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Hormetic Versus Toxic Effects of Vegetable Tannin in a Multitest Study

E. De Nicola,[1] M. Gallo,[2] M. Iaccarino,[1] S. Meriç,[3] R. Oral,[4] T. Russo,[1] T. Sorrentino,[2] O. Tünay,[3] E. Vuttariello,[1] M. Warnau,[5] G. Pagano[1]

[1] Italian National Cancer Institute, "G. Pascale" Foundation, v. M. Semmola, I-80131 Naples, and CROP, I-83013 Mercogliano (AV), Italy
[2] Campania Regional Agency for Environmental Protection (ARPAC), v. Don Bosco, I-80143 Naples, Italy
[3] Istanbul Technical University, Environmental Engineering Department, Maslak, TK-34649 Istanbul, Turkey
[4] Ege University, Faculty of Fisheries, TK-5100 Bornova, Izmir, Turkey
[5] International Atomic Energy Agency, Marine Environment Laboratory, MC-98012 Monaco

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Abstract. Tannin from mimosa trees (*Acacia* sp.) utilized in traditional leather tanning was tested for toxicity in sea urchin (*Sphaerechinus granularis* and *Paracentrotus lividus*) embryos and sperm, marine, and freshwater algae (*Selenastrum capricornutum* and *Dunaliella tertiolecta*), and *Daphnia magna*. Based on a two-step tanning procedure used in traditional tanneries, two mimosa tannin preparations, i.e., fresh tannin (FT) and used tannin (UT), were tested as suspensions. The early results in *S. granularis* embryos showed that UT exerted lower acute toxicity than FT, namely, 1 vs 100 mg/L, to obtain 100% mortality, respectively. Subsequent bioassays were conducted on fresh tannin water extracts (TWE) corresponding to nominal tannin concentrations ranging from 0.1 to 30 mg/L. Developmental toxicity, up to embryonic mortality was exerted by TWE at levels >1 mg/L, *S. granularis* being more sensitive than *P. lividus* embryos/larvae. At the concentration of 0.1 mg/L, the frequencies of larval malformations were significantly lower than in controls. This positive stimulatory effect (currently termed as hormesis) was observed in extended numbers of culture replicates (up to 14) and was significant in the embryo cultures characterized by a relatively poor control quality (with $<70\%$ viable larvae in controls), whereas this effect was not observed in good-quality cultures (with $\geq 70\%$ viable larvae in controls). Cytogenetic analysis of *S. granularis* embryos reared in FT or UT suspensions (1 mg/L to 1 g/L) showed mitotoxic effects (decrease in active mitoses per embryo) in FT-exposed, but not in UT-exposed embryos. Mitotic aberrations were significantly increased by 10 mg/L UT. Sperm fertilization success in both sea urchin species showed an increasing fertilization rate (FR) up to 0.3 mg/L TWE and a dose-related decrease in FR up to 30 mg/L. Again, the offspring of *P. lividus* sperm exposed to TWE (0.1 and 0.3 mg/L) showed a decrease in larval malformations compared to controls, whereas a dose-related increase in developmental defects was observed in the offspring of *P. lividus* sperm exposed to higher TWE levels (1 to 30 mg/L). Algal cell growth bioassays in two species (*S. capricornutum* and *D. tertiolecta*) also showed a maximum growth at TWE levels ranging from 0.3 to 3 mg/L and a subsequent decline up to 30 mg/L TWE. *D. magna* bioassays resulted in daphnid immobilization by TWE concentrations ranging from 100 to 300 mg/L. The results demonstrate that tannins utilized in traditional leather tanning industry may raise concern of environmental damage at relatively high concentrations, whereas low-level tannins may result in hormetic effects. The present study also points to the need for bioassay design that should rely on adequate criteria in control quality, allowing to detect both inhibitory and hormetic effects.

Correspondence to: Giovanni Pagano; *email*: gbpagano@tin.it

The leather tanning industry has been investigated for its environmental impact related to chromium toxicity (Anonymous 1987; Losi *et al.* 1994) and due to environmental and occupational health concern toward the complex mixtures associated with tannery wastewater and sludge (Chattopadhyay *et al.* 1999; Fay and Mumtaz 1996; Klinkow *et al.* 1998; Vijayaraghavan and Murthy 1997). In front of the extensive literature on the modern leather tanning processes, few, if any, studies have been devoted to evaluate the environmental impact of the traditional leather tanning process based on vegetable tannins, mostly derived from ground bark of mimosa trees (*Acacia* sp., most often *Acacia mollissima*) (M. Cheggour, personal communication), and utilizing other natural products such as vegetable oil, wheat flour, and feces from various animals. Tannin is reacted twice with skins and hides, by means of used tannin in a pretanning stage and then by using fresh tannin in a second batch tank for tanning. The traditional leather tanning process involves extensive facilities in developing countries, resulting in occupational exposures and in environmental release of wastewater and sludge, also used for irrigation purposes. Thus, questions should be raised in evaluating the environmental toxicity of complex mixtures associated with traditional leather tanning and a major issue relates to tannin mixtures. Vijayaraghavan and Murthy (1997) studied

the treatment of tannery wastewater using upflow anaerobic contact filter. They reported that the inhibiting effect of tannin on anaerobic contact filter performance was beyond 914 mg/L tannin, whereas a chromium(III) concentration up to 140 mg/L did not affect the reactor performance. However, in batch process 77% (w/v) tannin affected the anaerobic microbial growth and hydrolyzable tannin was present in tannery wastewaters.

The literature on tannins is mostly focused on individual chemical species, e.g., tannic acid, thus providing only scanty information on tannin complex mixtures (Anonymous 1974, 2001; Cunha *et al.* 1995; Chen and Chung 2000; Haslam 1981, 1988). Though with this limitation, the database on tannin-associated biological effects is quite extensive, as reported in Table 1. This literature is mostly focused on tannin analogues tested alone (Sasaki *et al.* 1990; Chen and Chung 2000; Lehmann *et al.* 2000; Pautou *et al.* 2000; Wang *et al.* 2001) or in association with mutagens/carcinogens (Cunha *et al.* 1994; Kaur *et al.* 2000; Lehmann *et al.* 2000) or evaluated as dietary components (Zeiger 1993; Anonymous 2001). It is interesting to note that a substantial part of this data set includes, for many of tested tannins, both adverse effects at relatively high concentrations and protective effects toward a number of toxicity endpoints associated with low-level tannin exposures. In this respect, tannins may be regarded as an example of the extensive literature reporting on the positive stimulatory (hormetic) effects toward several biological endpoints by low doses of a number of chemical and physical agents (Calabrese and Balwin 2002; Kitchin 2002).

The present study was designed to evaluate the toxicity, if any, of tannin preparations from a leather tannery located in Marrakesh, Morocco, as a part of a more extensive investigation focusing on the environmental impact of wastewater and sludge from tanning facilities utilizing the traditional tanning process.

The bioassay battery utilized in this study included (i) sea urchin embryo and sperm bioassays, namely, changes in embryogenesis, mitotic activity, fertilization success, and offspring quality (following sperm exposure) (Pagano *et al.* 1983, 1986, 1996; Guillou *et al.* 2000; Trieff *et al.* 1995); (ii) *Daphnia magna* immobilization bioassays (US EPA 1993), and (iii) growth bioassays on phytoplanktonic algae (*S. capricornutum* and *D. tertiolecta*) (ASTM 1986; US EPA 1988).

Early results obtained in tannin-exposed sea urchin embryos suggested that low-level tannin might result in improved larval differentiation compared to control cultures. However, a suitable method to quantify hormesis in developmental toxicity bioassays was lacking. The observation of hormetic effects in sea urchin bioassays has been confined, so far, to changes in fertilization success, by maintaining fertilization rate (FR) in controls at suboptimal levels, 50 to 70% (Pagano *et al.* 1986). In order to evaluate any hormetic effects in the embryotoxicity bioassays, we needed to modify the criteria in the acceptance or rejection of control cultures, unconfined to those controls as close as possible to 100% normal larvae (namely, ≥90% in *P. lividus* and ≥80% in *S. granularis*). Thus, by assuming the new criteria, by accepting "low-quality" control cultures we were able, for the first time, to evaluate both inhibitory (toxic) and hormetic outcomes in terms of either developmental toxicity or amelioration of larval quality.

Testing any hormetic vs. inhibitory effects in algal growth bioassays is made feasible by evaluating any positive or negative changes vs. control growth. In the case of *D. magna* bioassays, control cultures display immobilization rates equal or close to zero, hence preventing, by definition, any observation of hormetic effects and permitting only the observation of inhibitory effects, if any.

The study provided a consistent set of information on tannin-associated dose-related trends and suggested novel criteria in defining control quality.

Table 1. Selected information on tannin-related toxic and hormetic effects

Agent	Effect
Camelliin B	Induced apoptosis in HeLa cell line
Hypericum perforatum extract and oil	↑ immunostimulating activity; ↑ immunosuppressing activity
Gallic acid	Nontoxic up to 5 g/kg body weight in mice
Areca nut polyphenols and tannin	Oral cancer promotion
Terminalia arjuna tannin extract	↓ 2AF -induced mutagenicity
Tannic acid	↑ metabolic activation of a few mutagens; anticlastogenic and antimutagenic effects in vivo
Tannins	↑ inhibitory activity on lipid peroxidation

Materials and Methods

Preparation of Tannin Suspensions and Water Extract

Fresh tannin (FT) and used tannin (UT) from ground bark of mimosa trees (*Acacia* sp., *A. mollissima*) were obtained from tanning facilities in Marrakesh, Morocco. Both FT and UT were dehydrated at 60°C up to constant weight. Dry preparations were tested as suspensions in filtered seawater (FSW) in a first series of experiments. In the subsequent part of the study, FT was suspended in FSW (300 mg/L), stirred 1 h, and filtered (50 μm). The resulting tannin water extract (TWE) was then diluted to the desired final concentrations in FSW. For *D. magna* and algal toxicity bioassays, the stock FT suspension (1 g/L) was prepared in bidistilled water and processed as described above. Freshly prepared suspensions and extracts were used in each bioassay.

Sea Urchins

Sea urchins from the species *S. granularis* and *P. lividus* were collected in the Bay of Naples by the staff of the "A. Dohrn" Zoological Station, Naples. They were transferred to laboratory immediately and submitted to bioassays upon arrival. Gametes and embryos were obtained and processed as reported previously (Pagano *et al.* 1986, 1993). In established experimental protocols, gamete and larval quality was evaluated in order to select optimal ovocytes (for shape, lack of immature forms and of debris) and optimal control larvae (with normal larvae ≥90% in *P. lividus* or ≥80% in *S. granularis*). In the present study, experiments were conducted also using low-quality eggs, displaying abnormalities such as immature or decaying ovocytes. Control cultures resulted in a heterogeneous quality range, including "high-

quality" cultures, with ≥70% viable larvae (i.e., normal or retarded pluteus larvae lacking malformations or developmental arrest; see below), and "low-quality" cultures, with <70% viable larvae. Natural FSW and 2.5×10^{-4} *M* $CdSO_4$ were used as untreated negative control and positive control, respectively (Pagano *et al.* 1982, 1986).

In a first series of embryotoxicity experiments, FT and UT suspensions were tested in *S. granularis* embryos at levels ranging from 1 mg/L to 1 g/L (dry wt/vol). In the subsequent experiments, *S. granularis* and *P. lividus* embryos were reared in TWE at concentrations equivalent to FT ranging from 0.1 to 30 mg/L.

Exposure of embryos was carried out at 18°C in the dark and occurred throughout development from zygote (10 min after fertilization) up to the pluteus larval stage (72 h after fertilization). Embryo concentration in cultures was maintained at ~20–30 embryos/ml, in order to avoid any bias due to larval overcrowding potentially leading to unspecific developmental defects.

Sperm bioassays were conducted on sperm cell suspensions by exposure of a 0.1% suspension of "dry" sperm pellet for 10 min (*S. granularis*) or 2 h (*P. lividus*) in TWE. The sperm-containing supernatant was used to inseminate 10 ml of untreated egg suspension (~20–30 eggs/ml). This procedure allowed for a fertilization rate (FR) in controls ranging 50 to 70% fertilized eggs providing information, if any, either about inhibition or hormesis (Pagano *et al.* 1986). The endpoints were (a) sperm fertilization success (measured as FR), measured 1 to 3 h after fertilization on living embryos (2- to 32-cell stage), and (b) offspring quality (see below).

Morphologic analysis of larvae was performed 72 h after fertilization on living plutei immobilized in 10^{-4} *M* chromium sulfate prior to observation. The same procedure was utilized to evaluate developmental defects both in larvae exposed throughout development and in the offspring of exposed sperm. In each treatment schedule, 100 pluteus larvae were scored for the frequencies of (1) normal (N) larvae, (2) retarded (R) larvae (size, ≤1/2 N), (3) malformed larvae affected in skeletal or gut differentiation (P1), (4) embryos unable to attain the pluteus stage as abnormal blastulae or gastrulae (P2), (5) dead pluteus larvae (D1), identified as trasparent larval "ghosts," or (6) dead embryos prior to larval differentiation (D2). In order to simplify data evaluation, the percentage frequency of normally developed and viable larvae (N + R) were plotted, as reported in Figures 2, 3, and 5.

Cytogenetic analysis was carried out on 30 cleaving embryos from each of four replicate cultures, fixed in Carnoy's fluid 5 h after fertilization, then stained with acetocarmine and rinsed in 20% acetic acid. The parameters being analyzed included quantitative and morphological abnormalities. Quantitative abnormalities were the following: (a) mean number of mitoses per embryo (MPE), (b) percentage interphase embryos (% IE) lacking active mitoses, and (c) metaphase/anaphase ratio (M/A). The morphological abnormalities were scored as (i) anaphase bridges, (ii) lagging chromosomes, (iii) acentric fragments, (iv) scattered chromosomes, and (v) multipolar spindles. These abnormalities were both scored individually and reported as total mitotic aberrations (TMA) per embryo; the percentage of embryos having ≥1 mitotic aberrations [% E(Ab+)] was also scored.

Daphnia magna

Newborn daphnids (<24 h old) were exposed to TWE at concentrations ranging from 0.1 mg/L to 1 g/L for 24 h, and tests were conducted in quadruplicate. In each treatment schedule 20 daphnids were scored for their frequencies of immobilized daphnids. $K_2Cr_2O_7$ was used as a positive control in the concentration range of 0.6 to 1.7 mg/L. A negative control with standard water solution was also conducted (US EPA 1993). In the toxicity concentration range, $EC_{50(24\ h)}$ was calculated by probit analysis (US EPA 1993).

Algae

Unicellular algae from the species *S. capricornutum* and *D. tertiolecta* were used to evaluate the hormetic and/or toxic effects of TWE according to standard methods (ASTM 1986; US EPA 1988). Weekly transplantations maintained algal cultures in exponential growth, and blank controls were cultured in a salt solution dissolved in ultrapure water. Cultures were kept in Erlenmeyer flasks at 20 ± 1°C under light conditions of 4000 lux using cool light lamps and algae were exposed to TWE at concentrations ranging from 0.3 to 30 mg/L. The toxicity tests were initiated from a 2000 cell/ml algal concentration and conducted in three to six replicates. The endpoint consisted of algal growth, which was measured after 72 h in a Bürker cell counting chamber. Similar to *D. magna* toxicity tests, $K_2Cr_2O_7$ positive controls were conducted in parallel to TWE solution.

Statistical Analyses

Differences in the distributions of actual frequencies observed for each larval class (N, R, P1, P2, D1, and D2) between controls and experimental groups or among experimental groups were tested for significance by the *G* procedure (adapted from the log-likelihood ratio test) for $2 \times k$ contingency tables (Godfrey 1992).

Comparisons among viable larvae (N+R classes) in control batches and in those exposed to increasing TWE concentrations were tested for significance using the multiple comparison test of Dunnett after arcsin transformation of the data, using Statistica® Software.

Data on cytogenetic parameters in sea urchins and data on *Daphnia magna* immobilization were analyzed using ANOVA and Tukey's multiple-comparison test, after arcsin transformation of the data when appropriate (Whorton 1985). Data on algal growth were analyzed by one-way ANOVA followed by the Tukey honest significant difference (HSD) test (Whorton 1985).

Results

Sea Urchins

A first series of experiments was designed to compare the effects of FT vs. UT suspensions on sea urchin embryogenesis. As shown in Figure 1, developmental toxicity to *S. granularis* embryos was exerted by UT to a lesser extent compared to FT, as 10 mg/L FT induced 40% early embryonic mortality (D2), up to 100% D2 by 100 mg/L FT, whereas 100 mg/L UT only caused 41% larval malformations (P1), and induced 100% D2 at the level of 1 g/L.

In the subsequent embryotoxicity bioassays, *S. granularis* and *P. lividus* embryos were reared in TWE at concentrations equivalent to FT levels ranging from 0.1 to 30 mg/L. As shown in Figures 2 and 3, the observed dose-dependent trends of developmental defects and embryotoxicity displayed a shift in both species from hormesis at low TWE levels (0.1 mg/L) to developmental toxicity with increasing TWE levels ranging from 3 to 30 mg/L. *S. granularis* larvae proved to be more sensitive than *P. lividus* larvae. The statistical significance of these data changed dramatically by analyzing the results from the lots with "low-quality" (<70% viable larvae) controls versus "high-quality" (≥70% viable larvae) controls. The hormetic effect at 0.1 mg/L TWE was highly significant by considering the results from lots with "low-quality" controls

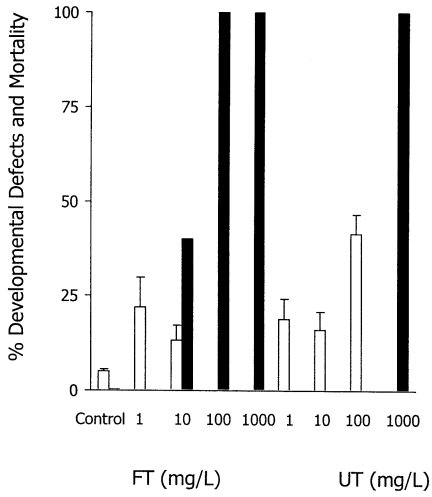

Fig. 1. Developmental defects (open bars) and embryonic mortality (solid bars) in *S. granularis* larvae exposed to fresh tannin (FT) and used tannin (UT)

($p_{\text{Dunnett}} = 0.009$), as shown in Figure 2a (*S. granularis*), and $p_{\text{Dunnett}} = 0.008$ as shown in Figure 3a (*P. lividus*). On the other hand, no significant hormetic effects were observed in cultures with >70% viable larvae (Figs. 2b and 3b), whereas only developmental toxicity was observed at TWE levels ranging 3 to 30 mg/L. Positive controls ($CdSO_4$ 2.5×10^{-4} *M*) invariably resulted in 100% developmental arrest (P2) or early embryonic mortality (D2), both in "low-quality" and in "high-quality" cultures (data not shown).

A series of experiments was performed to assess the effects, if any, on fertilization success following exposure of *S. granularis* and *P. lividus* sperm to TWE. Figure 4 shows the changes in sperm fertilization success, which was increased by TWE levels ranging from 0.1 to 0.3 mg/L and was then inhibited by increasing TWE concentrations (1 to 30 mg/L), in the same concentration range for *S. granularis* and for *P. lividus* sperm.

The offspring from TWE-exposed *P. lividus* sperm resulted in the same nonlinear dose-related trend. As shown in Figure 5a, a significant increase in viable larvae vs. controls was observed in the offspring of sperm exposed to TWE levels ranging from 0.1 mg/L ($p_{\text{Dunnett}} = 0.0014$) to 1 mg/L ($p_{\text{Dunnett}} = 0.008$), whereas following sperm exposure to TWE levels ranging from 3 to 30 mg/L, the viable offspring showed a dose-related decrease ($p_{\text{Dunnett}} = 0.037$ at 30 mg/L). Again, the significance of hormetic effects in the offspring of TWE-exposed sperm was dramatically affected according to the 70% cutoff in control quality as reported for TWE-exposed embryos. As shown in Figure 5b, no significant effect was detected in offspring quality with ≥70% viable larvae in control cultures.

Cytogenetic Analysis

A quadruplicate experiment was carried out by exposing developing *S. granularis* embryos to FT and UT suspensions at concentrations ranging from 1 mg/L to 1 g/L for 5 h after fertilization. While the results of embryologic analysis in the same experiment are reported in Figure 1, the effects of FT on mitotic activity are shown in Figure 6; MPE was affected by increasing tannin levels, with a slight, nonsignificant MPE increase in embryos exposed to 1 mg/L FT. By increasing FT levels, up to 1 g/L, a sharp decline in mitotic activity was observed, with MPE = 1.1 ± 0.7 (vs. MPE = 7.4 ± 0.5 in controls; $p < 0.0027$) and % IE = 62.5 ± 15.4 (vs. % IE = 33.1 ± 2.9 in controls; $p < 0.043$). No significant mitotoxic action was exerted by UT (data not shown).

The frequencies of the mitotic aberrations, TMA and E(Ab+), were significantly affected by exposure to 10 mg/L UT ($p = 0.018$ and $p = 0.036$, respectively), but not to FT, as shown in Table 2. By considering individual mitotic aberrations, 10 mg/L UT induced a significant increase in SC ($p = 0.037$), whereas FT (10 mg/L) increased AF frequency ($p = 0.032$).

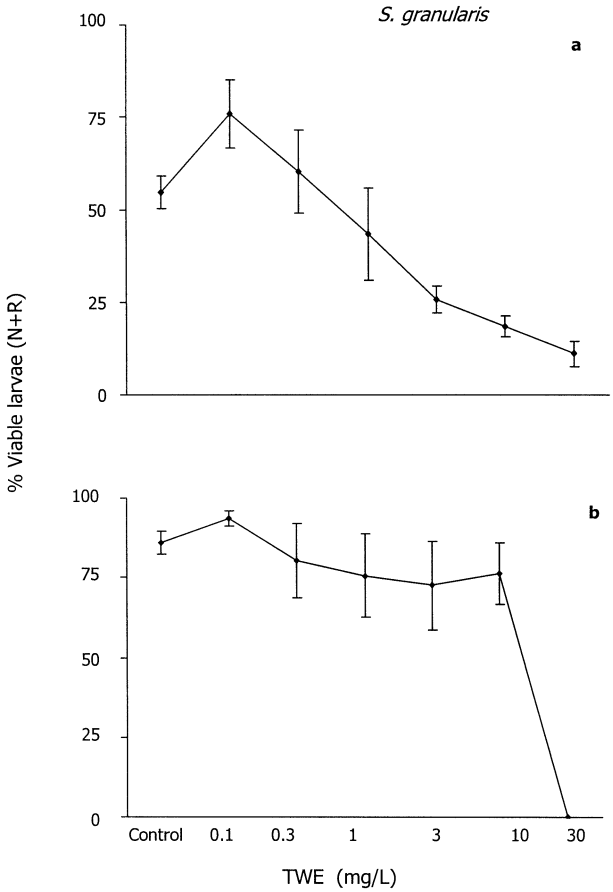

Fig. 2. Percentage *S. granularis* viable (normal + retarded) plutei following TWE exposure. (**a**) Cultures with <70% normal controls (nine replicates); (**b**) cultures with ≥70% normal controls (five replicates)

Daphnia magna

TWE did not cause any significant toxic effect up to 175 mg/L. A significant dose-related toxicity was observed in *D. magna* exposed to TWE levels ranging from 200 to 300 mg/L, up to

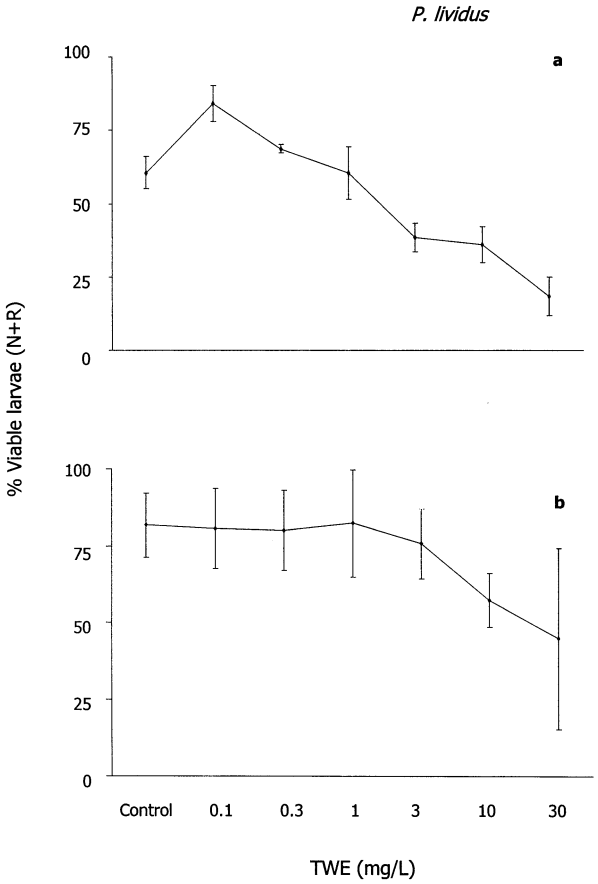

Fig. 3. Percentage *P. lividus* viable plutei following TWE exposure. (**a**) Cultures with <70% normal controls (three replicates); (**b**) cultures with ≥70% normal controls (three replicates)

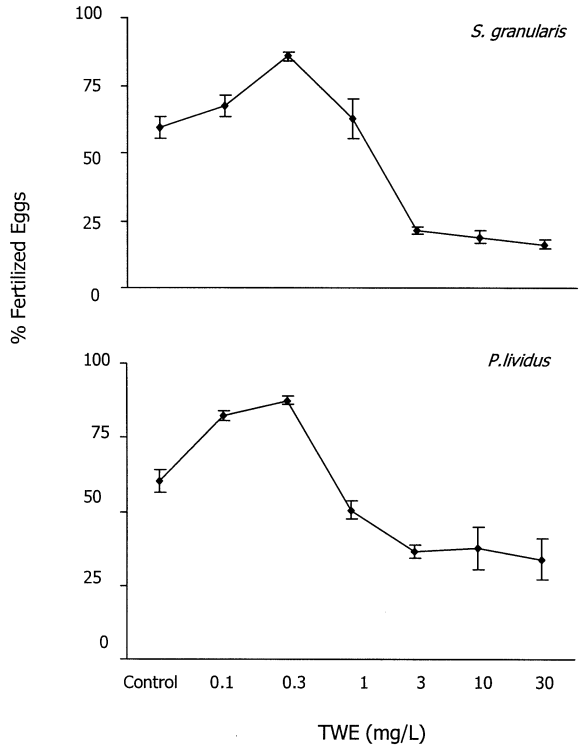

Fig. 4. Fertilization success of TWE-exposed *S. granularis* and *P. lividus* sperm

100% immobilized larvae (Fig. 7). The $EC_{50(24\ h)}$ value was calculated as 248 mg/L for TWE and 0.77 mg/L for positive controls at a 95% confidence level.

Algal Growth

As shown in Figure 8, both *D. tertiolecta* and *S. capricornutum* showed a biphasic dose–response trend when exposed to TWE levels ranging from 0.3 to 30 mg/L. Maximum algal growth was observed in *D. tertiolecta* exposed to 0.3 mg/L TWE, whereas *S. capricornutum* responded with maximum growth to 3 mg/L TWE. A dose-related growth inhibition was observed in both algal species exposed to TWE levels ranging from 3 to 30 mg/L.

Discussion

The present study has been focused on a vegetable tannin complex mixture from *Acacia* sp. utilized in the traditional leather tanneries in Morocco. The results point to consistent nonmonotonic dose–response trends in a number of toxicity endpoints in sea urchin bioassays, including developmental defects and mortality effects in tannin-exposed sea urchin embryos, as well as fertilization success and offspring quality following sperm exposure to TWE. A similar nonmonotonic trend in algal growth was observed in TWE-exposed *D. tertiolecta* and *S. capricornutum*. Only inhibitory effects were observed in *D. magna* due to lack of immobilized daphnids in control cultures, preventing the observation of any hormetic effects. The TWE levels used in this study ranged from five to three orders of magnitude below the FT concentrations (approx. 5 to 7 g/L) used in the tanning process (M. Cheggour, personal communication). By considering the two-stage process (treating skins with UT and then FT), it is presently difficult to assess the actual tannin levels occurring in tannery wastewater. However, Haslam (1988) reported that 50% of tannin remains in the effluent after application. By assuming an average 6 g/L FT concentration in batch tanks, tanning effluent will contain approximately 3 g/L of tannin. By assuming a dilution of tannery effluent in the municipal sewage system, the tannin concentration in sewer may remain in an inhibitory range, as measured in recent analyses of tannery wastewater and of sewage (unpublished). When a further dilution occurs in the receiving waters, the tannin concentration may decrease to levels resulting in hormetic effects. Thus, the TWE level range tested in the present study is expected to include environmentally realistic tannin levels. Any attempts to infer the actual environmental effects from the present results should include both hormetic and toxic potentials from wastewater to the exposed biota. Ongoing studies of the environmental tannin levels and of tannery wastewater toxicity are expected to clarify the prevailing events associated to tannin-contaminated wastewater.

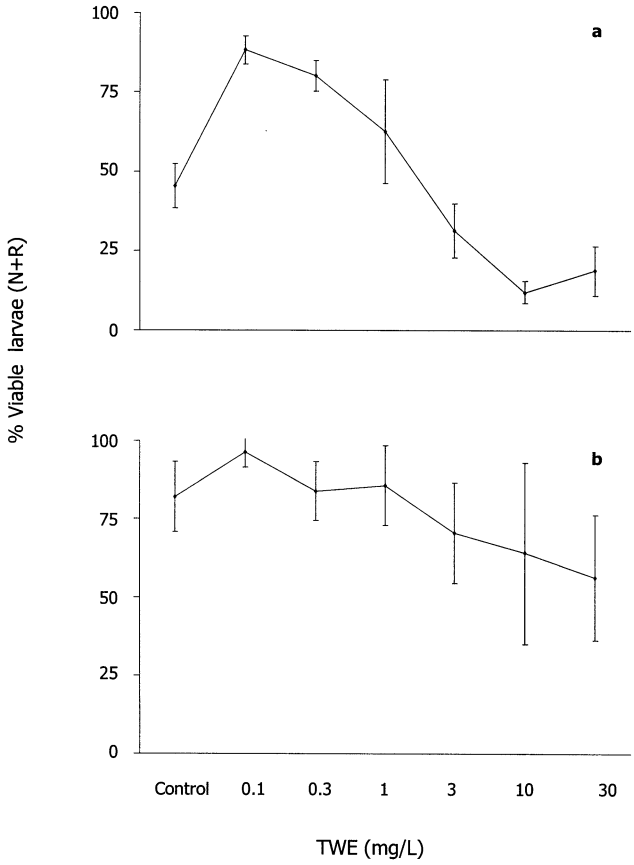

Fig. 5. Percentage viable plutei in the offspring of TWE-exposed *P. lividus* sperm. (**a**) Cultures with <70% normal controls (three replicates); (**b**) cultures with ≥70% normal controls (three replicates)

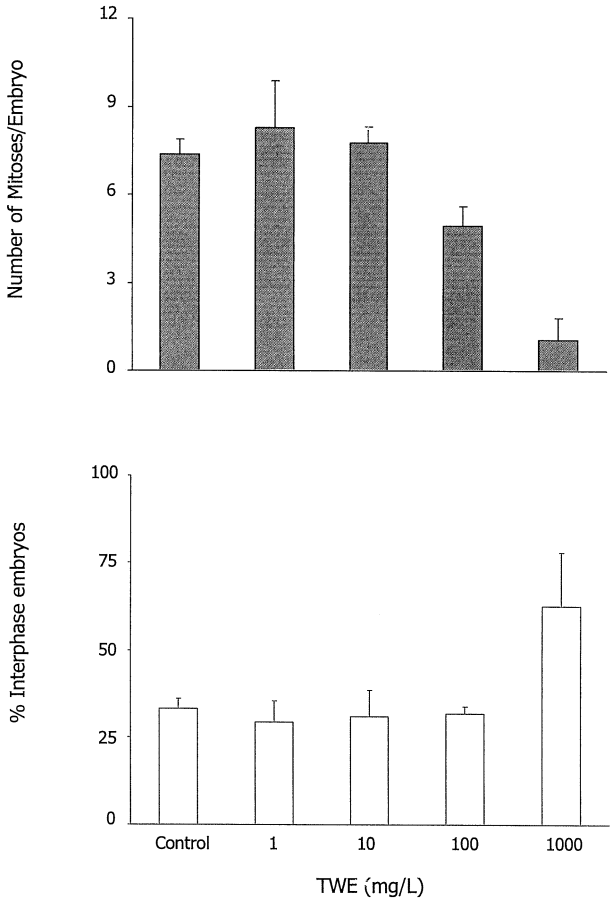

Fig. 6. Cytogenetic quantitative abnormalities in *S. granularis* embryos exposed to FT

A dose-related shift from hormesis to adverse effects has been reported for a number of chemicals and physical agents (Calabrese and Baldwin 2002; Kitchin 2002) and has been an extensive finding in previous studies of tannin-associated toxicity. This literature, however, has been focused mostly on individual tannins and related compounds as bioactive dietary components (Zeiger 1993; Anonymous 2001), as drugs (Kitchin 2002; Kaur *et al.* 2000), or as mutagenicity modulators of a number of agents (Hong *et al.* 1995; Kaur *et al.* 2000; Lehmann *et al.* 2000). To our best knowledge, no previous reports were specifically aimed at evaluating tannin-based complex mixtures as industrial pollutants. The subject deserves further investigations, by regarding tannin-associated pollution as a potential, though broadly unexplored subject in environmental studies. Further studies are expected to clarify the role(s) of tannin speciation in dose-related toxicity, by providing mechanistic explanation for biphasic trends causing hormesis and toxicity.

The present study has relied on novel criteria in defining control quality in sea urchin larval cultures, when the hypothesis of a hormetic effect is to be tested. In previous studies, sea urchin sperm bioassays had been designed by adjusting control fertilization rate at suboptimal values, 50 to 70% FR (Pagano *et al.* 1986), thus enabling the observation also of moderate spermiotoxic effects. In turn, reading a 100% FR might imply a "superoptimal" sperm:egg ratio, with a consequent loss of bioassay sensitivity. Moreover, any hormetic effect would be, by definition, impossible to

Table 2. Mitotic aberrations in *S. granularis* embryos exposed to FT or UT, quadruplicate experiments; exposure failed to result in any significant changes versus controls (data not shown)[a]

Treatment schedule	SC	AF	TMA
Control	0.03 ± 0.02	0.02 ± 0.01	0.24 ± 0.05
FT (mg/L)			
1	0.07 ± 0.05	0.25 ± 0.10	0.53 ± 0.21
10	0.37 ± 0.31	0.34 ± 0.21[b]	0.93 ± 0.48
100	0.41 ± 0.28	0.12 ± 0.06	0.80 ± 0.27
1000	0.43 ± 0.14	0.00 ± 0.00	0.48 ± 0.17
UT (mg/L)			
1	0.48 ± 0.31	0.11 ± 0.07	0.93 ± 0.23
10	0.77 ± 0.49[b]	0.00 ± 0.00	1.08 ± 0.49[c]
100	0.04 ± 0.02	0.11 ± 0.05	0.50 ± 0.07
1000	0.01 ± 0.01	0.02 ± 0.02	0.45 ± 0.18

[a] SC—scattered chromosomes; AF—acentric fragments; TMA—total mitotic aberrations/embryo. Data on other aberrations (anaphase bridges and others) were invariably nonsignificant.
[b] $p < 0.05$.
[c] $p < 0.02$.

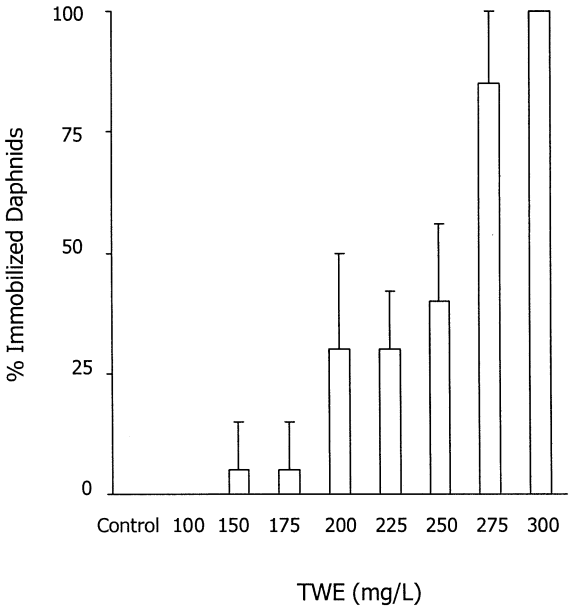

Fig. 7. Percentage immobilization in TWE-exposed *D. magna*. Quadruplicate experiment

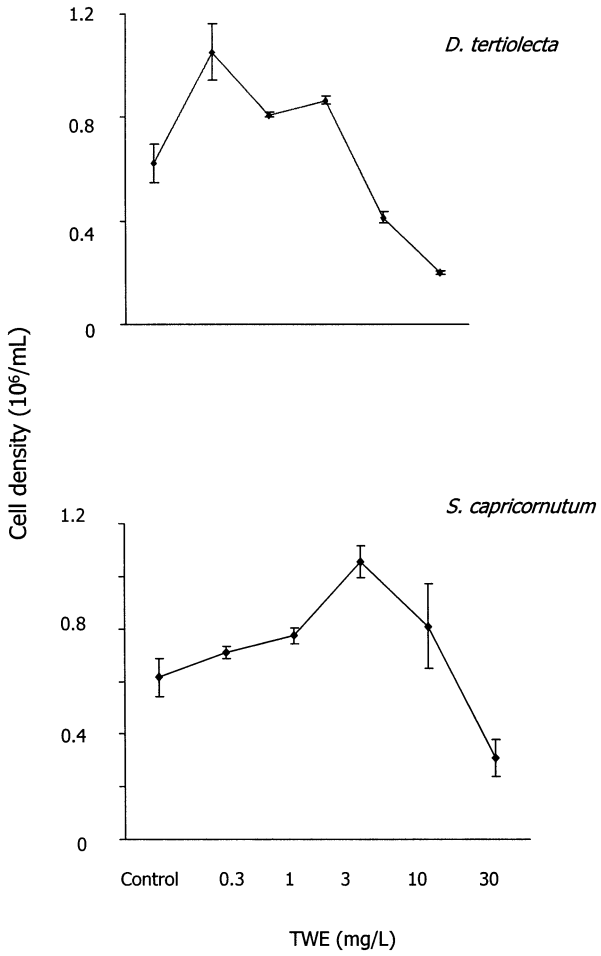

Fig. 8. TWE-modulated cell growth in *D. tertiolecta* and *S. capricornutum*. Six-replicate experiment

detect. It should be noted that in any sea urchin fertilization assays defining control values as 100% (see, e.g., Ghirardini *et al.* 2001), fertilized eggs result in an undesirable loss of sensitivity for spermiotoxic effects and are necessarily blind to any hormetic effects.

Based on a serendipitous observation in an early phase of this study, a hormetic effect in larval quality was suspected in sea urchin larvae exposed to low-level FT suspension. Hence, a new method was needed in evaluating any hormetic effects, based on the acceptance of low-quality control cultures, with relatively high (>30%) frequencies of spontaneous developmental defects (Figs. 2, 3, and 5). By referring to this control quality, both toxic and hormetic effects were detected, for the first time in sea urchin embryotoxicity bioassays. The results pointed to the occurrence of significant effects in terms of hormesis associated both with low-level TWE (0.1 mg/L) and with the induction of inhibitory effects (as decreased fertilization success or increased developmental defects) following high-level TWE (up to 30 mg/L). In terms of developmental defects, these effects were significant by evaluating TWE-induced effects in the cultures displaying <70% viable plutei, whereas "high-quality" cultures (with ≥70% viable plutei) failed to provide significant effects. Hence, one may conclude that such effects would be overlooked by applying the traditional criteria assuming that only cultures with high-quality controls should be taken into account. It should be noticed that the same sensitivity to toxicants was expressed by either low- or high-quality cultures exposed to TWE (1 to 30 mg/L) and in positive controls [Cd(II)-exposed cultures].

The data on cytogenetic analysis provided evidence for a significant mitotoxic effect of FT, not of UT, at the high level of 1 g/L (Fig. 6). This effect, again, showed a biphasic dose-related trend, although a low-level FT-associated mitogenic effect was not significant. An increase in mitotic aberrations was suggested by the results, yet statistical significance was observed in UT-exposed, rather than in FT-exposed embryos (Table 2). This apparent inconsistence might be accounted by the FT-associated mitotoxic action, preventing a suitable observation of mitotic aberrations (detected in active mitoses).

The results of *D. magna* bioassays provided evidence for a monotonic dose-related increase in immobilized daphnids (Fig. 7). Independently of the relatively lower sensitivity of *D. magna* versus sea urchins, the lack of any biphasic dose-related trend could be attributed to the optimal performance (100% motility) in *D. magna* control cultures since, by definition, any hormetic effects may only be detected by a positive shift versus control values.

Algal growth bioassays, both in *D. tertiolecta* and in *S. capricomutum*, provided evidence for a biphasic dose-related trend, with maximum algal growth at TWE levels ranging from 0.3 to 3 mg/L, in agreement with the sea urchin bioassay data. When attempting to transfer the present laboratory data to algal growth in environmentally realistic conditions, one might suggest that water contamination by low-level toxicants might partly contribute to algal blooms, in addition to or as a possible alternative to the well-established events of nutrient pollution.

Together, the present study may suggest a reappraisal in the design of a number of bioassay protocols based on the assumption that suboptimal control quality should be accepted as a current criterion leading to the observation of both hormetic and inhibitory outcomes. The growing relevance of hormesis in the impact of environmental contaminants and for regulatory purposes makes the introduction of these novel criteria in control quality as a mandatory task in the evaluation of environmental agents.

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