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Anti-inflammatory Activity in Rats and Mice of Phenolic Acids Isolated from *Scrophularia frutescens*

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Abstract

Different species of the Scrophularia genus (Scrophulariaceae) have been reported to have bacteriostatic and anti-inflammatory properties. In previous studies the anti-inflammatory and antibacterial activity of different extracts from *Scrophularia frutescens* were investigated and *p*-coumaric, caffeic, ferulic gentisic, protocatechuic, syringic and isovanillic acids were isolated and identified. In this work the anti-inflammatory activity of these compounds, administered orally, has been studied against carrageenan-induced rat paw oedema and, administered topically, against tetradecanoylphorbol acetate (TPA)-induced mouse ear oedema. The compounds' myeloperoxidase activity in inflamed ear was also investigated.

Some of the phenolic acids were remarkably active in the TPA test (protocatechuic 71·59% inhibition, $P < 0.001$; syringic 74·43%, $P < 0.001$; ferulic 71·02% $P < 0.001$) and all significantly inhibited mouse ear oedema. They were only moderately active, or were without activity, in the carrageenan test.

These results imply that the phenolic acids assayed are more effective topically than as oral anti-inflammatory agents and that their action is markedly influenced by the inhibition of neutrophil migration into inflamed tissue. This study has also enabled us to make some observations on the possible relationship between the chemical structure and anti-inflammatory activity of the compounds assayed.

Phenolic acids are derived from benzoic or cinnamic acids depending on the position of the carboxylic group on the benzene ring. They are natural constituents of many plant families. Different species of the Scrophularia genus (Scrophulariaceae) have been traditionally used for several skin inflammatory ailments, e.g. scrofulas and different types of dermatosis (including scabies, tumours and slough) (Font-Quer 1990). Some of these species are considered by different authors to have bacteriostatic (Kolodynska & Wieniaski 1966; Swiatek & Krazaczek 1976) and anti-inflammatory (Vigneau 1985) properties. We have previously performed a phytochemical and a pharmacological study of *Scrophularia frutescens*, identified several phenolic acids and reported their antibacterial activity (Fernández et al 1996).

The aim of this study was to determine the possible inhibitory effect of the *p*-coumaric, caffeic and ferulic acids (cinnamic acid derivatives) and gentisic, protocatechuic, syringic and isovanillic acids (benzoic acid derivatives), isolated from *S. frutescens*, on two different models of acute inflammation—carrageenan-induced rat-paw oedema and 12-*O*-tetradecanoylphorbol acetate (TPA)-induced mouse ear oedema. To determine the possible influence of these compounds on leukocyte migration, myeloperoxidase activity was also measured. With the results obtained we attempted to establish some structure–activity relationships.

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Materials and Methods

Compounds tested

p-Coumaric, caffeic, ferulic, gentisic, protocatechuic, isovanillic and syringic acids were isolated from an aqueous extract of *S. frutescens* (Fernández et al 1996). Indomethacin, 12-*O*-tetradecanoylphorbol acetate (TPA), carrageenan, acetylsalicylic acid, tetramethylbenzidine and

hexadecyltrimethylammonium bromide were purchased from Sigma.

Animals

Experiments were performed on male Swiss albino mice, 20–25 g, and male Wistar rats, 200–250 g. All animals (groups of six) were kept under controlled conditions during the experiments.

Carrageenan-induced rat paw oedema (Winter et al 1962)

Phenolic acids or vehicle (isotonic NaCl solution, 0·9%) were administered orally at doses of 200 mg kg^{-1}, 1 h before carrageenan injection. The volumes of the paws were measured by use of a plethysmometer (Letica LI-7500) before administering the inflammatory agent and 3 and 5 h after induction of inflammation; the oedema was expressed as the increase in paw volume as a result of carrageenan administration. Indomethacin (25 mg kg^{-1}) and acetylsalicylic acid (200 mg kg^{-1}) were used as standard drugs.

TPA-induced mouse ear oedema (De Young et al 1989)

Phenolic compounds dissolved in 70% aqueous ethanol were applied topically (0·5 mg/ear, 20 μL) after application of 2·5 μg TPA to the right ear. The left ear received the vehicle only. The control group received the phlogistic agent TPA. Inflammation was allowed to develop for 4 h after which the animals were killed by cervical dislocation and a section through the central portion of both ears (6 mm) was obtained and weighed. The oedema induced by TPA was expressed in terms of the increase in weight of the punch biopsies of the right ear compared with those of the left ear and the percentage inhibition of the oedema was also calculated.

Myeloperoxidase assay (Suzuki et al 1983)

Myeloperoxidase activity was determined by use of a modification of the method of Suzuki et al (1983). The tissue (ear punch) was homogenized for 45 s at 0°C, by means of a Polytron PT 1200, in sodium phosphate buffer (pH 5·4, 80 mM; 0·75 mL) containing hexadecyltrimethylammonium bromide (0·5%). The homogenate was centrifuged at 1200 *g* and 4°C for 15 min. For the assays supernatant (200 μL) was mixed with sodium phosphate buffer (pH 5·4, 80 mM), hexadecyltrimethylammonium bromide (0·5% w/v) and tetramethylbenzidine (1·6 mM added as 18·6 mM stock solution dissolved in *N,N'*-dimethylformamide). The mixture was then warmed to 37°C and the reaction started by addition of H_2O_2 (0·026%, 80 μL). Each tube containing the complete reaction mixture was incubated for exactly 3 min at 37°C and the reaction was then terminated by addition of 1·46 M sodium acetate (pH 3·0) and placed on ice. The absorbance of each tube was determined at 620 nm and corrected by subtracting the blank value. We define 1 unit of activity as the increase of absorbance min^{-1} in the final reaction volume containing 3 mL. The absorbance value for the control-untreated group (which received only TPA) was assigned as 100% activity. Percentage inhibition was calculated by comparison of results from drug-treated and control-untreated groups.

Statistics

Results are expressed as means ± s.e. Student's *t*-test was used for statistical evaluation (n = 6).

Results

The effects of the phenolic acids on carrageenan-induced paw oedema and TPA-induced ear oedema, after oral and topical administration, are summarized in Tables 1 and 2, respectively.

Table 1. Oral anti-inflammatory activity of the phenolic acids.

Compounds	Dose (mg kg^{-1})	Oedema volume (mL)		Inhibition (%)	
		3 h	5 h	3 h	5 h
Control	–	0·90 ± 0·03	0·83 ± 0·05	0	0
4-Hydroxycinnamic acid (*p*-coumaric acid)	200	0·79 ± 0·09	0·63 ± 0·07	12·2	23·9
3,4-Dihydroxycinnamic acid (caffeic acid)	200	0·96 ± 0·03	1·01 ± 0·05	0	0
4-Hydroxy-3-methoxycinnamic acid (ferulic acid)	200	0·66 ± 0·06	0·64 ± 0·05	26·6	22·2
2,5-Dihydroxybenzoic acid (gentisic acid)	200	0·62 ± 0·02†	0·61 ± 0·03	31·1	26·5
3,4-Dihydroxybenzoic acid (protocatechuic acid)	200	1·02 ± 0·01	0·79 ± 0·07	0	4·8
4-Hydroxy-3,4-dimethoxybenzoic acid (syringic acid)	200	0·53 ± 0·08†	0·56 ± 0·01	41·1	32·5
3-Hydroxy-4-methoxybenzoic acid (isovanillic acid)	200	0·65 ± 0·06	0·75 ± 0·09	27·7	9·6
Acetylsalicylic acid	200	0·47 ± 0·05†	0·46 ± 0·07	47·7	44·5
Indomethacin	25	0·21 ± 0·02*	0·34 ± 0·03	76·6	58·9

* $P < 0·05$, † $P < 0·001$, significantly different from control result.

Table 2. Topical anti-inflammatory activity of the phenolic acids.

Compounds	Dose ($mg\,kg^{-1}$)	Oedema weight (mg)	Inhibition (%)
Control	–	17·6 ± 0·6	0
4-Hydroxycinnamic acid (*p*-coumaric acid)	0·5	7·5 ± 0·5†	57·38
3,4-Dihydroxycinnamic acid (caffeic acid)	0·5	8·5 ± 0·4†	51·71
4-Hydroxy-3-methoxycinnamic acid (ferulic acid)	0·5	5·1 ± 0·8‡	71·02
2,5-Dihydroxybenzoic acid (gentisic acid)	0·5	7·4 ± 0·5†	57·95
3,4-Dihydroxybenzoic acid (protocatechuic acid)	0·5	5·0 ± 0·9‡	71·59
4-Hydroxy-3,4-dimethoxybenzoic acid (syringic acid)	0·5	4·5 ± 0·9‡	74·43
3-Hydroxy-4-methoxybenzoic acid (isovanillic acid)	0·5	11·5 ± 0·3*	34·65
Indomethacin	0·5	3·2 ± 0·1‡	81·82

‡ $P < 0.001$, † $P < 0.01$, * $P < 0.05$, significantly different from control result.

Effect of phenolic acids on carrageenan-induced rat paw oedema

The results indicate that most of the phenolic acids, at a dose of $200\,mg\,kg^{-1}$, have a moderate anti-inflammatory effect on carrageenan-induced rat paw oedema. Syringic acid has the highest anti-inflammatory activity 3 h after the injection of the phlogistic agents; its percentage inhibition (41·11%) was close to that of the same dose of acetylsalicylic acid (47·77%), a standard drug with similar structural features (both are derived from benzoic acid). Caffeic and protocatechuic acids had no anti-inflammatory effect and the other compounds did not have notable protective effects in this test.

Effect of phenolic acids on TPA-induced mouse ear oedema

Syringic and protocatechuic acids at a dose of 0·5 mg/ear afforded maximum inhibition of the TPA-induced ear oedema; the inhibition, 74·43% and 71·59%, respectively, was similar to that of indomethacin. The other compounds had a good inhibitory effect against the oedema.

Effect on myeloperoxidase-activity

All the compounds tested affected the migration of polymorphonuclear leukocytes to the inflamed tissue because all significantly inhibited myeloperoxidase activity (Table 3). For some of the compounds (e.g. protocatechuic, ferulic and gentisic acids) inhibition of the enzyme was similar to that exerted by indomethacin.

Discussion

All the compounds tested were active when assayed topically (ear oedema test). When administered orally (carrageenan oedema test) the compounds were only moderately effective or had no effect. The irritant effect of carrageenan is a result of activation of the kinin and complement cascades, and consequently of the release of inflammatory mediators such as vasoactive amines, eicosanoids, etc. (Lewis et al 1985). A positive response in the TPA test implies that inhibition of the oedema could be a result of blockage of protein kinase C. TPA stimulates the actions of protein kinase C in a manner similar to that of endogenous diacylglycerol, liberated from membrane phospholipids. Because subsequent activation of protein kinase C produces exocytosis, modulation of ion conductance, smooth-muscle contraction and

Table 3. Effect of the phenolic acids on myeloperoxidase activity.

Compound	Absorbance increase	Amount of myeloperoxidase (munits)	Inhibition of myeloperoxidase activity (%)
Control	0·751 ± 0·03	52·0	0
4-Hydroxycinnamic acid (*p*-coumaric acid)	0·085 ± 0·02‡	5·9	88·68
3,4-Dihydroxycinnamic acid (caffeic acid)	0·380 ± 0·07*	26·0	49·39
4-Hydroxy-3-methoxycinnamic acid (ferulic acid)	0·265 ± 0·05†	18·0	64·71
2,5-Dihydroxybenzoic acid (gentisic acid)	0·097 ± 0·01‡	6·7	87·08
3,4-Dihydroxybenzoic acid (protocatechuic acid)	0·098 ± 0·01‡	6·8	86·95
4-Hydroxy-3,4-dimethoxybenzoic acid (syringic acid)	0·193 ± 0·03†	13·4	74·31
3-Hydroxy-4-methoxybenzoic acid (isovanillic acid)	0·352 ± 0·06*	24·4	53·13
Indomethacin	0·017 ± 0·09‡	1·1	97·74

‡ $P < 0.001$, † $P < 0.01$, * $P < 0.05$, significantly different from control result.

cell proliferation (Nishizuka 1988), one explanation of the difference between the results could be the different oedematogenic mechanism of the phlogistic agents (Giner et al 1991; Lanhers et al 1992). Topical administration at the site of the inflammation is usually the most effective route because a much higher concentration of the drug can be attained (Carlson et al 1989), thus syringic acid is nearly twice as active when administered topically and protocatechuic acid is only active topically.

Myeloperoxidase activity was also strongly inhibited by most of the compounds, indicating that this kind of compound has a marked influence on the cellular response to the inflammation, mainly that of polymorphonuclear leukocytes. Even some of the compounds with moderate anti-inflammatory activity (e.g. *p*-coumaric and gentisic acids) elicited a substantial reduction in the activity of the enzyme.

Apparently compounds belonging to the benzene group have greater activity against the TPA-induced oedema than those of the cinnamic group, because protocatechuic acid (benzoic acid derivative) inhibits oedema by 71·59% compared with 51·71% for caffeic acid (cinnamic group) and they differ only in the position of the acid group. However, this feature does not seem to influence the effect against the carrageenan-induced oedema, which is similar for both compounds. Another structural feature that increases topical activity is the presence in the benzene ring of methoxy groups at C-3 or C-5, or both. Thus syringic (74·43%) and ferulic acids (71·02%) were the most active compounds in their respective groups. Positioning the methoxy group at C-4 has the opposite effect—isovanillic acid, the only compound with this substitution that was tested, had the lowest activity (34·65%). This result is not maintained in the carrageenan test, for which caffeic acid was least active (0%).

In summary, these results indicate that phenolic compounds are moderate systemic anti-inflammatory agents but have a strong anti-inflammatory effect when applied locally at the site of inflammation. In the topical model the best anti-inflammatory activity might be afforded by compounds related to benzoic acid derivatives, compounds with methoxy-group substitution at C-3 or C-5, or both.

It seems that the phenolic acids that are the active principles of some orally administered medicinal plants might merely act synergistically with other active substances, for example, harpagoside, isolated from *S. frutescens* (García et al 1996), might be another bioactive compound involved in its action. After topical administration the compounds, especially syringic, protocatechuic and ferulic acids, had good anti-inflammatory activity; their effect on leukocyte migration to the inflamed site might be an important aspect of their mechanism of action. The anti-inflammatory effects of these natural products might also be because of their behaviour as free-radical scavengers (Kroes et al 1992). These results are in accord with the popular uses of different species of the Scrophularia genus, traditionally used in skin troubles and imply that some of the phenolic acids assayed might offer promise for the therapy of inflammatory skin conditions.

References

Carlson, R. P., O'Neil-Davis, L., Calhoun, W., Datko, L., Musser, J. H., Kreft, A. F., Chang, J. Y. (1989) 5-LO Cyclooxygenase CO inhibitor, WY-47,288 on cutaneous models of inflammation. Agents Actions 26: 319–328

De Young, L. M., Kheifets, J. B., Ballaron, S. J., Young, J. M. (1989) Oedema and cell infiltration in the phorbol ester-treated mouse ear are temporally separated and can be differentially modulated by pharmacologic agents. Agents Actions 26: 335–341

Fernández, M. A., García, M. D., Sáenz, M. T. (1996) Antibacterial activity of the phenolic acids fractions of *Scrophularia frutescens* and *Scrophularia sambucifolia*. J. Ethnopharmacol. 53: 11–14

Font-Quer, P. (1990) Plantas Medicinales, 2nd edn. Labor, Spain

García, M. D., Fernández, M. A., Sáenz, M. T., Ahumada, M. C. (1996) Anti-inflammatory effects of different extracts and harpagoside from *Scrophularia frutescens*. Il Farmaco 51: 443–446

Giner, R. M., Sanz, M. J., Ferrandiz, M. L., Recio, M. C., Terencio, M. C., Rios, J. L. (1991) Topical anti-inflammatory activity of some iridoids and phenylpropanoids. Planta Med. 57 (Suppl. 2): A53

Kolodynska, Z., Wieniaski, W. (1966) Synthesis of hydroxycinnamides I. Synthesis of synapamide and caffeic amides. Diss. Pharm. Pharmacol. 18: 54–61

Kroes, B. H., Van den Berg, A. J. J., Quarles van Hufford, H. C., van Dijk, H., Labadie, R. P. (1992) Anti-inflammatory activity of gallic acid. Planta Med. 58: 499–503

Lanhers, M. C., Fleurentin, J., Mortier, F., Vinche, A., Younos, C. (1992) Anti-inflammatory and analgesic effects of an aqueous extract of *Harpagophytum procumbens*. Planta Med. 58: 117–123

Lewis, A. J., Carlson, R. P., Chang, J. (1985) Experimental models of inflammation. In: Bonta, I., Bray, M. A., Parnham, M. (eds) The Pharmacology of Inflammation. Elsevier, Amsterdam, pp 371–474

Nishizuka, Y. (1988) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308: 693–698

Suzuki, K., Otah, H., Sasagawa, T. S., Fujikura, T. (1983) Assay methods for myeloperoxidase in human polymorphonuclear leukocytes. Anal. Biochem. 132: 345–351

Swiatek, L., Krazaczek, T. (1976) Chemical composition of *Scrophularia vernalis*. Acta Pol. Pharm. 33: 653–658

Vigneau, C. (1985) Plantes Medicinales, Masson, Paris

Winter, C. A., Risley, E. A., Nuss, G. W. (1962) Carrageenan-induced oedema in hind paw of the rats as an assay for anti-inflammatory drugs. Proc. Soc. Exp. Biol. Med. 111: 544–547