CHEMICAL ENGINEERING TRANSACTIONS
VOL. 64, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Antonio Marzocchella, Tajalli Keshavarz

**ISBN** 978-88-95608- 56-3; **ISSN** 2283-9216
DOI: 10.3303/CET1864019

# Correlation Between Phenolic Compounds Contents, Anti-tyrosinase and Antioxidant Activities of Plant Extracts

Isaac de F. Corradi, Edward de Souza, Denise Sande, Jacqueline A. Takahashi*

Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais. Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte - MG, Brazil.
isaac.corradi@uol.com.br

Plants are a natural source of strategic bio-active chemicals able to inhibit tyrosinase and reduce reactive oxygen species helping to protect human body against diseases related to alterations in DNA and cancer. Anti-tyrosinase and antioxidant compounds are widely commercialized as components in foods, as well as in other products from pharmaceutical, cosmetics and health industries. Searching for plants containing natural antioxidants and bioactive compounds, we described a preliminary study in which eighteen extracts from thirteen plant species showed an interesting anti-tyrosinase potential. *Schinus terebinthifolius* was one of the most interesting plant species detected in the previous study. In this way, the present work aimed to evaluate the correlation between phenolic compounds contents and biological activities (anti-tyrosinase and antioxidant) of different extracts from *S. terebinthifolius* (barks and leaves). HPLC profile and antioxidant activities were determined for the extracts. Some secondary metabolites (caffeic and gallic acids as well as methyl gallate) were identified via HPLC and spectroscopic methods in *S. terebinthifolius* extracts. The *in vitro* anti-tyrosinase activity of these compounds was also evaluated and the extension of their activity was compared to the anti-tyrosinase activity of the crude extracts. Gallic and caffeic acid were found in both leaves and bark extracts. Gallic acid showed low anti-tyrosinase activity (30 %), in its pure form, but its presence was positively associated with improved degrees of anti-tyrosinase (69-75 %) and antioxidant activities (inhibition concentration – IC) of the extracts (IC 50 % ABTS: 16.6 - 23.9 $\mu g.mL^{-1}$ and IC 50 % DPPH: 295.2 – 392.4 $\mu g.mL^{-1}$). *Schinus terebinthifolius* extracts are, therefore, promising natural source of tyrosinase inhibitors (in bark extracts) and antioxidant agents (especially in leaves extracts), showing biotechnological potential to be used as functional food, nutraceutical or in cosmetic formulations.

## 1. Introduction

Tyrosinase is a polyphenol oxidase enzyme that plays a key role in the biosynthesis of melanin. Tyrosinase catalyzes o-hydroxylation of monophenol intermediaries and oxidation of o-diphenol compounds to produce o-quinones (Kim and Uyama, 2005). Therefore, when tyrosinase is inhibited, melanogenesis is stopped or some of its effects are reduced. Dysregulation in melanogenesis plays also an important role in neurodegenerative diseases like Parkinson's as well as in melanoma and skin stains. In addition, many formulations employed for treatment of stains and hyper-pigmentation contains one or more substances with anti-tyrosinase action. Many of stains removing cosmetic formulations are based on vegetal extracts in view of the fact that a great variety of botanic species contains tyrosinase inhibitory substances (Mapunya et al., 2012; Mocan et al., 2017), not to mention the current adoption of natural treatments by the population.
In food, *o*-quinones formed by tyrosinase action can be polymerized in pigments (dark, red and brown) causing enzymatic browning in plant-derived foods and seafood, leading to a decrease in appearance appeal, together with loss in nutritional and market value (Kubo and Kinst-Hori, 1998). Food additives are used to prevent this reaction, however, their use is limited because of low effectivity, off-flavors generation or allergenic properties (Loizzo et al., 2012). In this way, development of new tyrosinase inhibitory substances from natural sources with satisfactory efficacy and safety to be applied in food industry is an important industrial demand.

Please cite this article as: Corradi I., De Souza E., Sande D., Takahashi J.A., 2018, Correlation between phenolic compounds contents, anti-tyrosinase and antioxidant activities of plant extracts, Chemical Engineering Transactions, 64, 109-114 DOI: 10.3303/CET1864019

Oxidative stress is related to different human diseases associated with alterations in DNA, aging and cancer (Karim et al., 2017). It affects the skin, accelerating aging. Oxidation also represents a huge problem in food industries because it leads to reduction of products shelf-life. Many plant species have been investigated regarding to their antioxidant potential (Marchi et al., 2015). Crude plant extracts or their natural chemical constituents have been widely used in cosmetic formulations with the purpose of slowing skin aging and in food as preservatives (Di Petrillo et al., 2016).

Compounds with both, anti-tyrosinase and antioxidant activities, can be employed as functional foods and as dermo-cosmetics as well as by other fields of health industries. In this context, prospecting plant extracts possessing both tyrosinase inhibitors and antioxidant compounds represents an important step in the search for novel industrial developments for obtaining high tangible profits for adepts of natural-products solutions for usual health, cosmetic and food problems.

On a previous work, a large number of plants was collected, identified and classified accordingly to their botanic families and respective ethanol extracts were evaluated for their capacity of inhibiting tyrosinase via *in vitro* bioassays (Corradi et al., 2012). In the present work, extracts from *Schinus terebinthifolius*, a plant active as tyrosinase inhibitor in the previous screening, was deeply studied, to evaluate its antioxidant profile and to understand the correlation of some phenolic compounds present in the extracts and their biological activities profiles.

## 2. Materials and Methods

### 2.1 High performance liquid chromatography (HPLC) analysis

Ethanol extracts were obtained from *S. terebinthifolius* barks and leaves by percolation. Aliquots (10.0 mg) of each extract and of pure compounds (caffeic and gallic acids, and methyl gallate) were diluted in methanol and filtered (Millipore Millex® 0.45 µm filters). To obtain the chromatographic profiles, a HPLC system with two pumps (Shimadzu model LC-AT), with UV-Vis detector (Shimadzu SPD-20A Prominence), with two scanning channels, a manual injection valve (loop of 20 µL), syringe injection of samples SGE® 250 µL with without bezel needle was used. Column Agilent Zorbax® ODS (octadecylsilane) C18 (250 mm length x 4.6 mm internal diameter x 0.5 µm average particle diameter of the stationary phase) was used. Shimadzu LC Solution software was used to control the HPLC system.

Solvents A and B formed the mobile phase, the former comprised methanol and acetic acid (99.7:0.3) and the later was prepared using water, methanol and acetic acid (79.7:20:0.3). The chromatographic peaks were detected at 280 and 350 nm and the elution flow was 0.9 $cm^3.min^{-1}$. Samples of caffeic and gallic acids, and methyl gallate, purified from previous works were also injected in the Chromatograph. Chromatograms from extracts were compared to the retention times of the pure compounds to assure the presence of those compounds into the plant extracts.

### 2.2 Evaluation of *in vitro* tyrosinase inhibitory activity

Evaluation of inhibitory action of caffeic and gallic acids, and methyl gallate, and of *S. terebinthifolius* extracts, (barks and leaves extracts) was performed following the formation of dopachrome from L-Dopa (Aldrich®, St. Louis, MO, USA), in presence of tyrosinase (Sigma-Aldrich®, St. Louis, MO, USA) at room temperature (r.t) and in physiological pH (7.4). All test solutions were prepared with PBS (phosphate buffer solution) 0.1 $mol.dm^{-3}$, pH 7.4, (Synth®, Diadema, SP, Brazil). Dopachrome formation can be characterized, monitored and quantified by its typical absorption at 475 nm ($\varepsilon_{475}$ 475 = 3600 $M^{-1}.cm^{-1}$). Accordingly, several experiments were performed employing solutions of L-Dopa (4 $mg.cm^{-3}$) and tyrosinase (13 $U.cm^{-3}$), r.t, at physiological pH to obtain the free conversion curves; and L-Dopa (4 $mg.cm^{-3}$) and tyrosinase (13 $U.cm^{-3}$) plus kojic acid (standard inhibitor, used as a control at the concentration of 1 $mg.cm^{-3}$), r.t, at physiological pH to get the total inhibition curves. Experiments with pure compounds (1 $mg.cm^{-3}$) were carried out with L-Dopa (4 $mg.cm^{-3}$) and tyrosinase (13 $U.cm^{-3}$), r.t, at physiological pH (Corradi et al., 2012). Analysis were performed on a Hitachi U-2010 UV-Vis Spectrophotometer, series 9713-002, version room 2550 01.

Aliquots of 0.9 $cm^3$ of PBS, 1.0 $cm^3$ of each pure compound and 0.1 $cm^3$ of enzyme solution were added to cells with 1 cm optical path length and capacity of 4.5 $cm^3$ (polymethylmethacrylate disposable cells purchased from Kartell S.P.A., Noviglio, Italy). The contents of each cell were homogenized and subjected to pre-incubation for 300 s. After completion of pre-incubation, each cell received 1.0 $cm^3$ of L-Dopa, followed by rapid homogenization and immediate insertion of the cell in the spectrophotometer. PBS was employed as blank and a mixture of 1.9 $cm^3$ of PBS, 0.1 $cm^3$ of enzyme and 1 $cm^3$ of L-Dopa, prepared under the same conditions as the other samples, was utilized as control. Usually in those analysis, kojic acid, 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, a well-known tyrosinase inhibitor (Sendovski et al., 2011) is used as standard.

Formation and quantification of dopachrome produced in the reaction mixture were monitored at 475 nm in real time for 900 s. Anti-tyrosinase activity was calculated by the Eq(1):

$$\%\ Tyrosinase\ inhibition = \frac{(A - B) - (C - D)\ x\ 100}{(A - B)} \quad (1)$$

Where A = maximum absorbance of the reference solution (only enzyme and substrate); B = initial absorbance of the blank solution (absorbance at time 0); C = maximum absorbance of sample solution (sample + enzyme + substrate); and D = initial absorbance of sample solution (absorbance at time 0). The values utilized for A and C in the equation refer to maximum absorbance at 475 nm, indicating the maximum point of dopachrome formation, which occurred around 450 s after addition of L-Dopa to the system, according to observations of dopachrome formation from L-Dopa, catalyzed by tyrosinase in the absence of any inhibitor. The values of B and D were those determined in the beginning of each run, i.e., at time zero (t = 0 s), in order to correct interferences caused by partial transparence of the reaction mixture on that specific wavelength or nearby.

### 2.3 Radicals capture antioxidant activities (ABTS and DPPH)

Evaluation of radicals' capture antioxidant activities of *S. terebinthifolius* extracts followed two methodologies. In capture radical ABTS, the first step was the preparation of an aqueous ABTS solution (7 mM ABTS and potassium persulfate 2.4 mM incubated for 12 h in dark). Then, the solution was diluted (methanol) until absorbance at 734 nm reached 0.708 ± 0.001. The reaction took place with 1 mL of this solution and 1 mL of extracts solution in different concentrations (2.5-125 $\mu g.mL^{-1}$), in dark room, for 7 min. Absorbance values (734 nm) were taken. DPPH radical capture consisted in mixing the extracts (100-1000 $\mu g.mL^{-1}$) with DPPH 0.3 % in methanol. The reaction occurred for 30 min in dark room and the absorbance (517 nm) was taken. In both assays, the positive control was ascorbic acid, a blank was made with methanol and the results were expressed as inhibition percentage, calculated using the Eq(2).:

$$\%\ inhibition = \frac{(A - B)}{A}\ X\ 100 \quad (2)$$

Where A = blank absorbance; B = sample absorbance.
Sample concentration ($\mu g.mL^{-1}$) required for capturing 50 % of DPPH or ABTS radicals (IC 50 %) was obtained from an inhibition (%) vs. concentration curve (Sande et al., 2016).

## 3. Results and Discussion

Several phenolic compounds have been widely described in *S. terebinthifolius* extracts (Santos et al., 2002; El-Massry et al., 2009; Uliana et al., 2016; Carvalho et al., 2009; Cavalher-Machado et al., 2008; Rosas et al., 2015). However, the presence of secondary metabolites in plant extracts depends on several variables, such as seasonal variations, part extract, extraction solvent, among others. In the present work, the HPLC profiles of *S. terebinthifolius* barks extract and *S. terebinthifolius* leaves extract were obtained and compared to the chromatographic profiles of several phenolic compounds (caffeic acid (1), gallic acid (2) and methyl gallate(3)) (Figure 1), in order to evaluate presence of those compounds in the extracts.

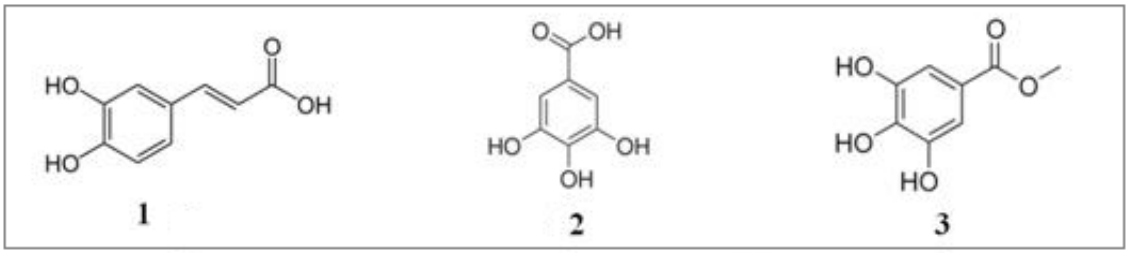


*Figure 1: Chemical structure of caffeic acid (1), gallic acid (2) and methyl gallate*

Caffeic and gallic acids were found in both extracts meanwhile methyl gallate was found only in leaves extracts (Table 1). Phenolic compounds found in the extracts were spectroscopically evaluated for *in vitro* tyrosinase inhibition (Table 1).

*Table 1: Phenolic compounds detected in S. terebinthifolius extracts and their tyrosinase inhibitory activity*

| Compound/ extract | Rt (min) | Tyrosinase Inhibition (%) | Part of the plant in which the compound was detected (reference) |
|---|---|---|---|
| Caffeic acid | 16 | 1 | Barks (Santos et al., 2002)<br>Leaves (El-Massry et al., 2009; Uliana et al., 2016) |
| Gallic acid | 5 | 30 | Barks (Carvalho et al., 2009)<br>leaves (Santos et al., 2002) |
| Methyl gallate | 12 | 53 | Leaves Cavalher-Machado et al., 2008; Rosas et al., 2015) |
| *S. terebinthifolius* barks | na | 79 | na |
| *S. terebinthifolius* leaves | na | 65 | na |

na: do not apply

Among the phenolic compounds detected in *S. terebinthifolius* extracts, gallic acid presented the second higher tyrosinase inhibition (30 % inhibition). Therefore, presence of gallic acid seems be related to the anti-tyrosinase activity detected for the extracts, which ranged from 65 % (leaves extract) and 79 % (barks extract) (Table 1). Surprising, caffeic acid showed only 1 % of tyrosinase inhibition. On the other side, methyl gallate, although presented the highest activity among the compounds detected in *S. terebinthifolius* extracts, did not provoke proportional increase of activity in leaves extract, where it was detected. Therefore, gallic acid was found to be the key compound related to the tyrosinase activity detected in the *S. terebinthifolius* extracts.
The antioxidant activity of a plant is also related to their fitocompounds, especially the phenolic ones (Ahmad et al., 2017). As well as on tyrosinase inhibitory activity, among the phenolic components detected in *S. terebinthifolius* extracts, gallic acid seems to influence more prominently the antioxidant activity. This acid was detected in both extracts with antioxidant activity.

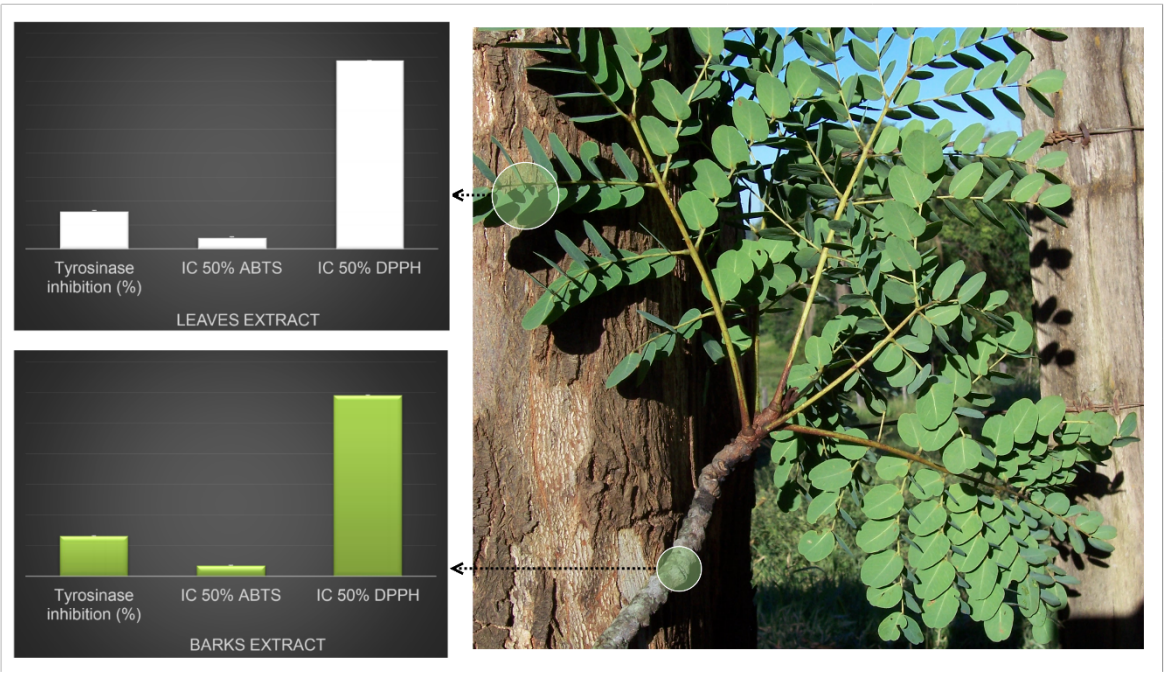


*Figure 2: Comparative profile of tyrosinase inhibition (%) and antioxidant activity by DPPH and ABTS methods of S. terebinthifolius barks and leaves extracts*

In the antioxidant assay measuring the capture of radicals DPPH, *S. terebinthifolius* extracts presented IC 50 % = 392.4 $\mu g.mL^{-1}$ (barks) and IC 50 % = 295.2 $\mu g.mL^{-1}$ (leaves), while, for ABTS radicals capture, the concentration necessary for IC 50 % of ABTS was 23.9 $\mu g.mL^{-1}$ (barks) 16.6 $\mu g.mL^{-1}$ (leaves) (Figure 2). The

association of gallic acid with antioxidant activity has been recently reported (Yahia et al., 2017). In the screening of both activities, gallic acid presented a more influential profile, although the results show that the anti-tyrosinase and antioxidant activities come from a synergy of the fitoconstituents present in the whole plant (Sande et al., 2016). A greater diversity of phenolic compounds was detected in leaves extract and this part of the plant presented an extract with better antioxidant activity than barks extract (Figure 2). This suggests a multi-component role in the antioxidant activity of *S. terebinthifolius* corroborating data reported in the literature (Mocan et al., 2017).

Currently, there is an increased popularity of natural food additives motivated by the knowledge of synthetic antioxidants toxicity (Sande et al., 2016) and food and cosmetic industries demand for natural antioxidants (Mocan et al., 2017). In this way, *S. terebinthifolius* revealed itself as source of phytochemicals that act as tyrosinase inhibitors (in bark extracts) and antioxidant agents (mostly in leaves extracts). Therefore, this species shows a high potential to be applied as antioxidants to preserve food. The role of *S. terebinthifolius* as functional food, nutraceutical or in cosmetic formulations is an innovative biotechnological promise.

## 4. Conclusions

From the natural products screened in this work, gallic acid was the metabolite most commonly found in the extracts. This compound, together with its methylate derivative, methyl gallate, showed anti-tyrosinase activity. However, the anti-tyrosinase and antioxidant activities of the crude extracts seem to be more dependent of gallic acid presence. Crossing the results, the inhibitory activity of the extracts was not exclusively associated with the presence of a single substance with anti-tyrosinase activity; this activity is mostly due to a combination of anti-tyrosinase agents present in the extracts composition. The same observation was found to antioxidant activity. Therefore, use of the whole extract of *S. terebinthifolius* has advantages over the use of a single compound and yet is a good choice to attend the global demand for natural bioactive products. Antioxidant and anti-tyrosinase effect of *S. terebinthifolius* extracts can bring various benefits to health, aiding in prevention of cancer, cardiovascular and neurodegenerative diseases. These natural components still have potential to be applied in functional cosmetics that help in treating aesthetic disorders.

**Acknowledgments**

The authors thank CNPq and FAPEMIG for grants and scholarships.

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