

Brine shrimp bio-assay of the leaves of some *Cordia* species

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INTRODUCTION

The genus *Cordia* (*Borraginaceæ*) is made up of a great number of species distributed in tropical and subtropical regions of the world¹. Most of these species originate from Asia (Palestine, Turkey, Iran, Mesopotamia, Saudi Arabia, India, Pakistan, South China, Indo-China, Indonesia), others are spontaneous in South and East Africa and in Abyssinia and sub-spontaneous in Egypt. The genus *Cordia* occurs also in Australia, in New Caledonia, in Middle America, in Guyana and in the tropical area of Brazil. Some species are naturalized in various countries where they are sometimes cultivated.

In the course of a pharmacognostic screening of drugs used in traditional medicine, we have studied some aspects of the biological activity of the leaves of *Cordia myxa* L., a plant indigenous to South Asia, cultivated in the Mediterranean region and North Africa, where it is sometimes spontaneous, extending our investigations also to other species of the genus *Cordia* cultivated in Sicily.

Since leaf preparations of several species of *Cordia* are used in traditional medicine as remedies for some tumoral formations², we studied the *in vitro* cytotoxic effects of different extracts obtained from the leaves of *Cordia francisci* Ten., *Cordia martinicensis* Roem. et Schult., *Cordia myxa* L., *Cordia serratifolia* H.B.K. and *Cordia ulmifolia* Juss., by means of the dye test using cells of the Yoshida ascites sarcoma finding that the ethanolic extracts show a significant cytotoxicity³.

As a method utilizing brine shrimp (*Artemia salina* Leach) was proposed as a simple bioassay for natural products⁴ whose

toxicity may be manifested towards the newly hatched nauplii, it seemed interesting to use it for a cross-monitoring.

With this procedure, LC₅₀ values in µg/ml were determined both for ethanolic macerates and for extracts obtained by hot ethanol in a Soxhlet-type extractor of the leaves of the above-mentioned *Cordia* species in the brine medium.

MATERIALS AND METHODS

Extract preparation: Our experiment was carried out by using different extracts of lyophilized and powdered leaves of *Cordia francisci*, *Cordia martinicensis*, *Cordia myxa*, *Cordia serratifolia* and *Cordia ulmifolia*: each extract was obtained by hot ethanol (95%) in a Soxhlet-type extractor; another extract was prepared by macerating the dried leaves in 70% ethanol for 7 days at room temperature. After filtration, the organic solvents were removed *in vacuo*.

Cytotoxicity testing: The cytotoxicity was evaluated *in vitro* by means of the dye test using cells of the Yoshida ascites sarcoma maintained in male Wistar Glaxo albino rats (b.w. 180-200 g)³.

Brine shrimp bioassay: Samples were prepared by dissolving 50 mg of each extract in 5 ml of methanol (Solution A). Solution B was prepared by diluting 0.5 ml of solution A to 10 ml with methanol. Appropriate amounts of solution (100 µl B, 50 µl A, and 500 µl A for 10, 100, and 1000 µg of extract/ml respectively) were transferred to 1.25 cm discs of filter paper which were first dried in air, and then dried further *in vacuo* for one hour. Control discs were prepared using only methanol. Five replicates were prepared for each dose level.

Brine shrimp eggs (*Euraquarium* S.p.A. - Bologna, I) were hatched in a shallow rectangular dish (25 x 35 cm) filled with artificial sea water which was prepared dissolving a commercial salt mixture (ECIS s.r.l. Bolzano, I) in double-distilled water (33.3 g/l). A plastic divider with several 2 mm holes was clamped in the dish to make two unequal compartments. The eggs (50 mg) were sprinkled into the larger compartment which was kept dark, while the smaller compartment was illuminated. After an incubation period of 48 hours at uniform temperature (28 °C), the phototropic nauplii were collected by pipette from the lighted side, having been separated by the divider from their shells.

Ten shrimps were transferred to each sample vial using a pipette, and artificial sea water was added to make 5 ml. The nauplii can be counted macroscopically in the stem of the pipette against a light background. A drop of dry yeast suspension (Liquifry-Interpet LTD - Dorking-Surrey, GB) (2 drops in 11 artificial sea water) was added as food to each vial. The vials were maintained under illumination. Survivors were counted, with the aid of a 3 x magnifying glass, after 6 and 24 hours, and the percent deaths at each dose and control were determined. In cases where control deaths occurred, the data were corrected using Abbott's formula⁵: % deaths = [(test-control)/control] x 100.

LC₅₀'s and 95% confidence intervals were determined from the 24 hour counts using the probit analysis method described by Finney⁶.

RESULTS AND CONCLUSIONS

The results presented, reported in Tab. 1, show that only the alcoholic macerates of *Cordia martinicensis* and *Cordia ulmifolia* leaves show a quite significant toxicity (LC₅₀ < 1000 µg/ml) in the brine shrimp bioassay, while in the *in vitro* dye test using cells of the Yoshida ascites sarcoma also the ethanolic macerate of *Cordia myxa* leaves and the ethanolic extracts (obtained by successive extractions in a Soxhlet) of *Cordia martinicensis* and *Cordia ulmifolia* leaves are active.

Chemical analysis has shown the presence of flavonoids structurally related to quercetin⁷, known to inhibit phosphatidylinositol kinase⁸, in all the ethanolic macerates of the leaves of *Cordia* species studied, whereas unsaturated pyrrolizidine alkaloids, also in N-oxide form (results in press), to which the toxicity may be attributed are present only in the extracts of *Cordia martinicensis* and *Cordia ulmifolia* leaves.

The different cytotoxic effect shown by the ethanolic extracts and by the macerates can be due to heating instability of the above-mentioned pyrrolizidine alkaloids⁹, molecules present in many taxa of *Boraginaceæ*, which have shown antimitotic and antitumoral activity in laboratory animals¹⁰.

The good proportionality between the results obtained by the brine shrimp bioassay and the *in vitro* dye test using cells of the Yoshida ascites sarcoma suggest that this bioassay system might be readily utilized for an initial toxicity screening of the natural products, both because it has the advantage of being rapid and because no aseptic techniques are required.

Table 1

Species	Brine shrimp bio-assay						DYE test using cells of the yoshioda Ascites sarcoma ³	
	Percent deaths at 24 hr			LC ₅₀ µg/ml crude plant extract	LC ₅₀ g/ml dried plant material	(95 % confidence interval)	LC ₅₀ µg/ml crude plant extract	LC ₅₀ g/ml dried plant material
	10 µg/ml	100 µg/ml	1000 µg/ml					
<i>C. francisci</i> (*)	0	2	34	> 1000	> 0.016	—	> 1000	> 0.016
<i>C. artinicensis</i> (*)	0	1	58	865	0.011	(611-1137)	523	0.007
<i>C. myxa</i> (*)	0	2	56	> 1000	> 0.012	—	961	0.012
<i>C. serratifolia</i> (*)	0	0	16	> 1000	> 0.013	—	> 1000	> 0.013
<i>C. ulmifolia</i> (*)	0	9	68	732	0.009	(549-960)	474	0.006
<i>C. francisci</i> (§)	0	0	10	> 1000	> 0.018	—	> 1000	> 0.018
<i>C. martinicensis</i> (§)	0	2	38	> 1000	> 0.017	—	837	0.015
<i>C. myxa</i> (§)	0	1	27	> 1000	> 0.018	—	> 1000	> 0.018
<i>C. serratifolia</i> (§)	0	0	4	> 1000	> 0.019	—	> 1000	> 0.019
<i>C. ulmifolia</i> (§)	0	4	46	> 1000	> 0.017	—	785	0.014

(*) Ethanolic macerates; (§) Ethanolic extract obtained by successive extraction in a Soxhlet.

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