Trans-3-Phenyl-2-Propenoic Acid (Cinnamic Acid) Derivatives: Structure-Activity Relationship as Hepatoprotective Agents

Eduardo Fernández-Martínez[1], Rosa A. Bobadilla[2,#], Martha S. Morales-Ríos[3], Pablo Muriel[4] and Víctor M. Pérez-Álvarez[4,*]

[1]*Centro de Investigación en Biología de la Reproducción, Universidad Autónoma del Estado de Hidalgo,* [2]*Departamento de Fisiología y Farmacología, Escuela Superior de Medicina del IPN,* [3]*Departamento de Química and* [4]*Sección Externa de Farmacología, Cinvestav-IPN, México*

Abstract: Among various phenolic compounds, caffeic acid (3,4-dihydroxycinnamic acid) exhibited pharmacological antioxidant, anticancer and antimutagenic activities. The antioxidant properties of phenolic compounds depend on their chemical structure, however, the role of the ethylenic side chain in the radical scavenging activity remains controversial. Thus, the aim of this study consisted to test cinnamic acid and 15 cinnamic acid derivatives in the well known CCl_4-induced acute liver damage model, which is dependent on oxidative stress mechanisms. Cinnamic acid and 15 cinnamic acid derivatives (50 mg/kg, p.o.) were administered to male Wistar rats intoxicated with CCl_4 (4 g/kg, p.o.). The activities of gamma-glutamyl transpeptidase, alkaline phosphatase and alanine aminotransferase were measured in serum. The lipid peroxidation products were determined in liver. Compounds with a methoxy group at position 3 or 4, or a 3,4-methylenedioxy moiety were the most active ones. Also, we observed that the monosubstituted 3 or 4 hydroxy, or the bulky 3,4 dibenzyloxy substituted compounds showed lower activity. The poorest activity was displayed by disubstituted 3,4-dihydroxy, dimethoxy or diacetyl cinnamic acid derivatives, the ester derived from cinnamic acid with an 8 carbon chain and *N*-dimethyl substituted compound. Thus, the methoxy substituted group at positions 3 or 4 or the 3,4-methylenedioxy moiety in the caffeic acid derivatives; seem to be the main features required for the hepatoprotection in this model.

Key Words: Carbon tetrachloride, cinnamic acid derivatives, liver injury, leukotrienes, free radicals, antioxidants.

INTRODUCTION

We have previously reported that acute liver damage induced by CCl_4 can be partially prevented by 3,4-dihydroxycinnamic acid, commonly known as caffeic acid [1]. We have also shown that this compound ameliorates liver fibrosis in experimentally induced cirrhosis in the rat [2]. Caffeic acid is known to be a potent antioxidant [3], probably due in part to its radical scavenging activity, although other mechanisms may contribute to its hepatoprotective properties [4]. The antiradical activity of phenolic compounds depends on their molecular structure, specifically on the availability of phenolic hydrogens and the possibility of the stabilization of the resulting phenoxyl radicals formed by hydrogen donation [5]. In fact, preliminary structure-activity relationship studies on cinnamic acid and derivatives have pointed out the importance of the catechol group in the antiradical efficacy [6,7]. In one study carried out by our group, we showed the importance of the 4-hydroxy group in producing the antioxidant and hepatoprotective activities of 4-hydroxycinnamic acid [8].

Nevertheless, the role of the ethylenic side chain of this type of phenolic compounds in the radical scavenging properties remains controversial. Some studies suggest that this structural feature is important for such scavenging activity due to of its participation in the stabilization, through resonance, of the phenoxy radical formed during the process. However, other studies claim [6,7] that the conjugated double bond is not a requirement for antiradical efficacy.

Therefore, the objective of the present work was to prepare a series of 14 cinnamic acid derivatives in order to determine the relative importance of the substituents in preventing experimental CCl_4-induced liver damage as evaluated through defined markers.

MATERIALS AND METHODS

Chemistry

The chemical structures of the compounds utilized in this study are shown in Table **1** and were already described. Compounds **1** and **2** (cinnamic and caffeic acids, respectively) were obtained from Sigma-Aldrich (St. Louis, MO), 3-hydroxycinnamic acid (**3**), 4-hydroxycinnamic acid (**4**), 3-methoxycinnamic acid (**5**), 4-methoxycinnamic acid (**6**), 3,4-dimethoxycinnamic acid (**7**), 3,4-diacetylcinnamic acid (**8**), 3,4-dibenzyloxycinnamic acid (**9**), 3,4-methylenedioxicinnamic acid (**10**) and 4-(*N*,*N*-dimethylamino)cinnamic acid (**16**) were obtained by Knoevenagel condensation of the corresponding benzaldehyde with malonic acid in anhydrous pyridine, catalyzed by traces of piperidine, under stirring at reflux for 3-6 h (see Fig. (**1**)). For derivatives with low water solubility (**5-10**), precipitation was induced by treating the reaction mixture with concentrated HCl, and separating the solid through filtration. For the remaining compounds, the

*Address correspondence to this author at the Sección Externa de Farmacología, Cinvestav-IPN, Apartado Postal 14-740, Mexico 07360 D.F., Mexico; Tel: (52) 50-61-3800. Ext 5422; Fax: (52) 50-61-3394;
E-mail: vperez@cinvestav.mx

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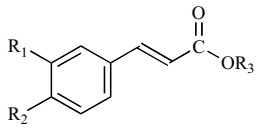
Table 1. Chemical Structures of Compounds Investigated



Compound	R_1	R_2	R_3
1	H	H	H
2	HO	HO	H
3	HO	H	H
4	H	HO	H
5	CH_3O	H	H
6	H	CH_3O	H
7	CH_3O	CH_3O	H
8	AcO	AcO	H
9	BnO	BnO	H
10	$-OCH_2-$	O	H
11	HO	HO	CH_3
12	HO	HO	C_2H_5
13	HO	HO	*n*-C_4H_9
14	HO	HO	*n*-C_6H_{13}
15	HO	HO	*n*-C_8H_{17}
16	H	$(CH_3)_2N$	H

reaction mixture was vacuum evaporated and the product purified by silica gel column chromatography.

Derivatization of caffeic acid (**2**) to the corresponding methyl-, ethyl-, butyl-, hexyl- and octyl esters **11-15** was accomplished by dissolving **2** in the corresponding alcohol with bubbled HCl gas for 1-3 h at reflux (see Fig (**2**)).

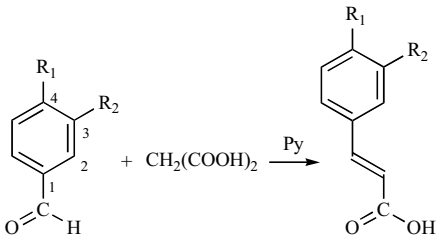


Fig. (1). General procedures for the preparation of cinnamic acid derivatives **3-10** and **16**.

Then, the solution was vacuum evaporated and the esters were purified by silica gel column chromatography.

Identity of compounds **3-16** was established by ^1H and ^{13}C NMR on Varian Mercury spectrometers working at 300 and 75 MHz, respectively.

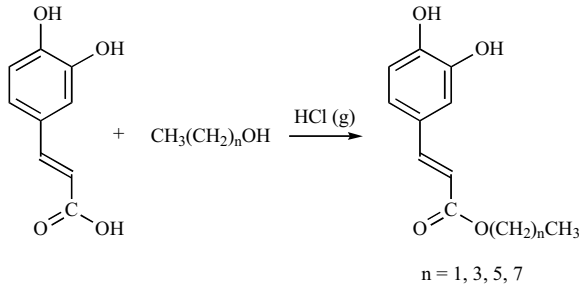


Fig. (2). General procedures for the preparation of cinnamic acid derivatives **11- 15**.

Treatments of Animals

All animals received human care and the study complies with the institution's guidelines and the Mexican official regulation (NOM-062-ZOO-1999) regarding technical specifications for production, care and use of laboratory animals.

In this study, 144 male Wistar rats weighing between 200 and 250 g were used. Animals were freely fed with Purina Chow diet. Rats were divided into 18 groups, 8 animals each one. Group 1 received a single dose of olive oil and the remaining groups received 4 g/100 g CCl_4 dissolved in olive oil (1:1 volume/volume). While group 2 received no addi-

tional treatment, groups 3-18 received cinnamic acid or its derivatives suspended in carboxymethylcellulose 0.7% (3 X 50 mg/kg, p.o.), 12 h and 2 h before and 12 h after the CCl_4 administration. All treatments were given by means of an intragastric tube and 1 ml of total volume of freshly prepared suspension was administered per animal. Experimental groups 3-18 received compounds **1-16,** respectively. Rats were anesthetized with diethyl ether 24 h after CCl_4 administration. Blood was obtained by heart puncture, and the liver was rapidly excised.

Biochemical Determinations

Serum was used for determination of the activities of gamma-glutamyl transpeptidase (γ-GTP, [9]), alkaline phosphatase (ALP, [10]), and alanine aminotransferase (ALT, [11]). The extent of lipid peroxidation was estimated in liver homogenates by using the thiobarbituric acid method to measure the formation of malondialdehyde (MDA, [12]). Protein determinations were performed by using serum bovine albumin as the standard, according to the method described by Bradford, [13].

Statistical Analysis

Data are expressed as mean ± SEM and were compared by the use of one way ANOVA, followed by the Student-Newman-Keuls test to detect statistical differences. Statistical significance was considered when $p < 0.05$.

RESULTS AND DISCUSSION

Protection Index

In order to evaluate the potency of the experimental compounds we defined a "protection index", which is the addition of the values of the protection levels produced by the experimental compounds on each of the plasmatic and liver damage markers (Table **2**). Complete protection (CP) = 2, partial protection (PP) = 1, no protection (NP) = 0, increased damage (ID) = -1.

The protection index ranking from the experimental compounds was as follows (Table **3**): 4-methoxycinnamic acid (**6**) = 3,4-methylenedioxicinnamic acid (**10**) > 3-methoxycinnamic acid (**5**) > 4-hydroxycinnamic acid (**4**) > cinnamic acid (**1**) = 3,4-dibenzyloxycinnamic acid (**9**) > 3-hydroxycinnamic acid (**3**) = 3,4-dihydroxycinnamic acid

Table 2. Effects of Experimental Compounds on the Plasmatic Enzyme Activities of Alkaline Phosphatase (ALP), Gamma-Glutamyl Transpeptidase (γ-GTP) and Alanine Aminotransferase (ALT) Determined in Rats Intoxicated with CCl_4. [a]different *vs* Control ($p < 0.05$), [b]different *vs* CCl_4 ($p < 0.05$)

Treatment	ALP µmol/l min	γ-GTP µmol/l min	ALT µmol/l min
Control	107.21 ± 7.36	8.67 ± 0.24	33.53 ± 1.23
CCl_4	161.03 ± 12.51 [a]	25.32 ± 1.27 [a]	64.19 ± 1.36 [a]
CCl_4 + Compound			
1	88.12 ± 4.05 [b]	18.66 ± 3.66	105.88 ± 10.58 [a,b]
2	103.15 ± 13.05 [b]	16.16 ± 2.47	107.49 ± 17.71 [a,b]
3	144.01 ± 18.12	12.66 ± 3.23	95.29 ± 8.23 [a,b]
4	148.11 ± 18.13	10.66 ± 1.66	56.47 ± 9.41
5	90.04 ± 2.84 [b]	13.26 ± 9.08	40.56 ± 0.85
6	150.33 ± 9.71	5.86 ± 2.14 [b]	14.12 ± 1.00 [b]
7	156.41 ± 10.89 [a]	15.06 ± 7.62	39.00 ± 1.17 [b]
8	153.97 ± 7.26 [a]	19.96 ± 2.99	22.78 ± 0.28 [b]
9	117.06 ± 9.86 [b]	24.59 ± 2.14 [a]	85.92 ± 3.36 [a]
10	105.86 ± 3.4 [b]	19.89 ± 0.802	26.83 ± 0.835 [b]
11	233.83 ± 11.73 [a,b]	29.37 ± 3.54 [a]	157.15 ± 2.21 [a,b]
12	164.3 ± 10.65 [a]	20.98 ± 3.35	77.74 ± 0.73 [a]
13	203.79 ± 9.06 [a]	25.4 ± 3.29 [a]	152.77 ± 1.79 [a,b]
14	142.74 ± 8.26	6.23 ± 0.91 [b]	55.24 ± 1.80
15	162.47 ± 6.28 [a]	21.10 ± 1.69	69.75 ± 0.76 [a]
16	154.16 ± 12.35 [a]	6.7 ± 0.54 [b]	48.64 ± 4.80

Table 3. Protection Index (Defined in Materials and Methods Section) on the Plasmatic Enzyme Activities of Alkaline Phosphatase (ALP), Gamma-Glutamyl Transpeptidase (γ-GTP) and Alanine Aminotransferase (ALT) Determined in Rats Intoxicated with CCl_4. [a]Different vs Control ($p< 0.05$), [B]different vs CCl_4 ($p<0.05$)

Treatment	ALP µmol/l min	γ-GTP µmol/l min	ALT µmol/l min	Lipid peroxidation nmol MDA/mg protein	Protection index
CCl_4 + Compound					
1	CP	PP	ID	PC	4
2	CP	PP	ID	NP	2
3	PP	PP	ID	CP	3
4	PP	PP	PP	CP	5
5	CP	PP	PP	CP	6
6	PP	CP	CP	CP	7
7	NP	PP	CP	ID	2
8	NP	PP	CP	ID	2
9	CP	NP	NP	CP	4
10	CP	PP	CP	CP	7
11	ID	ID	ID	CP	- 1
12	ID	ID	ID	PP	- 2
13	ID	NP	ID	ID	- 3
14	PP	CP	PP	ID	3
15	NP	PP	ID	CP	2
16	NP	CP	PP	ID	2

hexyl ester (**14**) > caffeic acid (**2**) = 3,4-dimethoxycinnamic acid (**7**) = 3,4-diacetylcinnamic acid (**8**) = 3,4-dihydroxycinnamic acid octyl ester (**15**) = 4-(*N*,*N*-dimethylamino)cinnamic acid (**16**).

On the other hand, the caffeic acid methyl- (**11**), ethyl- (**12**) and butyl- (**13**) ester derivatives significantly increased liver injury instead of protecting against it.

Structure-Activity Relationship

By analyzing a structure-activity relationship in rat liver damage prevention, it was found that compounds with the methoxy substituted group at positions 3 or 4 (**5** and **6**), or the 3,4-methylenedioxy (**10**) were the most active ones. Also, we observed that monosubstituted hydroxy compounds at positions 3 or 4, (**3** or **4**) or disubstituted with a bulky benzyloxy group (**9**) showed a lower but still significant activity, the hexyl ester derivative (**14**) showed low efficacy, the 3,4-disubstituted dihydroxy (**2**), dimethoxy (**7**) or diacetyl cinnamic (**8**) acid derivatives, the ester derived from cinnamic acid with an 8 carbon chain (**15**) and the compound with an *N*-dimethyl group (**16**), displayed the poorest activity.

Thus, methoxy substitution at positions 3 or 4 or 3,4-methylenedioxy moiety in the cinnamic acid derivatives, seem to be the main features required for the hepatoprotection in this model.

This finding is in agreement with the group of Lee, [14], who described a significant hepatoprotective activity for 4-methoxycinnamic acid in primary cultures of rat hepatocytes. Also, BHA (butylated hydroxyanisol) a powerful antioxidant agent, has shown protective activity against liver damage [15]. In the same way, all compounds in this work with hepatoprotective activity are monosubstituted derivatives with a methoxy or hydroxy group, while little or no activity was shown by disubstituted compounds, except for 3,4-methylenedioxycinnamic acid (**10**) and sterically impeded dibenzyloxycinnamic acid (**9**).

On the other hand, the protection against damage produced by the tested compounds on hepatic lipid peroxidation (Fig. (**3**)) can be attributed, at least partially, to their ability to form phenoxy free radicals stabilized by resonance, as has been suggested in previous reports [16].

The hypothesis of an antioxidant effect as a protective mechanism is further supported by the fact that it is known that CCl_4 liver damage is induced through its metabolite, the trichloromethyl radical. Nevertheless, the free radical scavenger effect can not completely explain the pharmacological effect observed, since compounds able to act as good antioxidants, due to the fact that their phenol structure can form phenoxy derivatives stabilized by resonance, did not display a more evident effect. The latter suggests that other mechanisms are involved in hepatoprotection, such as 5-lipoxygenase inhibition, leading to a decreased leukotriene formation, as has been previously suggested [1].

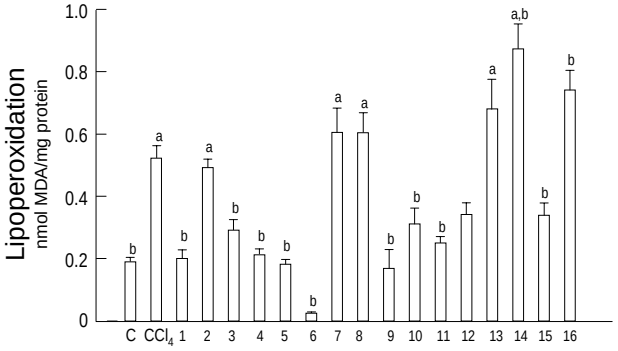


Fig. (3). Effects of experimental compounds on the lipid peroxidation level in CCl_4- challenged rat liver. [a]different *vs*. control ($p< 0.05$), [b]different *vs*. CCl_4 ($p<0.05$). MDA (malondialdehyde).

Caffeic acid (**2**), a compound widely present in the plant kingdom, has demonstrated a spectrum of pharmacological activities, including anti-inflammatory [17,18], anti-allergic [19], anti-tumor [20,21], anti-mutagenesis [22], cardio-protective [23], anti-hyperglycemic [3] and immunomodulatory [24] activities. These pharmacological properties may be associated to its antioxidant, anti NF-κB and anti 5-lipoxygenase activities [3, 4, 7, 15]. Therefore it would be very interesting to study the activity of the present compounds on NF-κB activation and 5-lipoxygenase activity (in addition to its antioxidant properties studied herein on liver damage) as pharmacological mechanisms in various disease processes.

CONCLUSIONS

In summary, the present work establishes a structure-activity relationship of a series of cinnamic acid derivatives using an acute rat liver damage model in complete animals. Evaluating the pharmacological effects of these derivatives in a rat experimental model gives the relative potency of each of these compounds. The most active derivatives of cinnamic acid were 3 and 4 methoxy substituted compounds **5** and **6** and 3,4-methylenedioxy substituted compound **10**. Interestingly, we also found that caffeic acid methyl- (**11**), ethyl- (**12**) and butyl (**13**) ester derivatives increased liver damage induced by CCl_4 intoxication. We can speculate pro-injure activity can be related to the alcohol metabolites that result from their biotransformation. We found that there was no direct relationship between the ability of a given compound to prevent lipid peroxidation production by CCl_4 and its ability to prevent liver damage; indicating that, other mechanisms are probably involved (Fig. (**3**)). Possible pharmacological targets include anti NF-κB and anti 5-lipoxygenase activities [3, 4, 7, 15]; that should be analyzed in future studies.

On the other hand, the present findings indicate that the most promising cinnamic acid derivatives **5**, **6** and **10** should be tested on other hepatic diseases, most importantly on experimental cirrhosis.

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