurring in Diabetes results in decreased cell deformability, diminished oxygen releasing capacity, etc. [6, 7]. Therefore, an improvement in the pathological physical state of diabetic membranes may help to prevent late diabetic complications. Aminoguanidine has been found to prevent or ameliorate the Diabetes-induced collagen cross-linking and vascular dysfunction [8, 9]. However, aminoguanidine toxicity limits its application. In search of new suitable non-toxic inhibitors of non-enzymatic glycation, resorcylidene aminoguanidine (RAG) was synthesised [10, 11]. At physiological pH, RAG exists in cationic as well as in neutral forms, with a pK_1 of 7.36 [10] (Scheme). It has been reported that the

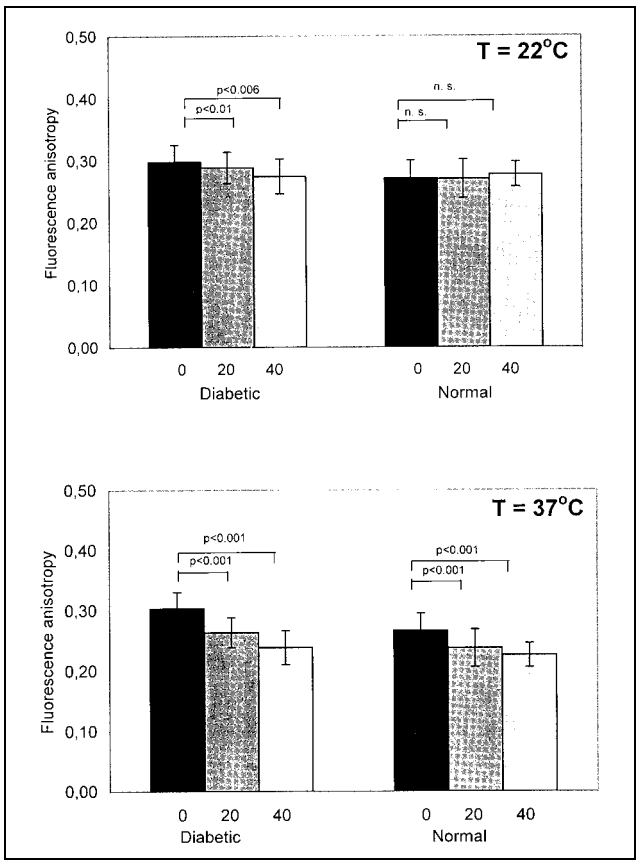

Fig.: Effect of RAG treatment on fluorescence anisotropy of DPH in human erythrocyte membranes (2%). 0 – no treatment, 20 – treatment with 20 μM RAG, 40 – treatment with 40 μM RAG. Data are given as means ± S.D. of 8 independent experiments