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TYROSINASE INHIBITORS FROM *ANACARDIUM OCCIDENTALE* FRUITS

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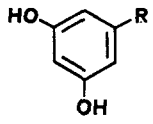
ABSTRACT.—Anacardic acids, 2-methylcardols, and cardols isolated from various parts of the cashew [*Anacardium occidentale*] (Anacardiaceae) fruit have been found to exhibit tyrosinase inhibitory activity. Kinetic studies with the two principal active compounds, 6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid and 5-[8(*Z*),11(*Z*),14-pentadecatrienyl]resorcinol, have indicated that both of these phenolic compounds exhibit characteristic competitive inhibition of the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by mushroom tyrosinase.

Tyrosinase is one of the most important key enzymes in the insect molting process (1) and investigating its inhibitors may be important in finding alternative insect control agents. Tyrosinase inhibitors have also become increasingly important for cosmetic products in relation to hyperpigmentation (2–4). Thus, tyrosinase inhibitors may also control production of the dermal melanin pigment since tyrosinase plays an important role in the process of melanin biosynthesis (5,6). In addition, disturbances in the amount and distribution of melanin formation might ultimately provide clues to systemic diseases (2). These observations led us to search for naturally occurring tyrosinase inhibitors (7). Because of its easy availability, mushroom tyrosinase was used for this study, although mushroom tyrosinase differs somewhat from animal tyrosinase. In our preliminary screening of the mushroom tyrosinase inhibitory activity, a MeOH extract of cashew nut shell, *Anacardium occidentale* L. (Anacardiaceae), was found to show potent inhibitory activity with an ID_{50} of 180 μg/ml.

We have recently reported 16 phenolic compounds [**1–16**] from cashew apple, nut, and nut shell oil with various biological effects such as antimicrobial (8,9), antitumor (10) and molluscicidal activities (11). The same phenolic compounds were examined for inhibitory activity against tyrosinase, since the activity was retained in the organic layer after partitioning of the MeOH extract of the cashew nut shell between EtOAc and H_2O. In addition to the 16 phenolic compounds, several related phenolic compounds, resorcinol, salicylic acid, as well as arbutin, a commercial skin whitening agent, were also tested for comparison purposes.

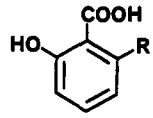
This paper describes the inhibitory activity of these phenolic compounds on mushroom tyrosinase; it also briefly discusses their structure-activity relationships. The emphasis for further study was placed on anacardic acids [**5–8**], especially 6-[8(*Z*),11(*Z*),14-pentadecatrienyl] salicylic acid [**5**], also known as anacardic acid. Compound **1**, 5-[8(*Z*),11(*Z*),14-pentadecatrienyl]resorcinol, otherwise known as cardol, was also studied in detail for comparison. The selection of these compounds was based largely on their availability.

The 16 phenolic compounds isolated from the cashew nut shell oil were first examined for their inhibitory activity to mushroom tyrosinase at a concentration of 0.8 mM. The results are presented in Table 1. Among the compounds tested, cardols [**1–4**], 2-methylcardols [**9–12**], and anacardic acids [**5–8**] exhibited inhibitory activity of this same order. In contrast, cardanols [**13–16**] did not show any activity at this concentration. The cardanols may exhibit some activity if tested at higher concentration, however,



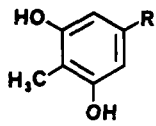
R=

1 $C_{15:3}$, 5-[8(*Z*),11(*Z*),14-pentadecatrienyl]resorcinol
2 $C_{15:2}$, 5-[8(*Z*),11(*Z*)-pentadecadienyl]resorcinol
3 $C_{15:1}$, 5-[8(*Z*)-pentadecenyl]resorcinol
4 $C_{15:0}$, 5-pentadecylresorcinol



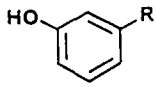
R=

5 $C_{15:3}$, 6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid
6 $C_{15:2}$, 6-[8(*Z*),11(*Z*)-pentadecadienyl]salicylic acid
7 $C_{15:1}$, 6-[8(*Z*)-pentadecenyl]salicylic acid
8 $C_{15:0}$, 6-pentadecylsalicylic acid



R=

9 $C_{15:3}$, 2-methyl-5-[8(*Z*),11(*Z*),14-pentadecatrienyl]resorcinol
10 $C_{15:2}$, 2-methyl-5-[8(*Z*),11(*Z*)-pentadecadienyl]resorcinol
11 $C_{15:1}$, 2-methyl-5-[8(*Z*)-pentadecenyl]resorcinol
12 $C_{15:0}$, 2-methyl-5-pentadecylresorcinol



R=

13 $C_{15:3}$, 3-[8(*Z*),11(*Z*),14-pentadecatrienyl]phenol
14 $C_{15:2}$, 3-[8(*Z*),11(*Z*)-pentadecadienyl]phenol
15 $C_{15:1}$, 3-[8(*Z*)-pentadecenyl]phenol
16 $C_{15:0}$, 3-pentadecylphenol

they are only slightly soluble in the H_2O-based test solution. In addition, it should be noted that 2-methylcardols [**9–12**] showed more potent activity than anacardic acids but they were not studied in detail because of their limited availability.

The above active compounds [**1–8**] showed a dose-dependent inhibitory effect on the mushroom tyrosinase oxidation of L-DOPA. The ID_{50} values of the active compounds [**1–8**] are listed in Table 1. Among the compounds tested, a cardol, 5-[8(*Z*),11(*Z*),14-pentadecatrienyl]resorcinol [**1**] exhibited the most potent tyrosinase inhibitory activity, with an ID_{50} of 0.04 mM, while an anacardic acid, 6-[8(*Z*)-pentadecenyl]salicylic acid [**7**] showed the least potency, with an ID_{50} of 7.5 mM.

The structure-activity relationships can be inferred as follows: The addition of a carboxylic group to the cardanols changes them to the corresponding anacardic acids [**13→5**, **14→6**, **15→7**, **16→8**]; the inactive cardanols change to

TABLE 1. Tyrosinase Inhibitory Activity of Some Phenolic Compounds from *Anacardium occidentale*.

Compound Tested	Inhibition % (0.8 mM)	ID_{50} (mM)
1	88	0.04
2	89	0.05
3	85	0.08
4	21	[a]
5	45	1.1
6	15	6.0
7	15	7.5
8	15	4.2
9	16	[a]
10	20	[a]
11	24	[a]
12	[a]	[a]
Resorcinol	34	1.2
Salicylic acid	23	3.9
Arbutin	[a]	6.2

[a]Not tested.

the active anacardic acids. Similarly, the addition of a hydroxy group to the cardanols alters them to the corresponding cardols [**13**→**1**, **14**→**2**, **15**→**3**, **16**→**4**], thereby dramatically increasing their activity. Thus, the inactive cardanols convert to the potent inhibitory cardols. Also, an additional methyl group on the cardols converts them to the corresponding 2-methylcardols [**1**→**9**, **2**→**10**, **3**→**11**, **4**→**12**] and there is some decrease in inhibitory activity.

All of these 16 natural phenolic compounds isolated from cashew nut shell oil have a C_{15}-alkyl side-chain with 0–3 double bonds. A modification of the number of double bonds in the side-chain of this series of the compounds seems to affect biological activities (11–13). In the case of the tyrosinase inhibitory activity of the anacardic acids, 6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid [**5**] exhibited slightly more activity than the other three congeners [**6**–**8**], which were all comparable to one another. More specifically, the ID_{50} of an anacardic acid, 6-[8(*Z*),11(*Z*),14-pentadecatrienyl]salicylic acid [**5**] was 1.1 mM and that of 6-pentadecylsalicylic acid [**8**] was 4.2 mM. Thus, the ID_{50} of the anacardic acid [**5**] possessing a $C_{15:3}$-unsaturated alkyl side-chain is about 4 times more potent than that of the anacardic acid [**8**] having a $C_{15:0}$-saturated alkyl side-chain. As a result, the cardol and anacardic acid possessing a $C_{15:0}$-saturated alkyl side-chain exhibited the least tyrosinase inhibitory activity.

In addition to determining the ID_{50}s of cardols, 2-methylcardols, and anacardic acids, those of resorcinol and salicylic acid were also established. Thus, by comparing the ID_{50} of salicylic acid with values for the corresponding anacardic acids [**5**–**8**], the addition of a C_{15}-alkyl side-chain does not seem directly related to tyrosinase inhibitory activity. As far as the ID_{50} is concerned, salicylic acid showed nearly the same ID_{50} value as those exhibited by the anacardic acids. However, in the case of the cardols, their tyrosinase inhibitory activity was found to be much more potent than resorcinol. For example, the ID_{50} of 5-[8(*Z*),11(*Z*),14-pentadecatrienyl]resorcinol [**1**] was 0.04 mM and that of resorcinol was 1.2 mM. This cardol [**1**] is therefore about 30 times more potent as a tyrosinase inhibitor compared to the corresponding resorcinol. Thus, the addition of a C_{15}-alkyl side-chain seems to affect tyrosinase activity.

Kinetic studies were also carried out

with the two principal active compounds, anacardic acid [**5**] and cardol [**1**], as well as the related compounds, salicylic acid and resorcinol. The results are shown in Figure 1, indicating that both anacardic acid [**5**] and cardol [**1**] are characteristic competitive inhibitors for the oxidation of L-DOPA by mushroom tyrosinase. These results are consistent with the pre-incubation experiments as shown in Figure 2. Thus, **5** and **1** are direct inhibitors of mushroom tyrosinase since both significantly decreased the enzyme activity, on pre-incubation with the enzyme in the absence of the substrate. On the other hand, salicylic acid was found to be a noncompetitive inhibitor, but resorcinol was neither a competitive nor a noncompetitive inhibitor. Interestingly, the latter phenolic compound exhibited some stimulatory activity to the enzyme at the low concentration. This will be described in detail elsewhere. The data obtained so far suggest that salicylic acid and resorcinol, and their corresponding anacardic acid and cardol possessing a C_{15}-alkyl side-chain, affect mushroom tyrosinase in different ways.

Mushroom tyrosinase is also known as polyphenol oxidase which oxidizes a wide range of substrates (14). Melanin formation is considered to be deleterious

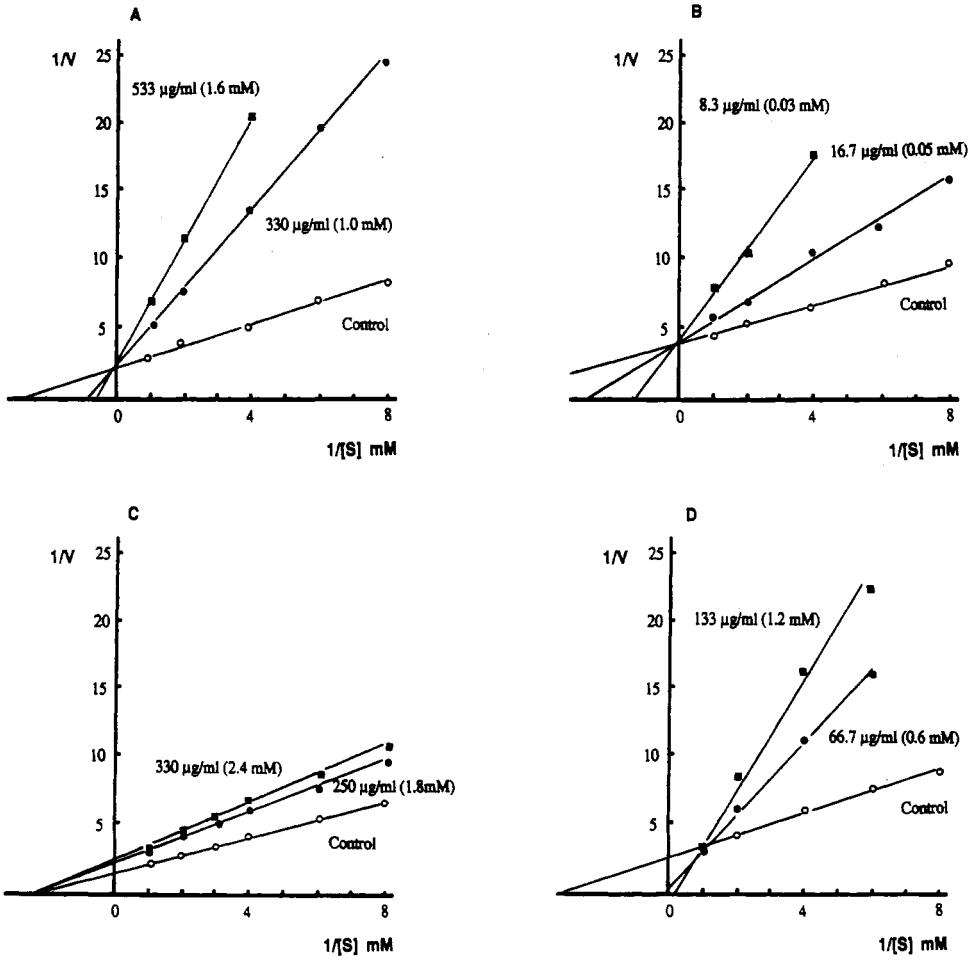


FIGURE 1. Lineweaver-Burk plots of mushroom tyrosinase and L-DOPA in the absence (○) or presence (●,■) of inhibitors (A, anacardic acid [**5**]; B, cardol [**1**]; C, salicylic acid; and D, resorcinol). 1/V: 1/Δ475 nm/min.

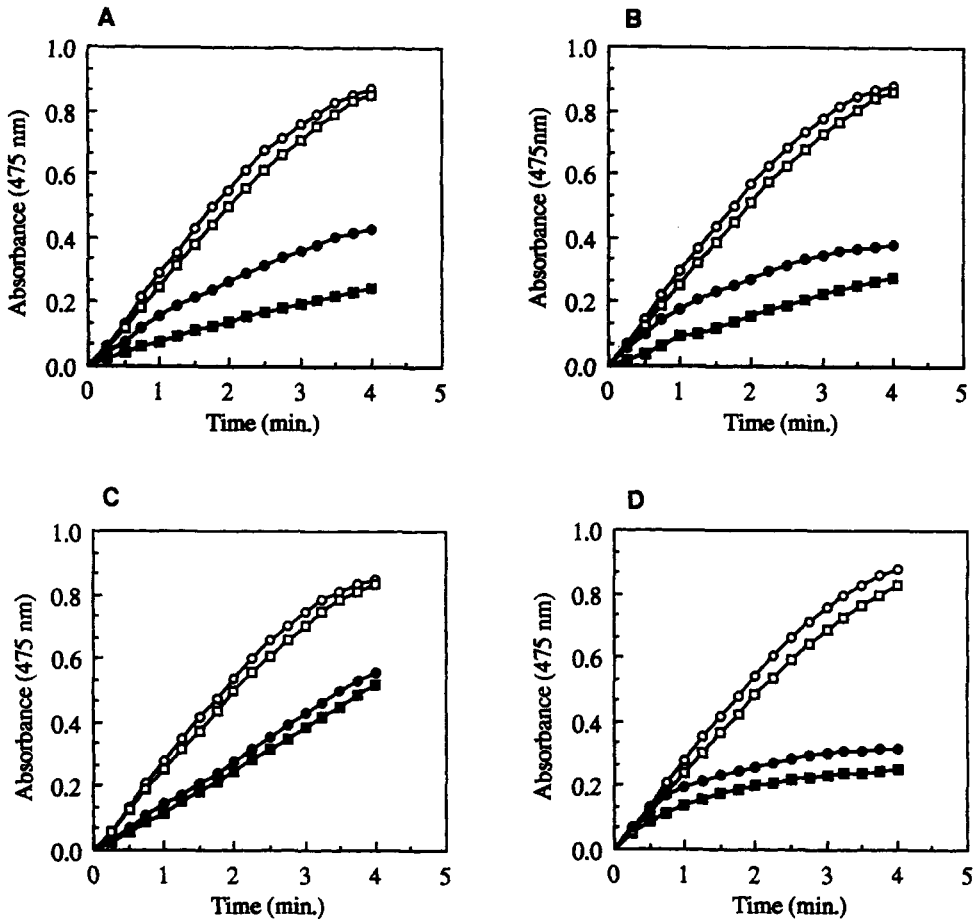


FIGURE 2. Pre-incubation effect of mushroom tyrosinase with inhibitors (A, anacardic acid [**5**]; B, cardol [**1**]; C, salicylic acid; and D, resorcinol). Test (■); pre-incubation of enzyme with inhibitors at 25° for 5 min, then L-DOPA was added. Control A (○); in the absence of inhibitors without preincubation. Control B (□); in the absence of inhibitors with pre-incubation. Control C (●); in the presence of inhibitors without pre-incubation.

to the color quality of plant-derived food. This broadens the possible use of tyrosinase inhibitors as food additives, in addition to insect control agents and whitening agents. In this communication we have focused on the possibility of using tyrosinase inhibitors as whitening agents for cosmetic products.

As mentioned above, cardols exhibited the most potent tyrosinase inhibitory activity. Despite this superior activity, cardols themselves can not be considered for practical application since they have been reported to cause skin irritation (15). However, they may still be useful as lead compounds. For example, arbutin (16), a hydroquinone glucoside, is used in this manner. More specifically, hydroquinone itself is known to be toxic, but its glucoside is not. The tyrosinase inhibitory arbutin is non-toxic and has been used as a commercial skin whitening agent.

The anacardic acids [**5**–**8**] were found to exhibit inhibitory activity against the tyrosinase, in addition to having potent antibacterial activity against several Gram-positive bacteria such as *Staphylococcus aureus* (8) and *Propionibacterium acnes* (17) which cause skin problems. The ID_{50} of **5** is about sixfold that of arbutin (ID_{50}=6.2 mM). The anacardic acids may,

therefore, be considered for practical use, especially in skin-care products. Furthermore, the previous report of prostaglandin synthetase inhibitory activity by an anacardic acid [**8**] (18) is another potentially important finding for skin-care product research.

We have recently reported the cytotoxic activity of anacardic acids [**5–8**] against several carcinoma cells (10). During the course of a screening for cytotoxicity, the anacardic acids were found to exhibit a depigmenting activity of mouse B16 melanoma cells (19) at dose levels causing no cytotoxicity. To use tyrosinase inhibitors for topical application as whitening agents they need to depress tyrosinase activity and intracellular melanin content at concentrations that are not cytotoxic to the cells. Hence, the above observation with the anacardic acids warrants further study.

In contrast to medicines which are used to heal ill people, skin-care products are repeatedly applied to healthy skin, often for long durations, so that safety is a prime consideration. Although cashew nut shells are not edible, the 16 phenolic compounds isolated from this source are also found in the nuts and apples of *A. occidentale* which are often consumed by the public, both in food and in beverages. Therefore, it would appear that their potential for human oral toxicity either is not serious or has been overlooked. However, a study of their safety in skin applications is certainly needed.

EXPERIMENTAL

TEST SUBSTANCES.—The 16 phenolic compounds [**1–16**] used for this study were previously isolated from cashew nut shell oil, *A. occidentale* (11). Repurification of some compounds by hplc (20) was carried out using an ODS C_{18} column. Arbutin was also previously isolated from the endosperm of the fresh fruits of California buckeye, *Aesculus californica* (Spach) Nutt. (Hippocastanaceae) (21). Resorcinol and salicylic acid were purchased from Sigma Chemical Co. (St. Louis, MO). L-DOPA was obtained from Aldrich Chemical Co. (Milwaukee, WI).

ENZYME ASSAYS.—Mushroom tyrosinase (EC 1.14.18.1) used for the bioassays was purchased from Sigma Chemical Co. All samples were first dissolved in DMSO and used for the experiments at 30 times dilution. The assay was performed as previously described (6,22). Thus, 1 ml of 2.5 mM L-DOPA was mixed with 0.1 ml of each sample solution and 1.8 ml of 0.1 M phosphate buffer (pH 6.8), and incubated at 25° for 10 min. Next, 0.1 ml of the aqueous solution of the mushroom tyrosinase (138 units) was added to the mixture to immediately measure the initial rate of linear increase in optical density at 475 nm, based on the formation of dopachrome. The extent of inhibition by the addition of samples is expressed as a percentage necessary for 50% inhibition (ID_{50}).

Pre-incubation mixtures consisted of 1.8 ml of 0.1 M phosphate buffer (pH 6.8), 0.6 ml of H_2O, 0.1 ml of the sample solution (equivalent amount of ID_{50}), and 0.1 ml of the aqueous solution of the mushroom tyrosinase (138 units). The mixture was pre-incubated at 25° for 5 min. Then, 0.4 ml of 6.3 mM L-DOPA was added and the reaction was monitored at 475 nm for 4 min.

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