^{13}C NMR spectral data and molecular descriptors to predict the antioxidant activity of flavonoids

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Tissue damage due to oxidative stress is directly linked to development of many, if not all, human morbidity factors and chronic diseases. In this context, the search for dietary natural occurring molecules with antioxidant activity, such as flavonoids, has become essential. In this study, we investigated a set of 41 flavonoids (23 flavones and 18 flavonols) analyzing their structures and biological antioxidant activity. The experimental data were submitted to a QSAR (quantitative structure-activity relationships) study. NMR ^{13}C data were used to perform a Kohonen self-organizing map study, analyzing the weight that each carbon has in the activity. Additionally, we performed MLR (multilinear regression) using GA (genetic algorithms) and molecular descriptors to analyze the role that specific carbons and substitutions play in the activity.

Uniterms: Flavonoids/antioxidant activity. ^{13}C NMR. Kohonen self-organizing map.

Danos aos tecidos devido ao estresse oxidativo estão diretamente ligados ao desenvolvimento de muitos, senão todos, os fatores de sedentarismo e de doenças crônicas. Neste contexto, a busca de moléculas naturais, que participam da nossa dieta e que possuam atividade antioxidante, flavonóides, torna-se de grande interesse. Neste estudo, nós investigamos um conjunto de 41 flavonóides (23 flavonas e 18 flavonóis), relacionando suas estruturas e atividade antioxidante. Os dados experimentais foram submetidos à análise de QSAR (relações quantitativas estrutura-atividade). Dados de RMN ^{13}C foram utilizados para realizar um estudo do mapa auto-organizável de Kohonen, analisando o peso que cada carbono tem na atividade. Além disso, realizamos uma MLR (regressão múltipla) usando GA (algoritmos genéticos) e descritores moleculares para avaliar a influência de carbonos e substituições na atividade.

Unitermos: RMN ^{13}C. Flavonóides/atividade antioxidante. Mapa auto-organizável de Kohonen.

INTRODUCTION

Flavonoids are phenolic compounds isolated from a wide range of vascular plants, with over 8000 individual compounds known (Halliwell *et al.*, 2000). They possess a wide variety of biological activities including antiinflammatory, antimicrobial, antiallergenic, antiviral, vasodilatory action and antioxidant activity. These compounds are able either to suppress free radical formation and chain initiation or to scavenge free radical and chain propagation (Fernández *et al.*, 2005).

These compounds contain a basic structure constituted of 15 carbon atoms arranged in three rings (C_6-C_3-C_6) and have shown the molecular structural requirements for marked radical scavenging activity against the diphenyl picrylhydrazyl (DPPH) radical. The presence of the galloyl group, as well as the number and position of hydroxyl groups, are important and further enhance the radical scavenging activity of flavonoids. On the other hand, the methoxylation or glycosylation of a free hydroxyl group may reduce greatly or even abolish the radical-scavenging capacity of flavonoids (Yokozawa *et al.,* 1998; Scotti *et al.*, 2007, 2009; Rastija *et al.,* 2009; Pasha *et al.*, 2007). The DPPH system is a stable radical-generating procedure that

simulates the biological free radical producing mechanism (equation 1) (Molyneux, 2004).

DPPH electron accepting mechanism.
$$DPPH\bullet + XH \rightarrow DPPHH + X\bullet \qquad (1)$$
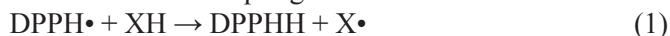
Quantitative structure-activity relationships (QSAR) of flavonoids are important due to their ease of analysis, since they are natural active substances that show small plane molecules with few functional groups, thus it is sometimes possible to directly find a relationship between the structure and the activity by analyzing the different functional groups attached to the flavonoid molecule. In this sense, the application of graph theory to chemical structures results in several molecular descriptors that are used to better comprehend important molecular aspects.

In addition to the descriptors that characterize the molecular structure, the ^{13}C NMR can also be used to describe the molecular structure. These data yield rich information about the molecular structure and are sufficiently sensitive to detect small differences in the molecule. These differences are measured by the variation of chemical shifts, and the values can be used to associate the chemical structure with the respective biological activity since this association can help understand the influence of the chemical environment on the biological activity selected. Thus, the ^{13}C NMR data may be useful in QSAR studies (Berger, 2006; Masui *et al.*, 2006; Vanderhoeven *et al.*, 2004).

Artificial Neural Networks (ANNs) are one of the most used approaches to achieve computer classification and pattern recognition. ANNs are an option for data set analysis that have unknown correlations, such as the prediction of the biological activity, taking into account the ^{13}C NMR. The most used ANN architecture for pattern recognition is the Kohonen network also known as the Self-Organizing Map (SOM) (Lawrence, 1994; Kohonen, 1989,1997,1998; Kohonen, Oja, 1998). Each neurone in the grid is associated to a weight, and similar patterns stimulate neurones with similar weight, so that similar patterns are mapped near each other, aiding QSAR analysis by simplifying visualisation and understanding (Gasteiger *et al.,* 1996).

The aim of this paper was to predict the antioxidant activity of flavonoids measured by the DPPH system using two methodologies. The first approach is based on the molecular information given by the ^{13}C NMR data, where the ANNs were used to predict the biological activity of the flavonoids, sorting them into active or inactive compounds. In the second approach, several molecular descriptors were computed and utilized to predict the antioxidant activity of flavonoids through genetic algorithms.

METHODOLOGY

Data set

In this study, a data set of 41 flavonoids (23 flavones and 18 flavonols), whose activities were reported in the literature by Yokozawa *et al.,* 1998, was employed. In the paper, antioxidant activity was measured spectrophotometrically by the DPPH method (Table I). The activity was reported as IC_{50} that indicates the concentration required to inhibit the DPPH radical by 50% (decrease of 50% in absorbance). Since the radical scavenging activity varied by orders of magnitude, as well as its respective errors, it was transformed into natural log values ($pIC_{50=}$ -$logIC_{50}$).

In order to design training and prediction sets, the complete data set was processed using cluster analysis. The compounds were first divided into two subsets: one training set composed of 20 molecules, and one external test set composed of 6 compounds (compounds 06, 10, 25, 29, 34, 36) (see Table I), suitable to analyze predictive performance. The size of the test set was around 20% of the whole set, taking care that the test set contains representative samples of the trained group and included the range of activity values of the training group.
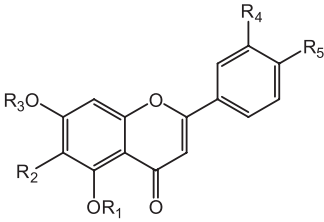
The active compounds, with pIC_{50} values, were selected to generate regression models utilizing genetic algorithms. Models for antiradical activity were constructed based on the training set and then validated internally (cross validation – leave one out) and externally.
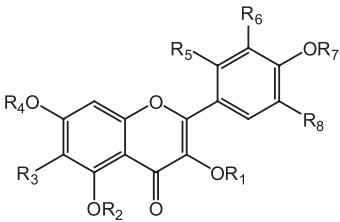
Molecular optimisation

All structures were extracted from the SISTEMAT database (Gastmans *et al.,* 1990a,b; Ferreira *et al.,* 2001). An in–house program for data extraction was written in Java and subsequently used to select the flavonoid compounds.

Molecular modeling computations were performed on SPARTAN for Windows v.4.0 software (Wavefunction, Inc., Irvine, Calif.). The molecules were subjected to geometry optimization and conformational analysis (systematic analysis with dihedral angle rotated every 30°). The semi-empirical quantum chemical method used was AM1 (Austin Model1) (Dewar *et al.*, 1985, 1990), which is suitable for this analysis (Bringmann *et al.*, 2000), despite the existence of more recent methods, and the root mean square (RMS) gradient value of 0.001 kcal/mol was used as the termination condition. Energy minimized molecules were saved as MDL MolFiles for computing various molecular descriptors using DRAGON Professional version 5.4 (Dragon, 2010).

TABLE I - List of compounds, substitutions and its respective log of biological activity. The compounds were classified as Active (A, $IC_{50}<200mM$) or Inactive (I, $IC_{50}>200mM$) for the Kohonen study: the respective training and test sets

Flavone   Flavonol

ID	Compound	pIC_{50}	Activity	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Set
1	Cirsiliol	5.15	A	H	OMe	Me	OH	OH	-	-	-	Training
2	6-Hydroxyluteolin	5.04	A	H	OH	H	OH	OH	-	-	-	Training
3	Luteolin-7-glucoside	5.00	A	H	H	glc	OH	OH	-	-	-	Training
4	Baicalein	4.97	A	H	OH	H	H	H	-	-	-	Training
5	Scutellarein	4.94	A	H	OH	H	H	OH	-	-	-	Training
6	Baicalin	4.90	A	H	OH	glcA	H	H	-	-	-	Test
7	Plantaginin	4.86	A	H	OH	glc	H	OH	-	-	-	Training
8	Luteolin	4.75	A	H	H	H	OH	OH	-	-	-	Training
9	Sorbarin	4.73	A	H	OH	rha	H	OH	-	-	-	Training
10	Apiin	4.19	A	H	H	glc²-api	H	OH	-	-	-	Test
11	Cosmosiin	3.63	I	H	H	glc	H	OH	-	-	-	Training
12	Acacetin	-	I	H	H	H	H	OMe	-	-	-	Training
13	Apigenin	-	I	H	H	H	H	OH	-	-	-	Training
14	Chrysoeriol	-	I	H	H	H	OMe	OH	-	-	-	Training
15	Cirsilineol	-	I	H	OMe	Me	OMe	OH	-	-	-	Training
16	Cirsimarin	-	I	H	OMe	Me	H	O-glc	-	-	-	Training
17	Cirsimaritin	-	I	H	OMe	Me	H	OH	-	-	-	Training
18	Linarin	-	I	H	H	glc⁶-rha	H	OMe	-	-	-	Training
19	Pectolinarigenin	-	I	H	OMe	H	H	OMe	-	-	-	Training
20	Pectolinarin	-	I	H	OMe	glc⁶-rha	H	OMe	-	-	-	Training
21	Rhoifolin	-	I	H	H	glc²-rha	H	OH	-	-	-	Training
22	Tectochrysin	-	I	H	H	Me	H	H	-	-	-	Training
23	Toringin	-	I	glc	H	H	H	H	-	-	-	Training
24	2''-O-Galloylmyricitrin	5.42	A	rha(2-galloyl)	H	H	H	H	OH	H	H	Training
25	Quercimeritrin	5.13	A	H	H	H	glc	H	OH	H	H	Test
26	Rhamnetin	5.11	A	H	H	H	Me	H	OH	H	H	Training
27	Quercetin	5.10	A	H	H	H	H	H	OH	H	H	Training
28	Rutin	5.04	A	glc⁶-rha	H	H	H	H	OH	H	H	Training
29	Chrysosplenol D	5.02	A	Me	H	OMe	Me	H	OH	H	H	Test
30	Hyperin	5.01	A	gal	H	H	H	H	OH	H	H	Training
31	Quercitrin	4.95	A	rha	H	H	H	H	OH	H	H	Training
32	Chrysosplenol C	4.95	A	Me	H	OH	Me	H	OMe	H	H	Training
33	Myricitrin	4.89	A	rha	H	H	H	H	OH	H	OH	Training
34	Isoquercitrin	4.88	A	glc	H	H	H	H	OH	H	H	Test
35	Isorhamnetin	4.78	A	H	H	H	H	H	OMe	H	H	Training
36	Oxyayanin A	4.71	A	Me	H	H	Me	OH	H	Me	OH	Test
37	Kaempferol	4.64	A	H	H	H	H	H	H	H	H	Training
38	Tetramethylquercetin	4.29	A	H	Me	H	Me	H	OMe	Me	H	Training
39	Afzelin	-	I	rha	H	H	H	H	H	H	H	Training
40	Kaempferitrin	-	I	rha	H	H	rha	H	H	H	H	Training
41	Kaempferol-7-glucoside	-	I	H	H	H	glc	H	H	H	H	Training

Molecular descriptors

DRAGON computer software (Dragon, 2010; Todeschini *et al.,* 2000) was employed to calculate 1664 molecular descriptors that were used for performing Multilinear regression (MLR) analysis. For all descriptors, the constant variables were excluded, just retaining those that presented a different value. For the remaining descriptors, pairwise correlation ($r < 0.99$) analysis was performed to exclude those which were highly correlated. Thus, the number of DRAGON descriptors used in our calculations was reduced to 548.

Genetic algorithm analysis

The MobyDigs program was used for the calculation of regression models utilizing genetic algorithms (GA). GA is a class of methods based on biological evolution roles. The first step is to create a population of linear regression models. These regression models mate with each other, mutate, cross-over, reproduce, and then evolve through successive generations towards an optimum solution (Leardi, 2001; Kubinyi, 1994a,b).

The search for the best subsets of models is calculated by using Ordinary Least Squares regression (OLS) under the Genetic Algorithm (GA) approach, i.e. by the Variable Subset Selection - Genetic Algorithm (VSS-GA)

method. Thus, by examining the regression coefficients, the standard deviations, significances and number of variables in the equation, the significance of the models could be determined.

^{13}C NMR data

For the analysis correlating the ^{13}C NMR with the flavonoid biological activities, the ^{13}C NMR data of most molecules were obtained from the literature (Agrawal, 1989). For a few structures (compounds 02, 16, 17, 24, 29 and 36), the ^{13}C NMR data were obtained using ACDlabs software (ACD/HNMR,2003). Since flavonoids are plane aromatic compounds, the additivity model can be applied satisfactorily to predict ^{13}C NMR data. To validate the method employed in the ^{13}C NMR data prediction, the chemical shifts of some flavonoids were predicted and compared against the literature data (Table II). The chemical shifts predicted by the software were very close to the literature reference (errors smaller than 3 ppm). Thus, for the six above-mentioned compounds, the ^{13}C NMR data were predicted by the ACD software.

To standardize the ^{13}C NMR analysis, since not all molecules have the glycosil and methyl groups, the ^{13}C NMR data used in the analysis included only the chemical shifts pertaining to the flavonoid skeleton. It is important to note that the even though the glycosil and the methyl

TABLE II - Use validation of the ACDlab software to predict NMR values

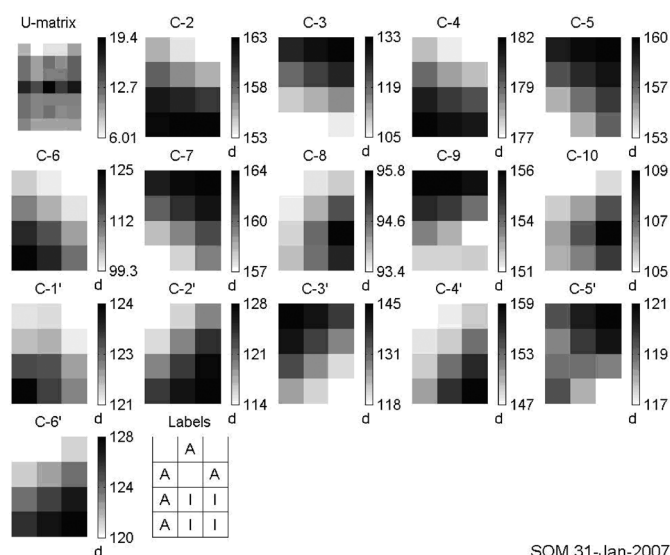|	Scutellarein (Flavone)		Luteolin (Flavone)		Kaempferol (Flavonol)	
	NMR (literature)	NMR (predicted)	NMR (literature)	NMR (predicted)	NMR (literature)	NMR (predicted)
C-2	164.2	163.6	164.5	164.5	146.1	146.4
C-3	102.9	104.1	103.3	103.3	135.5	135.6
C-4	182.7	182.1	182.2	182.2	175.7	175.8
C-5	154.0	148.1	162.1	160.0	156.0	157.6
C-6	129.9	128.7	99.2	99.2	98.2	98.3
C-7	147.7	153.6	164.7	164.2	163.8	163.9
C-8	94.6	94.0	94.2	94.2	93.4	93.5
C-9	150.4	151.3	157.9	160.0	160.5	159.1
C-10	104.7	104.3	104.2	104.2	102.9	103.0
C-1'	122.2	121.3	119.3	120.7	121.6	121.6
C-2'	129.1	128.1	113.8	113.8	129.3	129.4
C-3'	116.6	115.8	146.2	146.2	115.3	115.3
C-4'	161.7	160.9	150.1	150.2	159.0	159.1
C-5'	116.6	115.8	116.4	116.4	115.3	115.3
C-6'	129.1	128.1	122.1	122.7	129.3	129.4

FIGURE 1 - Contribution of each carbon to the map based on its ^{13}C NMR data, showing the contribution of the chemical shifts of the C-3' and C-4' carbons, which can be identified and characterised with high hit of similarity for both regions on the map (active and inactive compounds) supporting the relevance of these carbons to the antioxidant activity of flavonoids.

The significant linear adjustment for training and test sets is shown in Figure 2, and the small errors in Table IV confirm that the equation was capable of predicting and differentiating the most active compound from the others. Statistically, the validation of the model is shown

by analysing the significant values of the parameters r^2_{ext} (0.662) and Q_{ext}^2 (0.657).

Table V shows the correlation matrix between each pair of descriptors and shows low validated values, indicating low co-linearity descriptors since they are not highly correlated.

Radial distribution function, RDFRw, can be interpreted as the probability distribution of finding an atom in a spherical volume of radius ("R") in angstroms (equation 3), weighted by the characteristic properties "w" of the atom, where r_{ij} is the interatomic distance between atoms i and j, nAT is the number of atoms in the molecule, and β is a smoothing parameter. Besides information about interatomic distances in the entire molecule, these descriptors provide further information about bond distances, ring types, planar and non-planar systems and atom types.

RDF105m and RDF110v were selected in this study (by equation 2). The former indicates that in a radius of 10.5Å, the greater the atomic masses and number of atoms in this radius, the greater the RDF value (Hemmer *et al.,* 1999). The latter indicates that in a radius of 11.0Å, the greater the atomic van der Walls volumes as for the number of atoms in this radius, the greater the RDF value (Hemmer *et al.,* 1999).

$$RDFRw = \sum_{i=1}^{nAT-1} \sum_{j=i+1}^{nAT} w_i\, w_j.e^{-\beta(R-r_{ij})^2} \qquad (3)$$
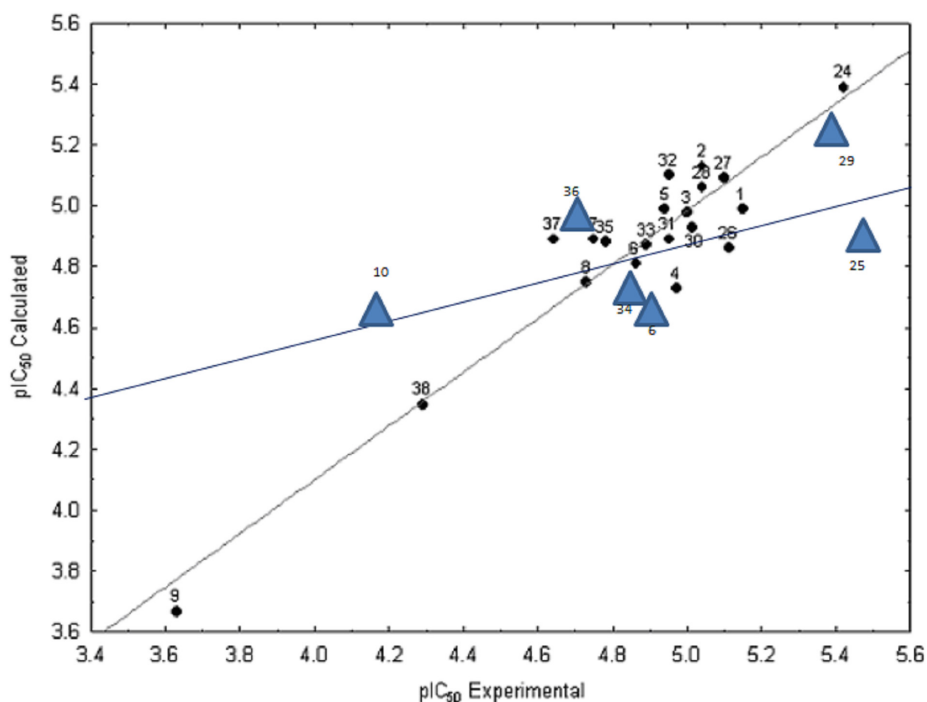
In Model I, RDF105m has a positive contribution

FIGURE 2 - Comparative plot experimental values versus calculated activity values (pIC$_{50}$) for the training (black balls) and test (blue triangles) sets.

TABLE IV - Calculated, predicted and error values for antioxidant activity

ID	Compound Name	pIC$_{50}$ Calculated	pIC$_{50}$ Predicted	Error
01	Cirsiliol	4.99	-	-0.16
02	6-Hydroxyluteolin	5.13	-	0.09
03	Luteolin-7-glucoside	4.98	-	-0.02
04	Baicalein	4.73	-	-0.23
05	Scutellarein	4.99	-	0.06
06	Baicalin	-	4.74	-0.16
07	Plantaginin	4.81	-	-0.05
08	Luteolin	4.89	-	0.14
09	Sorbarin	4.75	-	0.02
10	Apiin	-	4.48	0.29
11	Cosmosiin	3.67	-	0.03
24	2-*O*-Galloylmyricitrin	5.39	-	-0.02
25	Quercimeritrin	-	4.92	-0.21
26	Rhamnetin	4.86	-	-0.26
27	Quercetin	5.09	-	-0.01
28	Rutin	5.06	-	0.02
29	Chrysosplenol D	-	5.18	0.16
30	Hyperin	4.93	-	-0.07
31	Quercitrin	4.89	-	-0.07
32	Chrysosplenol C	5.10	-	0.15
33	Myricitrin	4.87	-	-0.02
34	Isoquercitrin	-	4.80	-0.08
35	Isorhamnetin	4.88	-	0.10
36	Oxyayanin A	-	4.82	0.11
37	Kaempferol	4.89	-	0.25
38	Tetramethylquercetin	4.35	-	0.06

TABLE V - Correlation (r) matrix between the descriptors

	RDF105m	RDF110v	O-057
RDF105m	1	.554	.014
RDF110v		1	.39
O-057			1

while RDF110v has a negative contribution. Thus, in a radius of 10.5Å, the greater the atomic masses, the greater the biological activity value, and in a radius of 11.0Å, the greater the atomic van der Waals volumes, the lower the biological activity value.

Analyzing the molecule structures, separating them into one set possessing the greatest values of biological activity, and another exhibiting the lowest values, and then comparing these compounds by groups that could be responsible for the high or low activity, gives rise to several considerations.

Compounds that showed the highest RDF105m values (compounds **3** and **25**) share a substitution pattern: O-glycosylation at C-7 and C-3' hydroxylation. These compounds also have a high biological activity value. Compound **37,** which does not have this pattern, has a low RDF105m value and a low biological activity value. As the radial distribution can characterize the solvation layers around a specific atom, this descriptor can be related to the access capability of both water molecules and the radical species involved in the reaction. In this study, this ability can be associated with the presence of the glycosyl group, which shows great hydrophilicity and has hydroxyl groups that participate in the biological activity. Thus, the greater the RDF value, the greater the activity.

RDF105m of molecule **3** is higher than **11** and the biological activity is also higher, which makes clear the influence of a single hydroxyl group at C-3'. Examining the compounds **3** and **7** and their activities and RDF105m, reveals that the absence of a hydroxyl at C-3' could be partially compensated by a hydroxyl at C-6. For compound **8** in comparison to **3,** the glycosyl group removal at C-7 decreases both activity and RDF105m value. In contrast, comparing the molecules **27** and **34** evidences that glycosyl removal at C-3 in flavonoids increases both activity and also RDF105m value. Moreover, each kind of sugar has a different effect in activity, as is seen when comparing compound **27** to compounds **30**, **31** and **34,** where higher activity is obtained with higher RDF105m value.

Molecules with bulky groups, especially at C-7, show the greatest probability of having a high value of RDF110v, and thus a lower value of antioxidant activity. Compound **10** has a high RDF110v and a low biological activity, whereas compound **27**, which is a small molecule, has a low RDF110v value and high biological activity. It is noteworthy that solely being a small molecule does not fulfil all the requirements for high activity, since the position of the bulky and hydroxyl groups usually impacts the effectiveness of the molecule against free radicals. For instance, molecules with greater activity and low RDF110v such as compounds **24** and **28** have bulky substituents but are at C-3.

Atom-centred fragments can be understood simply as being the number of specific fragment types in a molecule. For the O-057 molecular descriptor, the increase in the number of phenol, enol or acid groups present in the structure is directly proportional to the O-057 value observed. Molecules with acid groups, such as compounds **6** and **24,** have a high O-057 value and will exhibit a high antioxidant activity value. Polyhydroxylated flavonoids such as **2**, **4**, **5**, **8**, and **25** also have a high O-057 value, suggesting the positive effect of the hydroxyl group on the radical-scavenging activity.

CONCLUSIONS

Using molecular descriptors to perform a GA analysis, this study was able to show that sterical and fragment features are directly, and electronic features (van der Waals volume) indirectly, involved in the determination of the substitution patterns in the molecule that are responsible for a major effect on activity. For instance, hydroxyl at C-3', C-4' and C-6 are very important for high biological activity, while glycosylation at C-7 frequently decreases the activity.

The importance of these carbons was confirmed in the ^{13}C NMR analysis. Through the chemical shifts of the ^{13}C NMR, the ANN achieved a significant result, sorting the active and inactive molecules with minimal errors (20%). This supports the notion that ^{13}C NMR data can be used to perform classification of flavonoids taking into account the biological activity, making clear that radical scavenging activity is highly correlated with the chemical environment.

A future application could be the elucidation of the role that other carbons (i.e. C-2, C-3, C-5, C-8 and C-1') play on activity. In this context, a more extensive study is needed to compare a greater number of compounds and ^{13}C NMR data. Moreover, it would be useful to study other classes of flavonoids such as flavanones, flavanonols and anthocyanidins to determine which skeleton type is most active and which position in the structure has the greatest contribution to the activity.

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