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Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method

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The aim of this work was to propose a new antioxidant activity index (AAI) using the DPPH· method. Antioxidant activity was expressed as the antioxidant activity index (AAI) calculated as follows: AAI = final DPPH· concentration ($\mu g\ ml^{-1}$)/IC_{50} ($\mu g\ ml^{-1}$). The compounds, BHA, chlorogenic acid, ferulic acid, gallic acid, caffeic acid, quercetin, rutin, protocatechuic acid and *trans*-cinnamic acid were used, as well as the samples clove essential oil, eugenol and *Xanthium strumarium* extract. Three concentrations of DPPH· were used and no significant difference in the AAI for each compound tested was observed, indicating that the AAI found was appropriate to compare the antioxidant strength of plant extracts, as well as of pure compounds. Gallic acid showed the higher AAI value (AAI = 27) followed by protochatechuic acid (AAI = 20) and quercetin (AAI = 15). Clove essential oil showed very strong antioxidant activity (AAI = 9) while the *X. strumarium* extract presented strong antioxidant activity (AAI = 1.6).

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1. Introduction

There is consensus of opinion that free radicals induce oxidative damage to biomolecules. This damage causes atherosclerosis, aging, cancer and several other diseases (Aruoma, 1998). Moreover, free radicals are known to take part in lipid peroxidation in foods, which is responsible for rancid odours and flavours, which decrease the nutritional quality. Therefore, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butyhydro-quinone (TBHQ) are widely used in the food industry as potential inhibitors of lipid peroxidation. However, previous studies have demonstrated that BHA and BHT accumulate in the body and result in liver damage and carcinogenesis (Ito et al., 1986; Whysner, Wang, Zang, Iatropoulos, & Williams, 1994).

Interest in natural sources of antioxidant molecules for use in the food, beverage and cosmetic industries has resulted in a large body of research in recent years. It is well known that natural antioxidants extracted from herbs and spices have high antioxidant activity and are used in many food applications. Of these substances, the phenolic compounds, which are widely distributed, have the ability to scavenge free radicals by single-electron transfer (Hirano et al., 2001). Several studies have reported the antioxidant activity of plant extracts and their relationship with the phenolic compound content (Aaby, Hvattum, & Skrede, 2004; Silva, Ferreres, Malva, & Dias, 2005; Singh, Singh, Kumar, & Arora, 2007; Sun & Ho, 2005; Yuan, Bone, & Carrington, 2005). Stratil, Klejdus, and Kublán (2006) found high correlation between the content of phenolic substances and the total antioxidant activity of sets of samples.

Several methods have been proposed to measure the antioxidant activity of pure compounds and plant extracts, such as FRAP (Ferric Reducing Antioxidant Power), ORAC (Oxygen Radical Absorbance Capacity), ESR (Electron Spin Resonance), ABTS (2,2-azinobis (3-ethyl-benzothiazoline-6-sulphonate) and DPPH· (2,2-diphenyl-1-picrylhydrazyl). The DPPH· method is used worldwide in the quantification of free radical scavenging activity. The reaction is based on the colour decrease occurring when the odd electron of the nitrogen atom in DPPH· is reduced by receiving a hydrogen atom from antioxidant compounds. DPPH· is known as a stable free radical, but is sensitive to light, oxygen, pH and the type of solvent used (Ozcelik, Lee, & Min, 2003).

Several methods for the DPPH· assay have been reported, including different initial concentrations of the DPPH· solution such as 0.025 mM (Baydar, Özkan, & Yasar, 2007), 0.06 mM (Prakash, Singh, & Upadhyay, 2007) 0.1 mM (Sharififar, Moshafi, Mansouri, Khodashenas, & Khoshnoodi, 2007), 0.2 mM (Xu, Chen, & Hu, 2005), 0.3 mM (Umamaheswari et al., 2007) and 0.5 mM (Elzaawely, Xuan, & Tawata, 2007). Moreover, different aliquots of the extracts and the DPPH· solutions have been reported, resulting in different final concentrations of plant extract or pure compound and of the DPPH·. Reaction times (in the dark) of 10 min (Cui, Kim, & Park, 2005), 15 min (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005), 20 min (Chung, Chen, Hsu, Chang, & Chou, 2005), 30 min (Tepe, Sokmen, Akpulat, & Sokmen, 2005), 60 min (Akowuah, Ismail, Norhayati, & Sadikun, 2005), 100 min (Yuan et al., 2005) and 120 min (Sun & Ho, 2005) have been reported.

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The results of the DPPH· assays have been presented in many ways, such as inhibition of the free radical DPPH· in percent (I%) calculated in the following way: I% = $[(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 was the absorbance of the control and Abs_1 was the absorbance in the presence of the test compound (Guerrero, Guirado, Fuentes, & Pérez, 2006), percentage of residual DPPH· ($DPPH^{\cdot}_R$) calculated as follows: $\%DPPH^{\cdot}_R = [(DPPH^{\cdot})_t/(DPPH^{\cdot})_{t=0}] \times 100$, where $DPPH^{\cdot}_t$ was the concentration of DPPH· at steady-state and $DPPH^{\cdot}_{t=0}$ was the concentration of DPPH· at zero time (initial concentration) (Siddhuraju & Becker, 2007), antiradical activity calculated according to the formula: antiradical activity = 100 × (1−absorbance of sample/absorbance of control) (Baydar et al., 2007), ascorbic acid (AA) equivalent antioxidant capacity (AEAC) using the following equation: $AEAC(mgAA/100g) = (A_{control} - A_{sample})/(A_{control} - A_{(}AA)) \times conc.AA(mg/ml) \times vol.extract(ml) \times 100/g\,sample$ (Lim, Lim, & Tee, 2007). The majority of the studies express the results as the IC_{50} value defined as the amount of antioxidant necessary to decrease the initial DPPH· concentration by 50%, calculated using the graph by plotting inhibition percentage against extract concentration (Ani, Varadaraj, & Naidu, 2006; Elzaawely et al., 2007; Sokmen et al., 2004; Tepe et al., 2005).

Despite the worldwide use of the DPPH· method, the lack of standardization of the results makes it difficult to compare the antioxidant strength of different plant extracts as well as of pure compounds. Up to the present, no paper in the literature has proposed a universal index for the DPPH· assay. For plant extracts or pure compounds the data presented, such as *I*% or the IC_{50} value, change according to the final concentration of the DPPH· used. Therefore, the aim of this work was to propose a new antioxidant activity index (AAI) using the DPPH· method.

2. Materials and methods

2.1. Standards and reagents

Methanol (Ecibra, Brazil) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) (Sigma, USA), were used. The synthetic antioxidant butylated hydroxyanisole (BHA) and the compounds chlorogenic acid, ferulic acid, gallic acid, caffeic acid, quercetin, rutin, protocatechuic acid and *trans*-cinnamic acid were purchased from Sigma (USA).

2.2. Samples

The *Xanthium strumarium* used in this work was cultivated on the experimental farm of the Faculty of Agricultural Engineering (FEAGRI) of the State University of Campinas (UNICAMP, Campinas, São Paulo, Brazil). Voucher specimens were deposited at the State University of Campinas Herbarium denominated as 134865, and identified by Dr. Washington M.F. Neto (curator). The leaves were separated and dried in a tray drier with air circulation at 45 °C (Marconi, Model 035, Piracicaba, SP, Brazil), packed in dark plastic bags and stored in a domestic freezer at −20 °C until the extractions. Before being submitted to extraction, the leaves were triturated in a domestic food processor (Wallita, Model Master, São Paulo, SP) and the particles from 24 to 48 mesh selected using a magnetic agitator (Bertel, Model 1868, Caieiras, SP). The selected leaves were extracted ($n = 3$) with 80% methanol (20 g per 100 mL) for 7 days with periodic agitation. The extract was then filtered through filter paper and the residue resubmitted to agitation for 10 min with 100 mL of 80% methanol and filtered again. Both filtrates were mixed and the solvent evaporated to dryness under vacuum at 38 °C. The dry extract was stored in a freezer at 20 °C until assayed. The clove essential oil and eugenol were purchased from Dierberger Essential Oils S.A. (Brazil).

2.3. Antioxidant Activity

The antioxidant activity of the samples and standards was determined by way of the radical scavenging activity method using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·). About 0.1 ml aliquots of methanolic solutions of the samples or standards at different concentrations were each added to 3.9 ml of a DPPH· methanolic solution. Three DPPH· solutions were tested, 0.2000, 0.1242 and 0.0800 mM, prepared by dissolving 39.4, 24.5 or 15.8 mg in 500 ml of methanol, respectively. These concentrations were selected due the linearity range of DPPH· solutions, above 0.2 mM the concentration is very high and may be able to have a mistake due Beer's law, and below to 0.5 mM the color is very weak with limited range of absorbance reading. The blank sample consisted of 0.1 ml of methanol added to 3.9 ml of DPPH·. The tests were carried out in triplicate. After a 90 min incubation period at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated as follows: I% = $[(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 was the absorbance of the blank and Abs_1 was the absorbance in the presence of the test compound at different concentrations. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration vs the corresponding scavenging effect. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated as follows as:

$$AAI = \frac{\text{final concentration of DPPH}^{\cdot}(\mu g.ml^{-1})}{IC_{50}(\mu g.ml^{-1})}. \quad (1)$$

Thus, the AAI was calculated considering the mass of DPPH· and the mass of the tested compound in the reaction, resulting in a constant for each compound, independent of the concentration of DPPH· and sample used. In this work we considered the plant extracts to show poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when AAI between 0.5 and 1.0, strong antioxidant activity when AAI between 1.0 and 2.0, and very strong when AAI > 2.0. The assays were carried out in triplicate and all the samples and standard solutions, as well as the DPPH· solutions, were prepared daily.

2.4. Statistical analysis

The data obtained were analyzed using ANOVA/Tukey ($p < 0.05$). The statistical package used was Statistica™ 6.0 data analysis software by Statsoft, Inc., USA.

3. Results and discussion

Table 1 shows the results of the extracts and the pure compounds. For the IC_{50} determination, it is very important that this be carried out in the linear range for each compound. Therefore, every day the analysis was carried out a calibration curve was performed for all the compounds tested, and a good linear range was observed (Table 1). The stability and linearity ranges of the DPPH· solutions were evaluated and the results presented in Fig. 1. No difference in absorbance was observed between 0 and 90 min for any of the concentrations tested and good linear ranges were observed. A previous study reported that the absorbance of DPPH· at 517 nm in methanol and in acetone decreased by 20% and 35%, respectively at 25 °C in the light, however, in the dark, no significant change was observed during 150 min (Ozcelik et al., 2003).

The AAI was determined using Eq. (1), where the final concentrations of DPPH· solutions were 76.89, 47.75 and 30.75 μg ml^{-1} for the 0.2, 0.1242 and 0.08 mM solutions. Gallic acid showed the highest AAI value followed by protochatechuic acid and quercetin.

Table 1
Values for the antioxidant activity index (AAI) with different final concentrations of DPPH·

DPPH· 76.89 μg ml⁻¹	I			II			III			Mean IC_{50}	Mean AAI	SD
	r^2	$^{A}IC_{50}$	AAI	r^2	IC_{50}	AAI	r^2	IC_{50}	AAI			
Gallic acid	0.9984	2.90	26.51	0.9996	2.76	27.86	0.9999	2.84	27.07	2.83	27.15[a]	0.68
Protochatechuic acid	0.9987	3.80	20.23	0.9989	3.97	19.37	0.9938	3.68	20.89	3.82	20.17[b]	0.77
Quercetin	0.9995	5.48	14.03	0.9978	4.74	16.22	0.9997	4.39	17.51	4.88	15.92[c]	1.76
Eugenol	0.9987	7.35	10.46	0.9939	6.78	11.34	0.9985	6.83	11.26	6.99	11.02[d]	0.49
Chlorogenic acid	0.9999	7.56	10.17	0.9956	7.32	10.50	0.9991	7.43	10.35	7.44	10.34[d]	0.17
Clove essential oil	0.9988	8.15	9.43	0.9986	7.08	10.86	0.9999	8.27	9.30	7.83	9.86[d]	0.87
Caffeic acid	0.9937	8.00	9.61	0.9925	8.07	9.53	0.9986	8.57	8.97	8.21	9.37[d]	0.35
BHA	0.9976	7.83	9.82	0.9948	8.64	8.90	0.9963	8.22	9.35	8.23	9.36[d]	0.46
Rutin	0.9992	12.09	6.36	0.9993	11.39	6.75	1.0000	12.78	6.02	12.09	6.38[e]	0.37
Ferulic acid	0.9994	14.68	5.24	0.9990	13.70	5.61	0.9996	14.96	5.14	14.45	5.33[e]	0.25
X. strumarium	0.9953	44.70	1.72	0.9965	43.93	1.75	0.9960	46.81	1.64	45.15	1.70[f]	0.06
trans-Cinnamic acid	–	–	–	–	–	–	–	–	–	–	–	–
DPPH· 47.75 μg ml⁻¹												
Gallic acid	0.9992	1.83	26.09	1.0000	1.84	25.93	0.9998	1.96	24.36	1.89	25.46[a]	0.96
Protochatechuic acid	1.0000	2.35	20.30	0.9988	2.47	19.33	0.9993	2.23	21.44	2.35	20.36[b]	1.05
Quercetin	0.9998	3.22	14.83	0.9939	2.83	16.85	0.9997	3.05	15.66	3.03	15.78[c]	1.02
Eugenol	0.9994	4.45	10.73	0.9998	4.71	10.14	0.9991	4.66	10.25	4.61	10.37[d]	0.32
Chlorogenic acid	0.9995	4.89	9.76	0.9987	4.77	10.01	0.9986	4.57	10.45	4.74	10.08[d]	0.35
Clove essential oil	0.9992	4.98	9.59	0.9992	4.99	9.57	0.9996	4.79	9.97	4.92	9.71[d]	0.23
Caffeic acid	0.9983	4.60	10.38	0.9969	4.57	10.45	0.9966	4.25	11.23	4.47	10.69[d]	0.47
BHA	0.9987	5.28	9.04	0.9991	5.27	9.06	0.9969	5.15	9.27	5.23	9.12[d]	0.13
Rutin	0.9998	8.17	5.85	0.9997	7.30	6.54	0.9996	7.45	6.41	7.64	6.27[e]	0.37
Ferulic acid	0.9997	8.27	5.77	0.9998	8.93	5.34	0.9992	9.64	4.95	8.95	5.36[e]	0.41
X. strumarium	0.9973	29.31	1.63	0.9996	30.47	1.57	0.9958	29.99	1.59	29.92	1.60[f]	0.03
trans-Cinnamic acid	–	–	–	–	–	–	–	–	–	–	–	–
DPPH· 30.75 μg ml⁻¹												
Gallic acid	0.9997	1.17	26.38	0.9967	1.10	27.73	0.9967	1.11	27.66	1.12	27.25[a]	0.76
Protochatechuic acid	0.9893	1.41	21.86	0.9885	1.48	20.71	0.9908	1.48	20.80	1.45	21.12[b]	0.64
Quercetin	0.9968	1.98	15.56	0.9882	1.76	17.92	0.9862	1.97	15.60	1.90	16.36[c]	1.35
Eugenol	0.9982	3.02	10.19	0.9932	2.65	11.58	0.9842	2.83	10.86	2.83	10.88[d]	0.70
Chlorogenic acid	0.9989	2.82	10.92	0.9998	2.69	11.41	0.9999	2.73	11.28	2.75	11.20[d]	0.25
Clove essential oil	0.9938	3.28	9.36	0.9874	2.94	10.44	0.9928	2.94	10.45	3.05	10.08[d]	0.63
Caffeic acid	0.9957	2.89	10.64	0.9923	2.94	10.45	0.9996	2.78	11.07	2.87	10.72[d]	0.31
BHA	0.9994	3.51	8.77	0.9816	3.03	10.14	1.0000	2.69	11.44	3.08	10.12[d]	1.34
Rutin	0.9975	5.12	6.00	0.9940	4.28	7.18	0.9894	3.95	7.78	4.45	6.99[e]	0.91
Ferulic acid	0.9966	6.77	4.54	0.9992	5.66	5.43	0.9992	5.08	6.06	5.84	5.34[e]	0.76
X. strumarium	0.9923	20.23	1.52	0.9899	19.02	1.61	0.9991	18.12	1.69	19.12	1.60[f]	0.08
trans-Cinnamic acid	–	–	–	–	–	–	–	–	–	–	–	–

I, II and III: Different days of analysis; r^2: linearity coefficient; SD: standard deviation; –: not found. Different letters correspond to significant difference ($P < 0.05$).
[A] μg ml⁻¹.

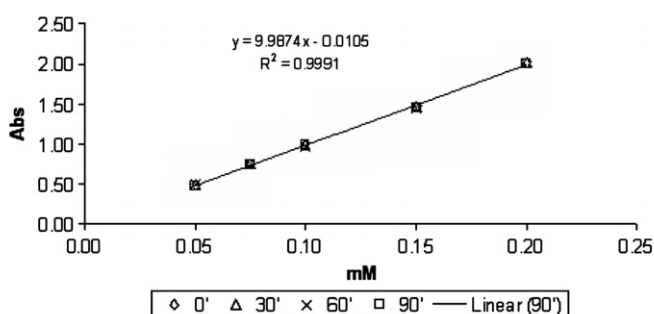

Fig. 1. Calibration curve and stability evaluation of the DPPH· solutions. The assays were carried out in triplicate with 3.9 ml of DPPH· solution plus 0.1 ml of methanol.

No significant differences were observed between eugenol, chlorogenic acid, clove essential oil, caffeic acid and BHA, which all showed higher AAI values than ferulic acid and rutin, which were similar to each other (Table 1). *trans*-Cinnamic acid presented no ability to reduce the DPPH· even when tested at a higher concentration (200 μg ml⁻¹ final concentration). The *Xanthium strumarium* extract showed a high AAI value and exhibited strong antioxidant activity. A previous study reported the presence of phenolic compounds, such as chlorogenic and ferulic acids in the *Xanthium strumarium* extracts (Han, Li, Zhang, Zheng, & Qin 2006). Clove essential oil had very strong antioxidant activity due to the presence of eugenol, which was reported as the majority compound (Jirovetz et al., 2006; Tomaino et al., 2005).

Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds (Bravo, 1998). The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activity, and this is referred to as structure–activity relationships (Balasundram, Sundram, & Samman, 2006). The antioxidant activity of phenolic acids increase with increasing degree of hydroxylation, as is the case of gallic acid (trihydroxylated) and protochatechuic acid (dihydroxylated) which show high AAI values. The substitution of the hydroxyl group in the aromatic ring with a methoxyl group, as in the case of caffeic acid and ferulic acid (Fig. 2) reduced the AAI value (Table 1), in agreement with Rice-Evans, Miller, and Paganga (1996) who reported that the substitution of hydroxyl by methoxyl groups reduced the activity. This can be explained by the reduction in the hydrogen atom donating capacity of the molecule. The absence of a hydroxyl group in the aromatic ring of *trans*-cinnamic acid (non phenolic) could explain its incapacity to reduce DPPH·. The structure–activity relationships (SAR) of flavonoids are generally more complicated than those of hydroxybenzoic and hydroxycinnamic acids due to the relative complexity of the flavonoid molecules. Van Acker et al. (1996) reported that the degree of hydroxylation as well as the position of the hydroxyl

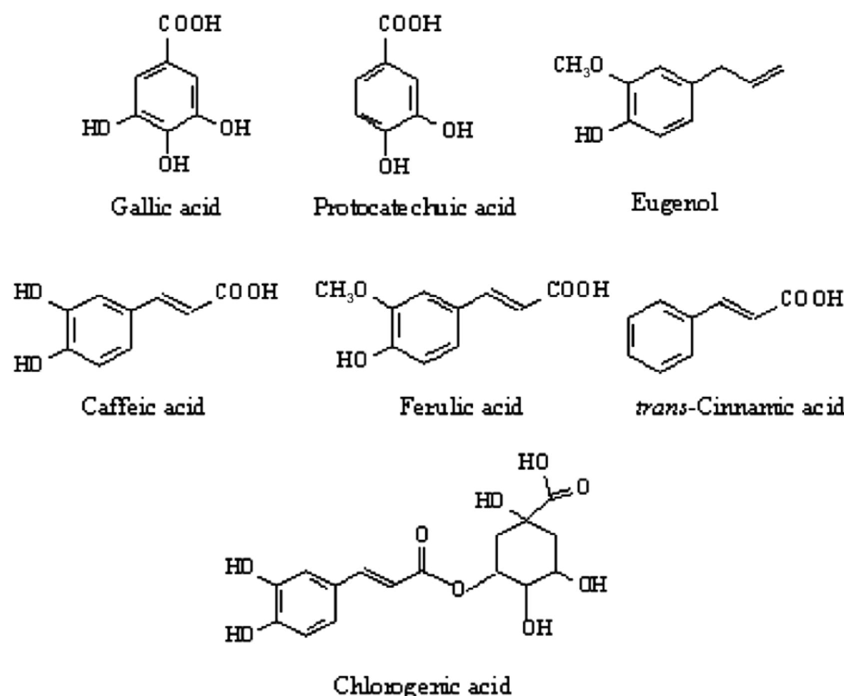

Fig. 2. Structure of some of the compounds used in the assays.

groups, and a double bond combined with a R1=OH (Fig. 3), increased the radical scavenging capacity of flavonoids. For this reason, quercetin has a higher AAI value than rutin (Table 1), in which the hydroxyl group is substituted by rutinose in the R1 position (Fig. 3).

When different DPPH· solutions were used for the same extract or pure compound, the IC_{50} value varied, although the AAI value remained constant, since no significant differences between the AAI values found for each compound tested were observed (Table 1). Meda et al. (2005) evaluated the antioxidant activity of honey by the DPPH· method and used quercetin as the control. The authors reported that the IC_{50} of quercetin was 0.87 $\mu g\ ml^{-1}$ when a final DPPH· concentration of 0.0338 mM was used. However, as cited before, the IC_{50} value varies with the final concentration of DPPH· used. So, applying Eq. (1) proposed in this study, the AAI for quercetin in the study of Meda et al. (2005) was 15.32, in agreement with the results of the present study. The AAI values for samples of honey (Sun and Ho, 2005) ranged from 0.45 to 9.8, since five samples presented AAI < 1.0 and 12 samples presented AAI between 1.0 and 2.0. Singh et al. (2007) evaluated the antioxidant activity of *Acacia auriculiformis*, the values of IC_{50} were 35.4 and 51.3 $\mu g\ ml^{-1}$ for water and ethyl acetate fractions, so that the AAI values (Eq. (1)) were 0.95 and 0.66, respectively. Sharififar et al. (2007) reported IC_{50} values of 11.7 and 16.2 $\mu g\ ml^{-1}$ for non-polar and polar fractions of endemic *Zataria multifolra* Boiss, applying the Eq. (1), the AAI values were 3.33 and 2.4, respectively. Elzaawely et al. (2007) studied the antioxidant activity of leaves and rhizomes of *Alpinia zerumbert*, the author's related values of IC_{50} from 70 to 700 $\mu g\ ml^{-1}$, according to the Eq. (1), the AAI values were 0.09 to 0.93 and 3.8, respectively.

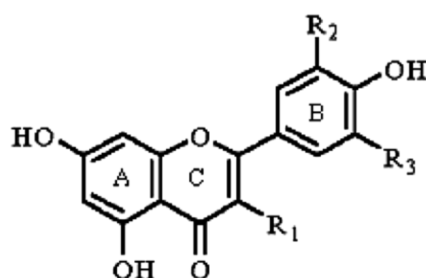

Fig. 3. Structure of the flavonoid molecule. Quercetin: R1 and R2=OH, and R3=H; rutin is a glycoside of quercetin where R1 = disaccharide, rutinose (β-1-L-rhamnosido-6-o-glucose).

As it was mentioned, there is a deficiency to compare the antioxidant potential between extracts due the several ways that of the results are presented. The DPPH· index (*I*%) only shows the capacity of the sample, in a fixed concentration, to reduce or not the DPPH· radicals, in which many cases, increasing the extract concentration the *I*% will be increased. The IC_{50} shows the extract concentration necessary to decrease the initial DPPH· concentration by 50%, however, using different DPPH· concentration the results will be different for the same sample. So, the AAI relate the DPPH· concentration used in the assay with IC_{50} of the sample, resulting in a constant data for each compound or plant extract, since has been obtained at the same conditions, because there is a consensus that different extraction procedure or different places of harvest, could give different results.

4. Conclusions

The proposed antioxidant activity index (AAI) was shown to be appropriate for the comparison of the antioxidant strength between plant extracts and essential oils, as well as between pure compounds, since no significant difference in AAI was observed when different solutions of DPPH· and different concentrations of tested compound, were used. Gallic acid, protochatechuic acid and quercetin showed higher AAI values. The clove essential oil presented very strong antioxidant activity, and no significant difference from the AAI of eugenol was observed. *Xanthium strumarium* extract presented strong antioxidant activity.

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