Chemistry and Pharmacology of Collinin, Active Principle of *Zanthoxylum* spp.

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Abstract: Collinin is a geranyloxycoumarins isolated in small amounts from plants of the Rutaceae family. Synthetic schemes were recently developed allowing to handle collinin in sufficient quantities to put in evidence valuable biological effects. The aim of this review is to examine the phytochemical and pharmacological properties of this compound.

Key Words: Anti-inflammatory activity, anti-cancer activity, collinin, geranyloxycoumarins, rutaceae.

INTRODUCTION

Coumarins represent a large class of natural compounds mainly found in the families of Rutaceae and Apiaceae. Although more than 1300 natural coumarins have been identified to date [1], most chemical and pharmacological studies have been carried out on coumarin itself or structurally simple derivatives.

Coumarins could be divided in 3 groups: a) substituted coumarins, b) ring-fused coumarins and c) *C*- and *O*-prenylcoumarins. In particular this third group comprises compounds in which a terpenyl side chain is attached to the benzopyrone ring directly or through one or more phenoxy group *via* an ethereal bond. While prenylcoumarins have been well studied both from a chemical and a pharmacological point of view, prenyloxycoumarins, considered for decades merely as biosynthetic intermediates of linear-, furano- and pyranocoumarins, have only in the last decade been characterized as secondary metabolites exerting valuable biological activities [1].

Collinin (**1**), more rarely known as schinifolin, is a geranyloxycoumarins isolated for the first time in 1949 [2].

This secondary metabolite has been found in nature in very small concentration. In order to get further insights into the pharmacological profile of this secondary metabolite, valuable synthetic schemes were developed during the last five years allowing to handle collinin in sufficient quantities to put in evidence interesting *in vitro* and *in vivo* biological properties. So (**1**) was shown to exert anti-platelet aggregation, anti-hepatitis B virus, anti-inflammatory and colon cancer chemopreventive effects. The aim of this review is to examine in detail the properties of the title compound so far reported in the literature from a chemical, phytochemical and pharmacological point of view.

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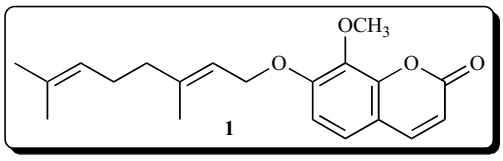
NATURAL SOURCES

The title coumarin has been found in nature only in plants belonging to the family of Rutaceae. Collinin took its name from the Australian plant *Flindersia collina* Bail., commonly known as "leopard ash", from the bark of which it was isolated for the first time in 1949 by Anet and coworkers [2]. Five years later collinin was isolated by Brown and coworkers from the bark of another plant of the genus *Flindersia*, namely *F. maculosa* (Lindl.) Benth. [3]. In 1977 Tikhomirova and coworkers extracted (**1**) from aerial parts and roots of *Haplophyllum alberty-regelii* [4]. Finally, starting form the beginning of 90's, several Authors reported the isolation of collinin from apolar extracts of bark and leaves of *Zanthoxylum schinifolium*, a plant that is commonly used in ethnomedical folk traditions of Korea, China, Japan and Taiwan and that up to now represents the main natural source of this natural geranyloxycoumarin [5-9].

However, until now collinin has been obtained from natural sources in very small amounts, being the best yield of extraction 0.028 ‰. This represents the main disadvantage that prevented for (**1**) a detailed characterization of its chemical and pharmacological properties.



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SYNTHESIS OF COLLININ

Low yields of extraction of collinin from natural sources have prompted studies to efficiently carry out the synthesis of (**1**). Up to now only two synthetic scheme were described in the literature. The first one was reported by Curini and coworkers in 2003 (Scheme **1**) [10].

This was a three-steps synthesis in which first the coumarin nucleus of daphnetin (**4**) was built by a Pechmann condensation of pyrogallol (**2**) and propiolic acid (**3**) at 120 °C in solvent-free conditions under the catalysis of concd. H_2SO_4 obtaining the desired product with a yield of 59%.

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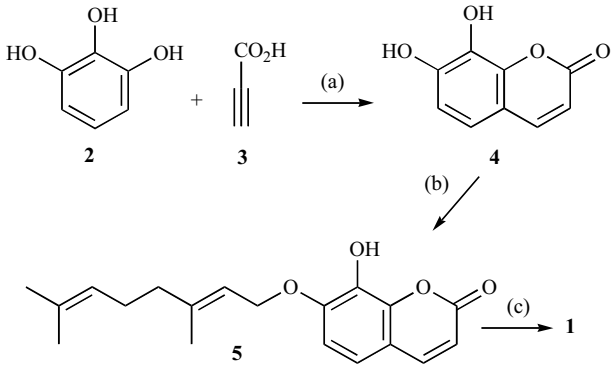


Scheme 1. (a) conc. H_2SO_4 (cat.), 120 °C; (b) geranyl bromide, DBU or Hunig's base, rt; (c) MeI, NaH 60%, DMF, rt.

Subsequently the selective geranylation of the OH group in position 7 yielding compound (**5**) was achieved by reaction of geranyl bromide in acetone at room temperature for 3 h in the presence of sterically hindered bases such di*iso*propylethyl amine (Hunig's base) or 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) with the latter performing better than the first base (65% yield *vs* 32% yield).

This selectivity may be explained by a preferential hydrogen abscission by bulky amines from the more accessible OH in position 7. The employment of sterically hindered bases was necessary as under standard geranylation conditions (e.g. using K_2CO_3 or NaH as base) an unseparable mixture of 8-, 7- and double geranylated products was obtained.

In the last step methylation of the OH group in position 8 of (**5**) with methyl iodide and NaH in *N*,*N*,-dimethylformamide at room temperature for 2 h was accomplished yielding collinin in 64% yield. The overall yield of this synthesis was 24.6%.

In 2007 De Kimpe and coworkers reported a synthesis of collinin in which the key step is the synthesis of the parent compound of (**1**), hydrangetin (**6**) [11], a coumarin that was isolated in 1961 for the first time from *Hydrangea macrophylla* by Towers and coworkers (Scheme **2**) [12,13].

The starting product for the synthesis of hydrangetin and collinin was commercially available 2,4,6-tribromophenol (**7**). The latter was converted in 83% yield into 2,4,6-tribromoanisole (**8**) by reaction with dimethyl sulphate and NaOH in refluxing water for 2h. Compound (**8**) was then lithiated with a solution in pentane of *n*-butyllithium at -20 °C, followed by quenching with trimethyl borate and oxidation of the resulting borane with peracetic acid furnishing 4-bromo-2,6-dihydroxyanisole (**9**) with a 89% yield. Compound (**9**) was then debrominated with H_2 in methanol using 10% Pd on activated charcoal at 50 °C for 17h giving 2,6-dihydroxyanisole (**10**) in nearly quantitative yield.

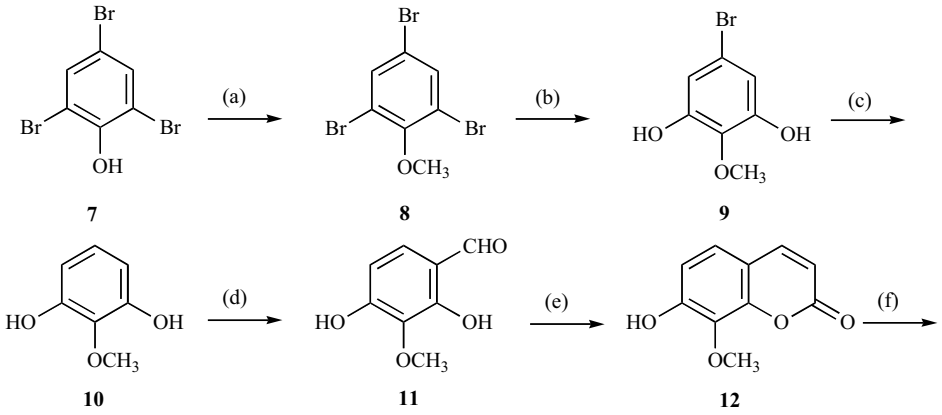
The Gattermann reaction, employing zinc (II) chloride, dry HCl and zinc (II) cyanide as formylating agent, gave 2,4-dihydroxy-3-methoxybenzaldehyde (**11**). Hydrangetin (**6**) was finally obtained by a Wittig reaction of compound (**10**) with methyl (triphenylphosphoranylidene)acetate in *N*,*N*-diethylaniline under heating for 4h. Standard reaction of alkylation of (**6**) with geranyl bromide and potassium carbonate at 50 °C for 15 h furnished collinin in 34 % yield.

PHARMACOLOGICAL PROPERTIES OF COLLININ

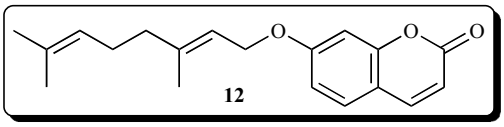
Anticancer Activity

Epidemiological surveys and animal experiments have suggested that adding to the diet some constituents of vegetables and fruits may contribute to decrease the incidence of several types od cancers in humans. Although collinin has been known for more than fifty years, only in 2006 the first and up to now only report concerning its anticancer activity was published in the literature by Tanaka and coworkers [14]. In this study Authors studied the effect of collinin and another closely structurally related geranyloxycoumarin, auraptene (**12**), extracted mainly from plant belonging to the genus *Citrus*, as orally active chemopreventive agents on AOM and dextrane sodium sulphate (DSS)-induced colon carcinogenesis in mice.

The experimental diets containing these two compounds at 2 dose levels (0.01 and 0.05%), were fed for 17 weeks to male CD-1 (ICR) mice that were initiated with a single in-



Scheme 2. (a) Me_2SO_4, NaOH, H_2O, rt, 2h; (b) 1) *n*-BuLi, pentane, -20 C° to – 10 °C, 15 min., 2) $B(OMe)_3$ -30 °C to 0 °C, 30 min., 3) AcOOH 32%; (c) H_2, 5% Pd/C, MeOh, 50 °C, 17 h; (d) 1) $Zn(CN)_2$, HCl, Et_2O, rt, 2) H_2O, H_2SO_4, Δ, 30 min; (e) $(Ph)_3P{=}CHCO_2Me$, Et_2NPh, Δ, 4h; (f) geranyl bormide, K_2CO_3, acetone, 50 °C, 15 h.



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traperitoneal injection of AOM, 10 mg/kg body weight) and promoted by 1% (w/v) DSS in drinking water for 7 days. Their tumor inhibitory effects were assessed at week 20 by counting the incidence and multiplicity of colonic neoplasms and the immunohistochemical expression of proliferating cell nuclear antigen (PCNA)-labeling index, apoptotic index, cyclooxygenase (COX-2), inducible nitric oxide (*i*NOS) and nitrotyrosine in colonic epithelial malignancy. Feeding with auraptene or collinin, at both doses, significantly inhibited the occurrence and multiplicity of colonic adenocarcinoma: 42.6% for 0.01% auraptene, 68.5% for 0.05% auraptene, 46.3% for 0.01% collinin and finally 74.1% for 0.05% collinin. Results are reported in Table **1**.

In addition, feeding with auraptene or collinin significantly lowered the positive rates of PCNA, COX-2, *i*NOS and nitrotyrosine in adenocarcinomas, while the treatment increased the apoptotic index in colonic malignancies (Table **2**).

These findings may suggest that prenyloxycoumarins, extracted from edible vegetables, could serve as effective colon cancer chemopreventive agents.

ANTI-PLATELET AGGREGATION ACTIVITY

Natural, semisynthetic or synthetic compounds containing a coumarin moiety, such warfarin and dicumarol rank as major anticoagulant drugs, through inhibition of platelet aggregation. Also for collinin degrees of inhibition at different levels were put in evidence by Taiwanese and Japanese authors [6,8].

The anti-platelet aggregation effects of collinin were revealed by a bioassay-guided fractionation of the chloroform-

Table 1. Incidence and Multiplicity of Colonic Neoplasia

Treatment	No. of mice	Incidence (no. of mice with neoplasms)			Multiplicity (no. of tumors/mice)		
		Total	AD[1]	ADC[2]	Total	AD	ADC
AOM + 1% DSS	10	10/10	10/10	10/10	5.40 ± 1.71[3]	2.40 ± 1.07	3.00 ± 1.41
AOM + 1% DSS/0.01% auraptene	10	8/10	8/10	5/10[4]	3.10 ± 2.28	2.10 ± 1.79	1.00 ± 1.33[5]
AOM + 1% DSS/0.05% auraptene	10	6/10[6]	6/10[6]	4/10[7]	1.70 ± 1.70[8]	1.10 ± 1.29	0.60 ± 0.84[8]
AOM + 1% DSS/0.01% collinin	10	7/10	6/10[6]	4/10[7]	2.90 ± 2.33	2.00 ± 1.83	0.90 ± 1.20[5]
AOM + 1% DSS/0.05% collinin	5	6/10[5]	5/10[3]	4/10[7]	1.43 ± 1.43[8]	0.80 ± 0.92	0.60 ± 0.84[8]
AOM	5	0/5	0/5	0/5	NR	NR	NR
1% DSS	5	0/5	0/5	0/5	NR	NR	NR
0.05% auraptene	5	0/5	0/5	0/5	NR	NR	NR
0.05% collinin	5	0/5	0/5	0/5	NR	NR	NR
none	5	0/5	0/5	0/5	NR	NR	NR

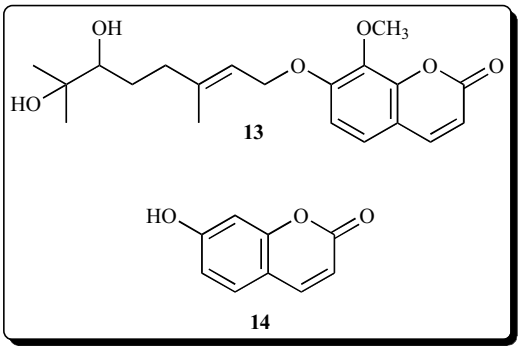
[1]AD , adenoma; [2]ADC, adenocarcinoma; [3]Mean ± SD; [4]Significantly different from "AOM + 1% DSS" group by Fischer's exact probability test or Bonferroni multiple comparison post test ([4]$p < 0.02$, [5]$p < 0.005$, [6]$p < 0.05$, [7]$p < 0.01$ and [8]$p < 0.001$); NR = not recorded.

Table 2. PCNA, Apoptosis Indices and Scores of COX-2, *i*NOS and Nitrotyrosine Expression in Colonic Adenocarcinomas

Treatment	PCNA-labeling index	Apoptotic index	COX-2	*i*NOS	Nitrotyrosine
AOM + 1% DSS	68.2 ± 10.5[1]	11.4 ± 5.8	3.6 ± 0.6	3.7 ± 0.5	3.5 ± 0.8
AOM + 1% DSS/0.01% auraptene	50.0 ± 12.6[2]	18.1 ± 5.0[3]	2.4 ± 1.2[2]	2.3 ± 0.8[4]	1.7 ± 0.8
AOM + 1% DSS/0.05% auraptene	47.2 ± 13.4[2]	20.7 ± 5.4	2.0 ± 0.9[3]	1.8 ± 1.0[4]	1.4 ± 0.7[4]
AOM + 1% DSS/0.01% collinin	51.8 ± 10.0[2]	19.1 ± 5.6[3]	2.6 ± 1.0	2.4 ± 0.7[2]	1.8 ± 0.8
AOM + 1% DSS/0.05% collinin	49.3 ± 13.2[3]	21.3 ± 6.9[2]	2.3 ± 1.2[3]	2.2 ± 1.3[2]	1.3 ± 0.5[3]

[1]Mean ± SD; [2-4]Significantly different from "AOM + 1% DSS" group by Bonferroni multiple comparison test ([2]$p < 0.01$, [3]$p < 0.05$ and [4]$p < 0.001$).

soluble portion of the root bark of *Z. schinifolium* allowing to identify (**1**) as the most potent aggregation inhibitor among a series of differently functionalized geranyloxy- and simple coumarins. The anti-platelet aggregation activity was measured *in vitro* by the turbidimetric method by which platelets were pre-incubated with the compound at 37 °C for 3 min. after which the inducers, arachidonic acid at a dosage of 100 μM, collagen at a dosage of 10 μg/mL and platelet aggregating factor (PAF) at a dosage of 2 ng/mL, were added to trigger aggregation. Results, comparing the activity of auraptene, collinin, its 6',7' diol, named schinilendiol (**13**), isolated from *Z. schinifolium* [6,8] and their unalkylated parent compounds hydrangetin (**6**) and umbelliferone (**14**), are reported in Table **3**.



As it can be seen from data reported in Table **3**, collinin was the most effective compound which, even at the dosage of 50 μg/mL caused complete inhibition of platelet aggregation induced by arachidonic acid and collagen. Values of inhibition reported in Table **3** allow also to state that the presence of a geranyloxy side chain, the presence of two double bonds in positions 2 and 6 the lack of oxygenated functionalities in this *O*-chain are essential for the observed activity. Moreover, as auraptene and collinin were very similar in potency, it' reasonable to hypothesize that the 8-methoxy group does not play a crucial role in this kind of pharmacological activity.

ANTI-INFLAMMATORY ACTIVITY

Several coumarins have been reported to exert valuable anti-inflammatory activity, although when prenyloxycoumarins were tested contrasting results were obtained [15]. For example it has been reported that auraptene (**12**) did not affect the oedematous response induced by TPA-dermatitis in mouse ears, but, on the contrary, double pre-treatment of mouse skin with compound (**12**) markedly suppressed oedema formation, hydrogen peroxide production and leukocyte infiltration induced by the same pro-inflammatory agent [16]. Moreover auraptene was seen to inhibit the expression of *i*NOS and COXs.

With the aim to depict a preliminary structure-activity relationship and to definitively assess the anti-inflammatory properties of prenyloxycoumarins by means of a suitable pharmacological model, Curini and coworkers studied the effects of collinin, auraptene and structurally-related semisynthetic coumarins using the Croton oil induced dermatitis in mouse ear as a model of acute inflammation. This *in vivo* inflammatory model possesses the advantage of using very small amount of pure compounds and consequently is particularly suitable in the biological screening of natural and semisynthetic compounds.

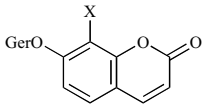
In this study inflammation was induced on the right ear (surface about 1 cm^2) of anaesthetised male CD-1 mice, weighting 28-32 g, by application of 80 μg of Croton oil dissolved in acetone. Control mice received only the irritant solution, whereas the others received both the irritant solution and the compounds under test. After being sacrificed after 6 hours, a plug of 6 mm diameter was excised from

Table 3. Inhibitory Effect of Collinin on the Aggregation of Washed Rabbit Platelets

Treatment	% Aggregation[1]			
	Concentration (μg/mL)	AA (100 μM)	Collagen (10 μg/mL)	PAF (2 ng/mL)
Control		86.4 ± 1.0	89.7 ± 1.1	90.5 ± 1.0
Collinin (1)	100	0.0 ± 0.0[2]	0.0 ± 0.0[2]	0.0 ± 0.0[2]
	50	0.0 ± 0.0[2]	0.0 ± 0.0[2]	--
	20	40.9 ± 20.5[3]	83.4 ± 1.0[2]	--
Auraptene (12)	100	0.0 ± 0.0[2]	2.9 ± 2.6[2]	0.0 ± 0.0[2]
	50	2.2 ± 1.9[2]	19.8 ± 17.1[2]	--
	20	86.7 ± 1.5	87.1 ± 0.4	--
Schinindiol (13)	100	73.5 ± 3.2	82.2 ± 1.2	76.4 ± 4.5
Hydrangetin (6)	100	79.1 ± 2.0[4]	81.0 ± 0.8[2]	91.0 ± 2.6
Umbelliferon (14)	100	76.6 ± 2.1[2]	76.3 ± 2.7[2]	87.1 ± 2.6

Abbreviations: AA = arachidonic acid, PAF = platelet aggregation factor; [1]Mean ± SD; [2]$p < 0.001$; [3]$p < 0.1$; [4]$p < 0.01$.

Table 4. Anti-Inflammatory Activity of Collinin, Auraptene and Semisynthetic Geranyloxycoumarins



Compound	Dose (μmol/cm^2)	No. of mice	Oedema reduction (%)
Controls	--	30	--
X = -H (auraptene)	1.00	10	51
X = -OCH_3 (collinin)	1.00	10	47
X = -OH	1.00	10	39
X = -O*n*pentyl	1.00	10	9
X = -OGer	1.00	10	27
X = -OAc	1.00	9	51
X = -CH_3	1.00	11	31
X = -I	1.00	10	29
X = -Br	1.00	10	43
X = -Cl	1.00	10	36
X = -F	1.00	11	27
Indomethacin	0.25	10	47

$p < 0{:}05$ at the analysis of variance, as compared with controls.

both the treated and untreated ears of animals. Oedema was quantified by the difference in weight between two plugs and the anti-inflammatory activity expressed as percent reduction of the control mice using indomethacin as reference drug.

Results of the inflammatory activity of collinin and other natural and semisynthetic prenyloxycoumarins are reported in Table **4**.

At the tested doses, only collinin, auraptene and its 8-acetoxy derivative were shown to exert an appreciable anti-inflammatory activity inhibiting the oedematous response by about 50%. So, from the data obtained in this test, it could be concluded that natural prenyloxycoumarins like collinin and auraptene inhibits the oedematous response induced by Croton oil. The substitution in position 8 of the benzopyrone ring affects the observed anti-inflammatory activity of this class of compounds and it seems to be higher when medium polarity substituents are present, such as the OCH_3 or acetoxy groups, while halogen atoms do not enhance the anti-inflammatory capacities.

ANTI-VIRAL ACTIVITY

Like it has been seen dealing with the anti-platelet aggregation inhibitory activity, the chloroform-soluble fraction of the bark of *Z. schinifolium* displayed a strong activity of anti-hepatitis B virus (HBV) DNA replication *in vitro* [7,8]. A bioassay-guided fractionation of the chloroform extract allowed to identify collinin as the only secondary metabolite exhibiting anti-HBV activity with a ID_{50} value of 17.1 μg/mL (Table **5**). Although not reported by Authors, the value recorded for (**1**) is similar to that displayed by 2',3'-dideoxycytidine, an anti-viral drug commonly used in the therapy of hepatitis B [7].

Table 5. Anti-HBV Inhibitory Activity and Cytotoxicity of Collinin

	ED_{50} (μg/mL)	$HBID_{50}$ (μg/mL)	SI
Collinin (1)	68.3	17.1	3.99
Auraptene (12)	NA	NA	--

NA = not active.

ED_{50} = concentration that caused a 50% reduction in cell number.

$HBID_{50}$ = concentration that inhibited HBV viral DNA yield in the medium by 50%.

SI = selective index ($ED_{50}/HBID_{50}$).

The fact that auraptene gave no results in the same test led to make the hypothesis that the 8-methoxy group in the coumarin moiety played an important role for the observed anti-viral activity.

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