

Tyrosinase Inhibitors from Galls of *Rhus javanica* Leaves and Their Effects on Insects

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As a defense mechanism of the leaves of *Rhus javanica* (Anacardiaceae) against the aphid *Melaphis chinensis* (Aphididae) attack, tannic acid is rapidly accumulated and forms galls along the midrib of the leaves resulting in a unique natural medicine Gallae Rhois. Tannic acid was found to inhibit the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by tyrosinase (EC 1.14.18.1) with an IC₅₀ of 22 μ M. The aphid would detoxify the ingested toxic tannic acid to relatively nontoxic gallic acid, whereas the non-adapted pink bollworm *Pectinophora gossypiella* larvae are sensitive to the ingested tannic acid.

Key words: Gallae Rhois, Tyrosinase Inhibitory Activity, Insect Growth Inhibitory Activity

Introduction

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO) (Mayer, 1987), is a copper containing enzyme widely distributed in microorganisms, animals and plants. This mixed function oxidase catalyzes two distinct reactions of melanin synthesis (Robb, 1984), the hydroxylation of a monophenol (monophenolase activity) and the conversion of an *o*-diphenol to the corresponding *o*-quinone (diphenolase activity). In our continuing search for alternative insect control agents from plants (Calderon *et al.*, 2001; Céspedes *et al.*, 2001a, 2001b; Kubo, 1993a), tyrosinase inhibitors have recently been targeted (Kubo, 1997) because tyrosinase is one of the key enzymes in the insect molting process (Andersen, 1990). Hence, tyrosinase inhibitors might ultimately provide clues to control insect pests by inhibiting tyrosinase, resulting in incomplete cuticle hardening and darkening (Kramer and Hopkins, 1987). For example, this enzyme is previously reported to be highly correlated with aphid resistance of the *Solanum* plants (Ryan *et al.*, 1982).

Gallic acid is derived from hydrolyzable tannin during the gall forming process in *R. javanica* by the insect *M. chinensis*, or by accompanying micro-

organisms within the parasites' body, or the plant itself. It appears therefore that the releasing mechanism of gallic acid may be one of the key processes to understanding plant defense and/or gall formation. The chemicals involved in gall formation are undoubtedly numerous. However, as an initial step to understanding this process, we focused the current study on the biological activity of gallic acid, especially its effects on insects as well as tyrosinase inhibitory activity. In order to facilitate it, tannic acid previously isolated from Gallae Rhois was also studied for comparison. It should be noted that the structure of tannic acid has not been yet established although it was reported as the mixture of at least seven related galloylglucoses by HPLC analysis. The basic structure is 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose to which the depside galloyl group is randomly distributed among C-2, C-3 and C-4 positions (Nishioka, 1983).

In our continuing search for alternative insect control agents, the ethanol extract of Gallae Rhois was noted to inhibit the oxidation of L-DOPA catalyzed by mushroom tyrosinase. The same extract was also found to exhibit growth inhibitory activity against the pink bollworm *Pectinophora gossy-*

piella in an artificial diet feeding assay with an ID_{50} of 2.2 mg/ml (Kubo, 1993b). Our original attempt to clarify the gall formation mechanism on a molecular level could not be achieved because of the lack of *M. chinensis* availability. Hence, the current study was emphasized characterizations of tyrosinase inhibitors as well as insect growth inhibitors against *P. gossypiella*.

Materials and Methods

Chemicals

An authentic tannic acid was provided by Dr. T. Yoshida, Okayama University, Okayama, Japan. Since tannic acid is a mixture of similar gallate analogues (Nishioka, 1983), the experiment was set up based on its average (1700) molecular weight. Gallic acid and its methyl and ethyl esters, 1,1-diphenyl-2-*p*-picrylhydrazyl (DPPH) and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). L-Tyrosine and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO).

Plant materials

Gallae Rhois was generous gifts by Mr. S. Kawamichi, Kawamichi Pharmaceutical Industries, Co. Ltd., Kyoto, Japan. The fresh leaves of *R. javanica* were collected near Yamashina, Kyoto in April 1987 and were immediately immersed into methanol. A voucher specimen of the plant was deposited to the Herbarium of Kyoto Pharmaceutical University, Kyoto, Japan and identified by Dr. S. Kiyosawa, Kyoto Pharmaceutical University, Kyoto, Japan.

Extraction and isolation

The galls were milled as a white powder (350 g) and extracted with 95 % aqueous EtOH for a week (3 ×) at room temperature, the solvent was evaporated under vacuum and concentrated at 40 °C to give a slightly yellowish residue which weighed 41.8 g and was used for chemical analysis. The crude extract was successively partitioned between *n*-hexane, CH_2Cl_2 , EtOAc and H_2O in this order and subsequent bioassay revealed the biological activity to be retained in the EtOAc fraction. The solvent was evaporated from the bioac-

tive EtOAc fraction *in vacuo* to give a residue (11.3 g). Then, 2.02 g of this residue were dissolved in 5 ml of the mobile phase and were injected onto the DCCC. The ascending method was used with one fraction equivalent to 300 drops. The solvent system consisted of $CHCl_3$ -MeOH- H_2O -AcOH-MeCoEt (23:42:26:3:6, v/v/v/v/v). Fractions were collected and checked by TLC plates. Three active compounds were obtained and the further purification by LH-20 column chromatography yielded gallic acid (83 mg), methyl gallate (17 mg) and ethyl gallate (134 mg). They were identified by spectroscopic methods and compared with authentic samples.

Since ethanol was used in the initial extraction, this group is probably an artifact. This was confirmed by the fact that ethyl gallate was not detected when methanol was used for extraction. Similarly, methyl gallate may also be an artifact since methanol was used as a solvent during purification procedure although this was not confirmed. As a result, the polar part of the ethanol extractable fraction of the white powder consists almost exclusively of gallic acid. In addition, the fractionation was also guided by the artificial diet feeding assay against the pink bollworm, and the same compounds were characterized as active principles.

Tyrosinase assay

The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO) and was purified by the procedure as previously reported (Espín and Wichers, 1999), this fungal source was used for the entire experiment because it is readily available. The preliminary assay was tested at 167 μ g/ml, unless otherwise specified. All the samples were first dissolved in DMSO and used for the experiment at 30 times dilution. Tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, but the assay was carried out in air-saturated aqueous solutions. The enzyme activity was monitored by dopachrome formation at 475 nm up to the appropriate time (not exceeding 10 min, unless otherwise specified.). The extent of inhibition by the addition of samples is expressed as the percentage necessary for 50 % inhibition (IC_{50}).

The assay was performed as previously described (Kubo and Kinst-Hori, 1998). First, 1 ml of a 2.5 mM L-DOPA or L-tyrosine solution was mixed with 1.8 ml of 0.1 M phosphate buffer (pH 6.8), and incubated at 25 °C for 10 min. Then, 0.1 ml of the sample solution and 0.1 ml of the aqueous solution of the mushroom tyrosinase (138 units) were added in this order to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm.

The pre-incubation mixture consisted of 1.8 ml of 0.1 M phosphate buffer (pH 6.8), 0.6 ml of water, 0.1 ml of the samples solution (equivalent amount of IC₅₀), and 0.1 ml of the aqueous solution of the mushroom tyrosinase (138 units, one unit = ΔA_{280} of 0.001 per min at pH 6.5 at 25 °C in 3 ml reaction mix containing L-tyrosine). The mixture was pre-incubated at 25 °C for 5 min. Then, 0.4 ml of 6.3 mM L-DOPA solution was added and the reaction was monitored at 475 nm for 2 min.

Protease assay

Type II fungal protease of *Aspergillus oryzae* and casein powder used for the assay were purchased from Sigma Chemical Co. (St. Louis, MO). Bacto-agar was obtained from Difco Laboratories (Detroit, MI). The assay was performed using previous methods according to Stauffer (1989). Agar plate was prepared as follows: 10 ml of solution of M/15 phosphate buffer (pH 7.4), 1 % of agar and 4 mg/ml of casein as substrate were placed in petri dishes (100 mm × 15 mm) and allowed to harden. The samples were dissolved in DMSO and used for the experiment at 10 times dilution. Protease (2.5 mg) was dissolved in 1 ml of 10 % DMSO-M/15 phosphate buffer (pH 7.4) or 1 ml of 10 % sample solution-M/15 phosphate buffer (pH 7.4) as control and test solutions, respectively. Filter paper disks were dipped in the control or test solution and placed on the agar plates. The plates were covered and incubated at 37 °C overnight and then flooded with 5 % trichloroacetic acid solution. The casein which had not been hydrolyzed precipitated *in situ*, giving a milky white background. Clear zones were measured and percent inhibitions were calcu-

lated from a standard curve made from different concentrations of enzyme solutions.

Radical scavenging assay

The assay was performed as previously described (Haraguchi *et al.*, 1997). The reaction mixture consisted of 1 ml of 100 mM acetate buffer (pH 5.5), 1 ml of ethanol and 0.5 ml ethanolic solution of DPPH. After allowing the mixture to stand at room temperature for 20 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity was measured as the decrease in absorbance of the DPPH expressed as percent of the absorbance of a control DPPH solution (Blois, 1958). Inhibitory activity was expressed as the mean 50 % inhibitory concentration of triplicate determinations, obtained by interpolation of concentration-inhibition curves.

Insect feeding assay

The artificial diet feeding assay was carried out as previously described with some modifications (Kubo, 1993b).

Statistical analysis

Data shown in figures and tables are the mean results obtained with means of three replicates and independent tyrosinase and DPPH preparations. Data were subjected to analysis of variance (ANOVA) with significant differences between means identified by GLM procedures. The results are given in the text as probability values, with $p < 0.05$ adopted as the criterion of significance, differences between treatment means were established with a Student-Newman-Keuls (SNK) test. The IC₅₀ values for each activity were calculated by Probit analysis on the basis of the percentage of inhibition obtained at each concentration of the samples.

Results and Discussion

Compounds assayed

Gallic acid characterized in the white powder could not be detected in *R. javanica* leaves, prior to attack by the gall-forming aphid *M. chinensis*. In contrast, hydrolyzable tannin is abundant instead in the fresh leaves. More importantly, this

tannin is rapidly accumulated in the wall after attack by *M. chinensis* and produces Gallae Rhois. This process can be termed as a proteinase inhibitor inducing factor (PIIF) (Ryan, 1979) since the aqueous extract of this Gallae Rhois much more strongly inhibited tyrosinase as well as two digestive proteinases tested, chymotrypsin and leucine-aminopeptidase, than that of the fresh leaves of *R. javanica*.

Tyrosinase activity

The tyrosinase bioassay with the purified gallic acid showed a concentration-dependent inhibitory effect on the oxidation of L-DOPA by mushroom tyrosinase. The IC_{50} was determined as 4.5 mM (767 μ g/ml) which is about 7-fold less potent activity than that of benzoic acid (Table I), a well documented tyrosinase inhibitor (Conrad *et al.*, 1994). Interestingly, gallic acid itself was oxidized as a substrate by the enzyme. The oxidation was characterized by a new peak with the maxima at 383 nm, characteristic of *o*-benzoquinones. It should be noted that this oxidation was at an extremely slower rate but did not have the lag period. In contrast, there was a progressive increase in the observed rate of this oxidation as soon as catalytic amounts (0.01 mM) of L-DOPA became available as a cofactor. Thus, upon addition of both tyrosinase and L-DOPA, a yellow color was immediately detected optically. As long as catalytic amounts of L-DOPA was present gallic acid was oxidized much faster than without this cofactor since the peak at 383 nm reached its plateau faster than 475 nm. Because of this enzymatically oxidizable nature, the mode of inhibition of gallic acid could not be analyzed by Lineweaver-Burk plots. Similarly, methyl gallate was also oxidized by the enzyme, yielding the yellowish oxida-

tion products. This oxidation rate was also significantly increased as soon as the catalytic amounts of L-DOPA became available as a cofactor. However, the oxidation products of methyl gallate did not show noticeable absorption between 350 and 550 nm. It is therefore obvious that the oxidation products were different from those of gallic acid. In addition, similar results were also obtained with ethyl gallate.

The oxidation products are a complex mixture of polar compounds and the unstable nature of the intermediates makes their characterization difficult. Despite our efforts, an attempt to characterize them failed. However, the resulting *o*-quinone of gallic acid may condense with one another through a Michael type addition, yielding a relatively stable quinol-quinone intermediate (Sayre and Nadkarni, 1994), though the possibility that the *o*-quinone may form adducts with different nucleophilic groups in the enzyme and inactivates it cannot be entirely ruled out. The former case seems to be more likely since the remaining L-DOPA in the cuvette was oxidized when oxygen was supplied by mixing as shown in Fig. 1. This result indicates that the enzyme was not inactivated by a K_{cat} type inhibition (inactivation of the enzyme by products of the reaction) (Golan-Goldhirsh and Whitaker, 1985) as long as the current experiment is concerned. It appears that gallic acid was isolated as a tyrosinase inhibitor by bioassay-guided fractionation using mushroom tyrosinase similar to the previous report (Matsuo *et al.*,

Table I. Tyrosinase inhibitory activity of gallic acid and related compounds^a.

Compounds	IC_{50} [mM]	K_I [nM]
Gallic acid	4.5	N. T.
Benzoic acid	0.64	0.29 ± 0.015
Tannic acid	0.022	0.007 ± 0.002

^a The experiment was performed in triplicate.

* With respect to L-DOPA.

N. T. = Not tested.

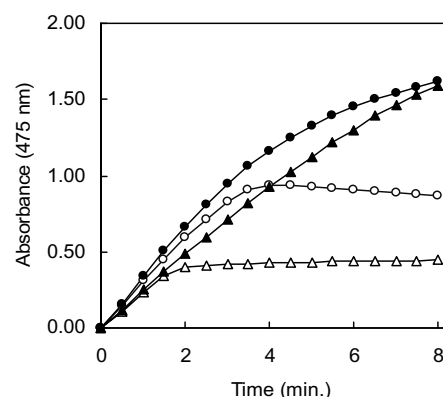


Fig. 1. Mixing effect of gallic acid (4.5 mM); (○) control without gallic acid and without mixing; (●) control without gallic acid and with mixing; (△) with gallic acid and without mixing; (▲) with gallic acid and with mixing.

1997), but this phenolic acid can also be termed as an alternate substrate if enough oxygen is available. In connection with this, the detectable quinol-quinone intermediate formation seems to be specific to gallic acid since the rather stable peak at 383 nm was observed only with the oxidation product of gallic acid.

On the other hand, tannic acid isolated from *Gallae Rhois* was found to exhibit tyrosinase inhibitory activity with an IC_{50} of $22 \mu M$ that is about 200-fold more potent than that of gallic acid. The inhibition kinetics of tannic acid for the oxidation of L-DOPA by mushroom tyrosinase was analyzed by Lineweaver-Burk plots and found to be a characteristic competitive inhibitor as shown in Fig. 2. Since tannins are known to react with proteins by cross-links, a process known as tanning, tannic acid should somehow irreversibly inactivate the tyrosinase (protein). However, pre-incubation of the enzyme in the presence of $22 \mu M$ of tannic acid, but in the absence of the substrate did not indicate that tannic acid is a direct inactivator of the enzyme since it did not significantly decrease the activity of the enzyme as far as the current experiment is concerned. The inhibition was increased only from 45 % to 50 %. On the basis of this, tannic acid may be a rather rare example of hydrolysable tannins that are not easily oxidized, enzymatically and non-enzymatically.

It appears that tannic acid exhibits much more potent tyrosinase inhibitory activity compared to that of gallic acid.

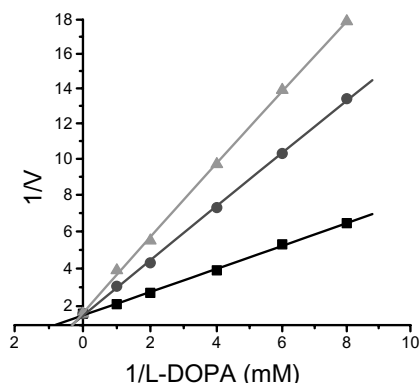


Fig. 2. Lineweaver-Burk plots of tyrosinase and L-DOPA without (■) and with tannic acid [(●): $13.3 \mu g/ml$ and (▲): $26.7 \mu g/ml$]. $1/V$: $1/(\Delta 475 \cdot min)$.

Insect bioassay

In the artificial diet feeding assay against *P. gossypiella* larvae, both gallic acid and tannic acid showed growth inhibitory activity and the ED_{50} of gallic acid was found to be 2000 ppm while that of tannic acid was 50 ppm. In other words, tannic acid showed 40-fold more potent growth inhibitory activity in this feeding assay, indicating that *P. gossypiella* larvae are sensitive to ingested tannic acid but somewhat tolerate gallic acid. It appears that the gallic acid characterized is responsible for the original insect growth inhibitory activity of the ethanol extract of the white powder observed in the preliminary screening. Similarly, tannic acid is responsible for that of the *Gallae Rhois* ethanol extract. Their insect growth inhibitory activity against the pink bollworm seems to correlate with their tyrosinase inhibitory activity, however this may be coincidence. It should be borne in mind that plant secondary metabolites can act in general by a variety of different mechanisms in insects.

The sensitivity of *P. gossypiella* to ingested tannic acid may be a consequence of its extensive chemical modification in the midgut and oxidation is the first thinkable chemical modification. If so, quinones should be the first oxidized structure. Needless to say, quinones are usually highly toxic to insect as well as to many other organisms. It should be noted that benzoic acid inhibited tyrosinase (Table I) but did not inhibit insect growth. This result may support that the oxidized products are responsible for the activity. In addition, oxidation also produces reactive oxygen species (ROS) in the gut lumen, and this ROS damages biological systems (Pardini, 1995). It seems that the detrimental effects of tannic acid on the non-adapted pink bollworm are result of ineffective defenses in a consequence of chemical modification in the midgut and/or against ROS generated in the gut lumen (Barbehenn *et al.*, 1996).

Antioxidant assay

The radical scavenging activity, which can be measured as decolorizing activity following the trapping of the unpaired electron of DPPH, was examined. In fact, gallic acid and methyl gallate exhibited almost equally potent radical scavenging

Table II. Antioxidative activity of gallic acid and related compounds^a.

Compounds	DPPH consumption ^b
Gallic acid	6.51 ± 0.30
Methyl gallate	6.02 ± 0.12
Ethyl gallate	6.18 ± 0.18
Tannic acid	78.8 ± 4.8

^a The experiment was performed in triplicate.^b The values indicate that one molecule of the test compound scavenges how many molecules of DPPH.

activity as shown in Table II. Tannic acid also showed potent radical scavenging activity. Thus, one molecule of tannic acid scavenges nearly eighty molecules of DPPH.

The possibility that their adverse effects are a consequence of their potential to act as a prooxidant may need to be considered. In fact, gallic acid is known to produce superoxide anion (Serrano *et al.*, 1998). Furthermore, tannic acid may somewhat irreversibly inactivate enzymes (proteins) in the midgut by cross-links prior to being oxidized.

The prooxidant or irreversible inactivation enzymes process also needs to be taken into con-

sideration since tannic acid may bind with proteins in the gut and, as a result, inhibit digestive enzymes as well as protein digestion (Feeny, 1976). This can be supported by the observation that tannic acid showed significant inhibitory activity against fungal protease. At the concentration of 3 mg/ml, tannic acid inhibited the enzyme activity 68 % while gallic acid did not show any inhibitory activity up to 6 mg/ml.

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Andersen S. O. (1990), Sclerotization of insect cuticle. In: Molting and Metamorphosis (E. Ohnishi and H. Ishizaki, eds.). Japan Sci. Soc. Press, Tokyo, pp. 133–155.

Barbehenn R. V., Martin M. M., and Hagerman A. E. (1996), Reassessment of the roles of the peritrophic envelope and hydrolysis in protecting polyphagous grasshopper from ingested hydrolyzable tannins. *J. Chem. Ecol.* **22**, 1901–1920.

Blois M. S. (1958), Antioxidant determination by the use of a stable free radical. *Nature* **181**, 1199–1200.

Calderón J. S., Céspedes C. L., Rosas R., Gómez-Garibay F., Salazar J. R., Lina L., Aranda E., and Kubo I. (2001), Acetylcholinesterase and insect growth inhibitory activities of *Gutierrezia microcephala* on fall armyworm *Spodoptera frugiperda* J. E. Smith. *Z. Naturforsch.* **56c**, 382–394.

Céspedes C. L., Alarcón J., Aranda E., Becerra J., and Silva M. (2001a), Insect growth regulator and insecticidal activity of β -dihydroagarofurans from *Maytenus spp.* (Celastraceae). *Z. Naturforsch.* **56c**, 603–613.

Céspedes C. L., Martínez-Vázquez M., Calderón J. S., Salazar J. R., and Aranda E. (2001b), Insect growth regulatory activity of some extracts and compounds from *Parthenium argentatum* on fall army worm *Spodoptera frugiperda*. *Z. Naturforsch.* **56c**, 95–105.

Conrad J. S., Dawso S. R., Hubbard E. R., Meyers T. E., and Strothkamp K. G. (1994), Inhibitor binding to the binuclear active site of tyrosinase: temperature, pH, and solvent deuterium isotope effects. *Biochemistry* **33**, 5739–5744.

Espín J. C. and Wichers H. J. (1999), Slow-binding inhibition of mushroom (*Agaricus bisporus*) tyrosinase isoforms by tropolone. *J. Agric. Food Chem.* **47**, 2638–2644.

Feeny P. (1976), Plant apparency and chemical defense. In: *Biochemical Interactions Between Plants and Insects* (J. W. Wallace and Masell R. L., eds.). Plenum Press, New York, pp. 1–40.

Golan-Goldhirsh A. and Whitaker J. R. (1985), Kcat inactivation of mushroom polyphenol oxidase. *J. Mol. Catal.* **32**, 141–147.

- Haraguchi H., Ishikawa H., Sanchez Y., Ogura T., Kubo Y., and Kubo I. (1997), Antioxidative constituents in *Heterotheca inuloides*. *Bioorg. Med. Chem.* **5**, 865–871.
- Kramer K. J. and Hopkins T. L. (1987), Tyrosine metabolism for insect cuticle tanning. *Arch. Insect Biochem. Physiol.* **6**, 279–301.
- Kubo I. (1997), Tyrosinase inhibitors from plants. In: *Phytochemicals for Pest Control* (P. Hedin, R. Hellingworth, E. Masler, J. Miyamoto and D. Thompson, eds.). ACS Symposium Series 658, American Chemical Society, Washington, DC, pp. 310–326.
- Kubo I. (1993a), Insect control agents from tropical plants. In: *Recent Advances in Phytochemistry* (K. R. Downum, J. T. Romeo and H. A. Stafford, eds.). *Phytochemical Potential of Tropical Plants*. Plenum, New York, Vol. 27, pp. 133–151.
- Kubo I. (1993b), Screening techniques for plant-insect interactions. In: *Methods in Plant Biochemistry* (K. Hostettmann, ed.). Academic Press, London, Vol. 6, pp. 179–193.
- Kubo I. and Kinst-Hori I. (1998), Tyrosinase inhibitors from cumin. *J. Agric. Food Chem.* **46**, 5338–5341.
- Matsuo K., Kobayashi M., Takubo Y., Kuwajima H., Ito H., and Yoshida T. (1997), Anti-tyrosinase activity constituents of *Arctostaphylos uva-ursi*. *Yakugaku Zasshi* **117**, 1028–1032.
- Mayer A. M. (1987), Polyphenol oxidases in plants – Recent progress. *Phytochemistry* **26**, 11–20.
- Nishioka I. (1983), Chemistry and biological activities of tannins. *Yakugaku Zasshi* **103**, 125–142. (In Japanese).
- Pardini R. S. (1995), Toxicity of oxygen from naturally occurring redox-active pro-oxidants. *Arch. Insect Biochem. Physiol.* **29**, 101–118.
- Robb D. A. (1984), Tyrosinase. In: *Copper Proteins and Copper Enzymes* (R. Lontie, ed.). CRC Press, Florida, Vol. II, pp. 207–240.
- Ryan J. D. (1979), Proteinase inhibitors. In: *Herbivores: Their Interaction with Secondary Plant Metabolites* (G. A. Rosenthal and D. H. Janzen, eds.). Academic Press, New York, pp. 599–618.
- Ryan J. D., Gregory P., and Tingey W. M. (1982), Phenolic oxidase activities in glandular trichomes of *Solanum berthaultii*. *Phytochemistry* **21**, 1885–1887.
- Sayre L. M., and Nadkarni D. V. (1994), Direct conversion of phenols to *o*-quinones by copper(I) dioxygen. Questions regarding the monophenolase activity of tyrosinase mimics. *J. Am. Chem. Soc.* **116**, 3157–3158.
- Serrano A., Palacios C., Roy G., Cespón C., Villar M. L., Nocito M., and González-Porqué P. (1998), Derivatives of gallic acid induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation. *Arch. Biochem. Biophys.* **350**, 49–54.
- Stauffer C. E. (1989), Measurement methods. In: *Enzyme Assays for Food Scientists*. Van Nostrand Reinhold, New York, pp. 128–130.