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(54) Titre : UTILISATION DE FLAVANOLIGNANES DANS LA PREPARATION DE MEDICAMENTS AYANT UNE ACTIVITE ANTI-PROLIFERATIVE DANS L'UTERUS, LES OVAIRES ET LES SEINS

(54) Title: USE OF FLAVANOLIGNANES FOR THE PREPARATION OF MEDICAMENTS WITH ANTIPROLIFERATIVE ACTIVITY IN UTERUS, OVARY AND BREAST

(57) Abrégé/Abstract:
The invention relates to the use of flavanolignanes such as silymarin, silybin, silidianin, silicristin, mixtures thereof, extracts and formulations containing them, in the therapy (and prophylaxis) of uterus, ovary and breast tumors. These flavanolignanes have already been used in therapy for the treatment of hepatopathias of different origin, and they surprisingly show a marked affinity to the estrogen receptors of type II and an antiproliferative activity on tumor cell lines of uterus, ovary and breast.

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(57) Abstract

The invention relates to the use of flavanolignanes such as silymarin, silybin, silidianin, silicristin, mixtures thereof, extracts and formulations containing them, in the therapy (and prophylaxis) of uterus, ovary and breast tumors. These flavanolignanes have already been used in therapy for the treatment of hepatopathias of different origin, and they surprisingly show a marked affinity to the estrogen receptors of type II and an antiproliferative activity on tumor cell lines of uterus, ovary and breast.

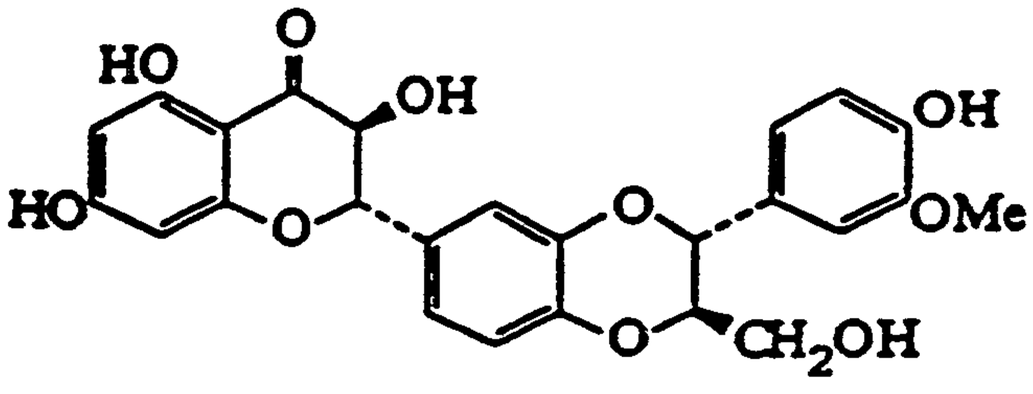
USE OF FLAVANOLIGNANES FOR THE PREPARATION OF MEDICAMENTS WITH ANTIPROLIFERATIVE ACTIVITY IN UTERUS, OVARY AND BREAST

The present invention relates to the use of flavanolignanes, alone or combined with known chemotherapeutic agents, for the preparation of medicaments for the therapy and prophylaxis of uterus, ovary and breast tumors.

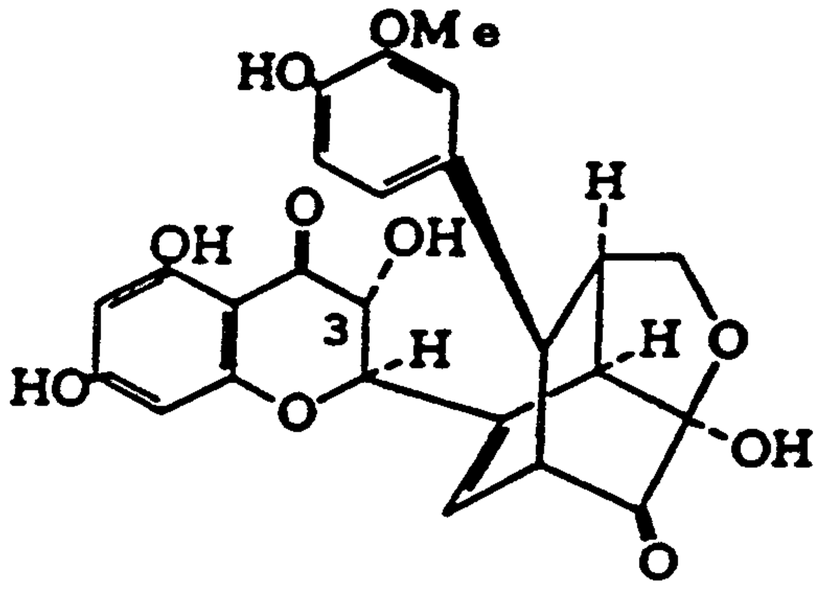
Recently, some flavonoids have been found to have antitumoral activity (Verna, Cancer Research 48, 5754, 1988) and chemoprophylactic activity in some tumors (Cassady, J. Nat. Prod. 53, 23, 1990). Particularly quercetin, a flavonoid which is almost ubiquitous in plants, has proved some inhibiting activity on the proliferation of human leukemia cells (Larocca, Br. J. of Haematology 75, 489, 1990) and on other cell lines (Scambia, Br. J. Cancer 62, 942, 1990 - Int. J. Cancer 46, 1112, 1990 - Cancer Chemother. Pharmacol. 28, 255, 1991 - Gynecologic Oncology 45, 13, 1992) besides having a synergistic activity with the usual chemotherapeuticals. Though the mechanism of such an inhibiting action on proliferation is unknown, it seems to be connected with the interaction of this flavonoid with the estrogen receptors of type II (Markaverich, J. steroid Biochem. 30, 71, 1988). These receptors, first described by Clark (J. Biol. Chem. 253, 7630, 1978) in the rat uterus, are different from the real estrogen receptors (ER) since these are present in a higher concentration and have a dissociation affinity constant (K_D: 10-20 nM) for estradiol lower than that of the

estrogen receptors (K_D: 0.2-1 nM).

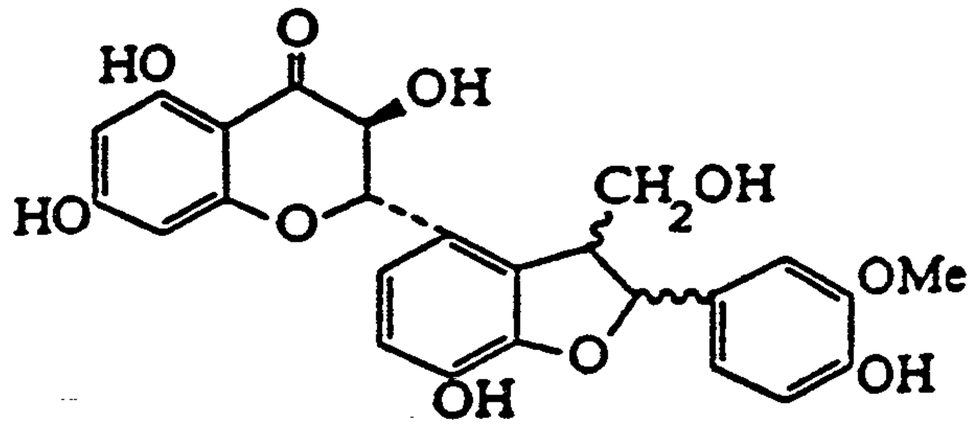
Now it has surprisingly been found that flavanolignanes, among which silymarin, already widely used in therapy for the treatment of hepatopathias of various origin, the three main components thereof being known under the names silybin, silidianin, silicristin and dehydrosilybin and having the structures reported below:



Silybin



Silidianin



Silicristin

have a high affinity to the estrogen receptors of type II and a very marked antiproliferative activity on uterus, ovary and breast tumoral cell lines resistant to Cis-platin and adriamycin. In order to verify the

antiproliferative effect of flavanolignanes, the growth curves of different stabilized cell lines deriving from various human tumors have been examined in the presence of the compounds and their capability to interact with the Type II EBS in ovary carcinoma samples has been evaluated.

The determination of the binding to the estrogen receptor has been carried out on cells of ovary tumor or of other organ tumors, cultured in monolayers using as medium the Dulbecco Modified Medium added with calf serum and with 200 unities/ml of penicillin. The cells used for the tests have been trypsinated every week and placed on a plate at a $8x10^{-4}$/ml density and incubated at 37°C under air atmosphere containing 5% CO_2 and humidity. For the control of the antiproliferative activity of the products, the cells have been placed into wells (Falcon 3046, Becton Dickinson NY) at a concentration of $4x10^4$/ml. After 24 hours the medium is substituted with fresh medium and the flavanolignanes dissolved in absolute ethanol are added. The controls are treated in the same experimental conditions only with the vehicle in the absence of the active ingredient. The treatment described above is repeated at 24 hour intervals during the 72 hours of the test time. The inhibition of the cell proliferation is evaluated by direct count of the cells, comparing the growth of the controls versus that of the treated samples.

For the dosage of the receptors, the cells after 24 hours are incubated with scalar amounts of labelled estradiol (^{3}H-E2 40Ci/mmol, Amershan UK) alone or in the presence of a 100-fold amount of diethylstilbestrol at

4°C for 2.5 hours.

At the end of the incubation time, the cells are quickly washed with fresh substrate and incubated for 30 minutes with 1M NaOH. Radioactivity is measured by means of a scintiller and binding specificity is calculated from the difference between the preparations containing or not diethylstilbestrol. Results are expressed as the number of binding sites per cell, according to conventional methods of the literature (Raneletti, 1988).

The inhibition on cell proliferation is evaluated by direct count of the cells, comparing the growth of controls versus that of the treated ones.

The results on different cell lines are reported in Fig. 1 and in Tables I and V. In particular, Fig. 1 shows the antiproliferative activity of silybin on A2780 WT, an ovary carcinoma cell line ER negative, type II positive.

The results are the average of two experiments carried out in triplicated. Standard deviations are less than 10 %.

The antiproliferative activity is dose-dependent. Table I reports the data relating silybin used at concentrations from 0.01 µM to 20 µM in cell proliferation; silybin exerts a dose-dependent antiproliferative effect on the different cell lines, including those resistant to chemotherapeuticals (MCF-7 ADRr, A2780 CIS) with a IC_{50} from 4.8 to 24 µM.

Table I Silybin antiproliferative effect on different stabilized cell lines

CELL LINES	TEST* NUMBER	CELL TYPE	EXPOSURE TIME	IC_{50} µM
A2780 WT	8	ovary ca	72 h	12
A2780 CIS	5	ovary ca CIS resistant	72 h	14
OVCA-433	3	ovary ca	72 h	4.8
MCF-7 ADRr	5	breast ca ADR resistant	72 h	24

* each test was carried out in triplicated.

In order to further study the antiproliferative activity of silybin, the effect of this substance on A2780 WT cell cycle has been studied. As shown in Table II, cytofluorimetric analysis reveals that silybin causes a decrease in the percentage of phase S cells and a mild increase in those in phase G0/G1.

Table II Effect of silybin on the distribution of A2780 WT in different phases of the cell cycle.

	TREATMENT*	
	Control	Silybin (10 µM)
G0/G1	58.7**	70.5
S	31	20.9
G2/M	10.3	8.6

* A2780 WT cells were cultured for 2 days with or

without 10 µM silybin.

** Results are expressed as the cell percentage in each phase of the cell cycle.

On the basis of these results, silybin was tested for any enhancement on the effect of some chemotherapeuticals, particularly cisplatin (CIS) and adriamycin (ADR). As reported in Table III, when silybin is used in combination with CIS, a synergistic inhibition effect on the growth is observed, compared with corresponding doses of the medicaments used alone.

Similar results are obtained using silybin in combination with ADR (Tab. IV).

In order to verify wether such an effect of a combination of silybin with adriamycin or cisplatin is due to a synergistic or an additive action, the data have been analyzed with the isobolic method by Berenbau (Adv. Cancer Res., 25, 269, 1981). The resulting combination index was less than 1 in both parental (A2780 WT, Tab. III) and resistant (MCF7, ADRr, Tab. IV) cell lines, thus proving that the combination of the two medicaments exerts a synergistic antiproliferative activity.

Table III Synergistic antiproliferative effect of the CIS-silybin combination on A2780 WT cell line.

CIS (µg/ml)	Silybin (µM)	% control	CIS (µg/ml)	Silybin (µM)	Combination index
0.1	0.1	67	0.23	2.9	0.46
0.25	0.1	58	0.31	6.4	0.81
0.5	0.1	37	0.54	>50	<0.92
1	0.1	18	>1	>50	<1.00
0.1	1	54	0.34	8.2	0.41
0.25	1	35	0.56	>50	<0.46
0.5	1	25	0.82	>50	<0.62
1	1	12	>1	>50	<1.02

Table IV Synergistic antiproliferative effect of a combination of ADR and silybin on MCF-7 ADRr line.

ADR (µg/ml)	Silybin (µM)	% of control	ADR (µM/ml)	Silybin (µM)	Combination index
0.1	0.1	80	0.6	0.1	1.16
0.5	0.1	76	0.9	0.36	0.77
1	0.1	69	2.5	2.2	0.44
2.5	0.1	62	5.4	7	0.51
5	0.1	47	>10	35	<0.50
10	0.1	39	>10	>50	<1.00

The antiproliferative activity of silybin and its analogous is observed not only on stabilized cell lines but also on human tumor primary cultures. A plurality of the tested flavanolignanes have a similar behaviour. In Table V, the data relating to silybin, silidianin, silymarin and dehydrosilybin are reported. The diastereomeric forms of silybin and isosilybin are as well active in these tests.

Table V Effect of different flavanolignanes on the growth of A2780 WT cells.

COMPOUND	TEST NUMBER	EXPOSURE TIME	IC_{50} µM
SILYBIN	8	72 h	12
DEHYDROSILYBIN	3	72 h	2.88
SILIDIANIN	3	72 h	12
SILYMARIN	3	72 h	15

Moreover, the above flavanolignanes were evidenced to inhibit <u>in vivo</u> the cell proliferation, by measuring the size of tumors implanted in the nude athymic mouse according to the conventional conditions of literature. The treatment of the animals at doses from 1 to 100 mg/Kg evidenced a marked regression of the studied tumors until their disappearance in a high percentage of individuals. The products in man proved to have an activity in ovary, breast and uterus tumors higher than that of known medicaments, such as Tamoxifen.

Silidianin, dehydrosilybin and the two diastereomeric forms of silybin showed a particularly marked

activity. In the in vitro tests flavanolignanes have been used as such, whereas in in vivo tests the complexes thereof with phospholipids, described in EP-A-0209038, have been used.

According to the invention, flavanolignanes can be administered orally or by infusion: for the oral route, natural or synthetic phospholipids proved to be particularly useful as carriers, since they form the above cited liposoluble stable complexes with said compounds; as well as liquid semi-synthetic glycerides containing medium-chain fatty acid triglycerides or analogues which can enhance the bioavailability of the single compounds, of the natural mixtures thereof or of the extracts containing them.

The flavanolignanes dosage in man range from 50 to 1500 mg/day, mainly administered by the oral route.

The invention also relates to compositions containing silymarin flavanolignanes and an antitumoral agent in the form of combinations for the simultaneous, sequential or separated use, in the antitumoral therapy.

The following examples further illustrate the invention.

Example 1

Hard gelatin capsules containing the complex of silybin with soy phosphatidylcholine.

Composition:

silybin-soy phosphatidylcholine complex	300 mg
sodium carboxymethylcellulose	16 mg
talc	6 mg
magnesium stearate	3 mg

Example 2

Soft gelatin capsules containing dehydrosilybin.

Composition:

dehydrosilybin	250 mg
liquid semi-synthetic glycerides	300 mg
partially hydrogenated vegetable oils	49 mg
soy lecithin	1 mg

Example 3

Hard gelatin capsules containing the complex of silymarin with soy natural phospholipids.

Composition:

silymarin-soy natural phospholipids complex	360 mg
sodium carboxymethylcellulose	21 mg
talc	6 mg
magnesium stearate	3 mg

Example 4

Hard gelatin capsules containing the complex of silidianin with soy phosphatidylcholine.

Composition:

silidianin-soy phosphatidylcholine complex	200 mg
sodium carboxymethylcellulose	10 mg
talc	3 mg
magnesium stearate	2 mg

CLAIMS

1. The use of flavanolignanes selected from silymarin, silybin, silidianin, silicristin, dehydrosilybin, mixtures thereof or extracts containing them for the preparation of medicaments with antagonistic activity on the estrogen receptors of type II and antiproliferative activity.

2. The use of complexes of flavanolignanes selected from silymarin, silybin, silidianin, silicristin, dehydrosilybin, mixtures thereof or extracts containing them, with phospholipids for the preparation of medicaments with antagonistic activity on the estrogen receptors of type II and antiproliferative activity.

3. Pharmaceutical compositions with antagonistic activity on the estrogen receptors of type II and antiproliferative activity, containing as the active ingredient flavanolignanes selected from silymarin, silybin, silidianin, silicristin, dehydrosilybin, mixtures thereof or extracts containing them optionally in the form of complexes with phospholipids, ~~and~~ in combination with antitumoral agents, for the simultaneous, sequential or separate use.

4. Compositions according to claim 3 suitable for the oral administration.

5. Compositions according to claim 4 containing as the carriers liquid semi-synthetic glycerides of average-chain fatty acids.

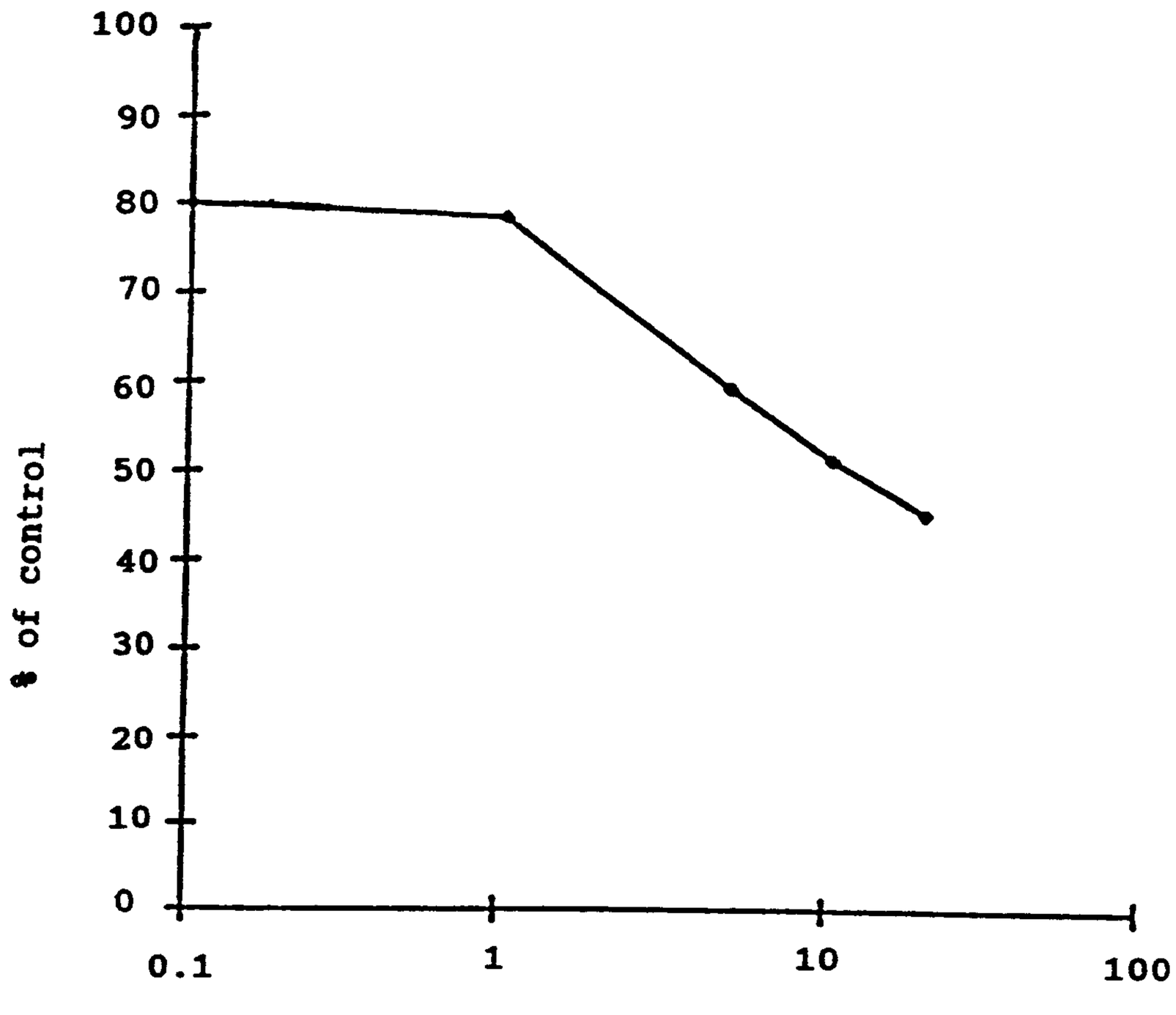


Figure 1 Inhibition curve of the growth of A2780 WT treated with silybin