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# INVESTIGATION OF T-2 MYCOTOXIN-INDUCED CYTOTOXICITY *IN VITRO* AND PROTECTIVE EFFECTS OF FLAVONOID COMPOUNDS (U)

by

R.J.F. Markham, V.L. DiNinno, N.P. Erhardt,
D. Penman and A.R. Bhatti



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ABSTRACT

Aspects of the in vitro cytotoxic effects of T-2 mycotoxin on murine thymocytes were investigated. Cytotoxicity was found to be consistent when tested bi-weekly for a four month period. Cytotoxicity reached maximal values over a narrow range of doses and was dependent on temperature (37°C optimum) and number of cells in the reaction mixtures. Quercetin, a flavonoid compound was able to decrease the effect of T-2 toxin when the drug was added within an hour of mixing the T-2 toxin with the thymocytes.

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## INTRODUCTION

Trichothecene mycotoxins are a chemically related group of secondary metabolites of Fusarium and some other fungal species which have been shown to be toxic in both man and animals. Mycotoxins have been implicated as the cause of inadvertent food intoxication following fungal contamination of foodstuffs (1-3) and recent studies have suggested their deliberate use as biological warfare agents (4, 5). Many organ systems in the body can be

affected by this toxin through its radiomimetic effect i.e. its ability to inhibit protein, RNA and DNA synthesis in actively growing cells (6). Hence, a major target of its action is the haematopoietic system resulting in anemia, leukopenia and immunological aberrations (reviewed in 7). To date there are no effective drugs or treatment regimens for this type of poisoning.

Previous work from this laboratory has shown that mycotoxins, in particular T-2 toxin, have potent cytotoxic capabilities in vitro expressed as a loss of viability of thymocytes and other leukocytes (8). The intent of the present communication is to suggest possible mechanisms of this cytotoxicity and to describe compounds which offer some protection against T-2 induced cytotoxicity.

## MATERIALS AND METHODS

### Animals

C-57 black male mice, 3-6 weeks of age, were used in all but one experiment, in which case mice up to 24 weeks of age were used. They were housed and cared for in accordance with guidelines prescribed by the Canadian Council on Animal Care.

### Collection of Cells and Culture

Isolated thymus cells were prepared as described by Mischell and Shiigi (9). Briefly, mice were sacrificed by $CO_2$ inhalation and the thorax was flooded with 70% alcohol prior to removal of the thymus. For each assay at least two thymuses were collected. After removal, the thymuses were placed in a petri dish containing Joklik's media (Flow Laboratories, McLean, Virginia) and minced with scissors to dissociate cells. The cell suspension was washed by centrifugation and the pellet was resuspended in the same medium to give a concentration of $2 \times 10^7$ cells/ml.

## Cytotoxicity Assay

T-2 toxin and other mycotoxins (Sigma Chemical Company) were dissolved in dimethylsulphoxide (DMSO) to give a stock solution of 20 mg/ml. Further dilution in DMSO allowed addition of specified amounts of mycotoxin to 0.5 ml of cell suspension ($1 \times 10^7$ cells). The amount of DMSO added did not exceed 25 µl and controls, containing cells and DMSO alone, were tested to detect any inherent cytotoxicity of DMSO.

Cytotoxicity was assessed by determining viability of thymocytes using dye exclusion with 0.2% eosin (9). At specified times after addition of T-2 toxin to the cells, an aliquot of the mixture was removed and mixed with an equal volume of eosin solution. Cells were examined microscopically using a Neubauer hemocytometer and viability of at least 200 cells was determined.

Quercetin or other flavonoid compounds were dissolved in DMSO and added to the T-2/thymocyte mixture at specified times and concentrations. Controls were included to determine if quercetin and additional DMSO had a cytotoxic effect on the thymocytes.

## RESULTS

Figure 1 shows the results of 8 separate experiments performed at 2 week intervals for 14 weeks. Despite possible variation between mice of the same and different ages and week to week variation there was a consistant pattern of cytotoxicity induced by T-2 toxin. For the controls, the mean percentages ($n = 8$) of viable cells at 0, 2 and 4 hours of incubation were 89.4 ± 5, 93.0 ± 5 and 91.0 ± 6, respectively. In the presence of T-2 toxin the mean percentage of viable cells at these time points were 84.9 ± 6, 36.3 ± 10 and 6.6 ± 4.

Previous work performed in this laboratory determined that 400 µg/ml T-2 mixed with 1 x $10^7$ cells could produce significant cytotoxicity in the thymocytes (8). To further define the dose response relationship of T-2 toxin cytotoxicity, thymocytes were incubated with a lower range of doses of T-2 toxin. Figure 2 indicates that T-2 toxin has a narrow dose response curve. Cytotoxicity is evident at 175 µg/ml and reaches maximal levels at 200 µg/ml. Similarily, increasing the numbers of cells decreased the cytotoxicity induced by a constant amount of T-2 toxin (Figure 3) suggesting that a critical concentration is required before cytotoxicity can be expressed.

The temperature dependence of T-2 induced cytotoxicity is shown in Figure 4. Full expression of cytotoxicity was not apparent until the incubation temperature was raised to 37°C.

T-2 proved to be the most toxic of the mycotoxins tested in this system (Figure 5) and subsequently was used in the evaluation of protective compounds.

Addition of flavonoid compounds afforded protection against T-2 induced cytotoxicity. In Figure 6A, quercetin is shown to give a significant reduction of 44% in the cytotoxicity induced by 400 µg/ml T-2 toxin. In separate experiments and with a different preparation of T-2 toxin, quercetin was able to decrease the cytotoxic effect of 200 µg/ml T-2 by 37% (Figure 6B). Quercetin was most effective when added before or at the time of addition of the T-2 toxin to the thymocytes. Delaying the addition of quercetin by 2 hours significantly reduced its protective effect (Figure 7).

Quercetin was active over a range of concentrations (Table 1) with an apparent decrease in effect with increasing concentration. This may be due to an observed cytotoxic effect of the drug itself (Figure 8).

## DISCUSSION

Although the toxic effects of T-2 toxin and other trichothecene mycotoxins have been known for some time, it is only recently that research has focused on the cellular and molecular basis of this toxicity. It is important that this knowledge be obtained as it will allow for a more logical and fruitful search for compounds which may be able to counteract the effects of T-2 toxin.

It has been suggested that inhibition of protein synthesis and, to a lesser degree, inhibition of DNA and RNA synthesis is responsible for the observed toxic effects of T-2 toxin (10, 11). More recent studies, however, have focused on possible membrane effects as mechanisms of cytotoxicity. Ethanol, detergents and other membrane modifying agents increase sensitivity of yeast to T-2 toxin as measured by inhibition of growth (12). Decreased temperatures, known to decrease membrane fluidity, also decrease the effect of T-2 toxin. It was also found that a yeast with reduced plasma membrane fluidity was resistant to T-2 toxin. Additional evidence for a major role of membranes in T-2 toxin induced cytotoxicity comes from studies of mycoplasma (13). Although T-2 toxin inhibited growth of the organism, no changes in gross protein DNA or RNA synthesis could be observed although precursors of these macromolecules were inhibited from crossing the cell membrane into the cytosol.

Membrane changes have also been implicated in T-2 toxin induced damage in eukaryotic cells. Hemolysis of red blood cells has been studied and an effect on the cell membrane has been described (14, 15). Hemolysis was evident over a narrow dose range and was temperature dependent. It was concluded that T-2 toxin induced membrane lesions of less than 5.5 A which precipitated osmotic lysis.

Our studies suggest that interaction of T-2 toxin with thymocyte membranes is involved in the observed cytotoxicity. The narrow range of doses which distinguish between cytotoxicity and non-cytotoxicity (as observed in red cells) is similar to the effect of certain anesthetics (16, 17) and polyene antibiotics (18). These agents are incorporated into cell membranes up to a critical point after which damage quickly ensues. Their ability to intercollate into the membrane structure is likely responsible for their action. Likewise, due to the amphipathic nature of the T-2 toxin molecule (15), it too may have membrane reactivity and be able to bind to hydrophobic sites in that structure. T-2 toxin has been shown to bind to the membranes of thymocytes although the exact nature of the receptor was not identified (20). The dependence on temperature for cytotoxicity supports the concept that membranes could be involved as fluidity is dramatically altered by decreased temperature. The fact that T-2 toxin is the most active of the mycotoxins tested adds further evidence for the membrane being targets for its action. The HT-2 toxin has hydroxyl group at C4, whereas the T-2 toxin has a acetyl group at this position. It may be that increasing the hydrophobicity of the T-2 toxin molecule in this manner allowed better membrane interaction. A reported age difference in the susceptability to T-2 toxin cytotoxicity in mice (19) may reside in membrane differences. Although we could not see any age effects between recently weaned and older mice, there may have been a difference if cells from newborn animals had been used.

Quercetin (3,3'4,5,7-pentahydroxyflavone) is among a large number of flavonoid compounds commonly found in a variety of fruits and vegetables. Flavonoid compounds have been reported to have substantial biological activity and have been used as antitumor drugs, heart stimulants, diuretics, antivirals and antihistaminics (21). Some of these compounds have been shown to protective against whole body irradiation which is significant with the knowledge of the radiomimetic effects of the mycotoxins (22). Quercetin itself has been investigated for its ability to reduce histamine release from mast cells as well as other anti-inflammatory properties (23). Quercetin, in

our experiments, clearly had a protective effect against T-2 toxin induced cytotoxicity. It was necessary for quercetin to be present during the early stages for full protection to be manifested.

There is no clear evidence of how these flavonoids, including quercetin, exert these effects. Quercetin has been shown to decrease calcium transport across membranes (24), inhibit NADPH oxidase in neutrophils (25) and decrease spontaneous lipid peroxidation following glutathione depletion in rats (26). Other flavonoids have similar antioxidant activity (27). From evidence implicating participation of cell membranes in the cytotoxicity, it may be possible that activities relating to inhibition of lipid peroxidation or cellular enzyme activation may be involved. This protection does not appear to be a general property of the flavonoids as related compounds do not possess the same protective effect as quercetin (data not shown). Rutin, in particular, a precursor molecule of quercetin, shows little activity. This molecule is far more hydrophilic than is quercetin and it may be that the interaction of quercetin with membranes in a competitive manner may be a mechanism of protection against membrane active T-2 toxin. Similar structure/function relationships have been found for other biological activities of flavonoid compounds (28).

It is imperative that the mechanisms of cytotoxicity of mycotoxins be elucidated so appropriate countermeasures can be devised.

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TABLE 1 Relation Between Concentration and Protective Effect of Quercetin on Cytotoxicity of T-2 Toxin on Murine Thymocytes.

| Concentration of Quercetin (µg/ml) | Protection* (%) |
|---|---|
| 5 | 29 ± 6** |
| 10 | 37 ± 4 |
| 30 | 33 ± 4 |
| 50 | 23 |

* Present reduction in observed cytotoxicity of T-2 toxin

** Data represents mean ± 1 standard deviation of at least three (3) separate trials

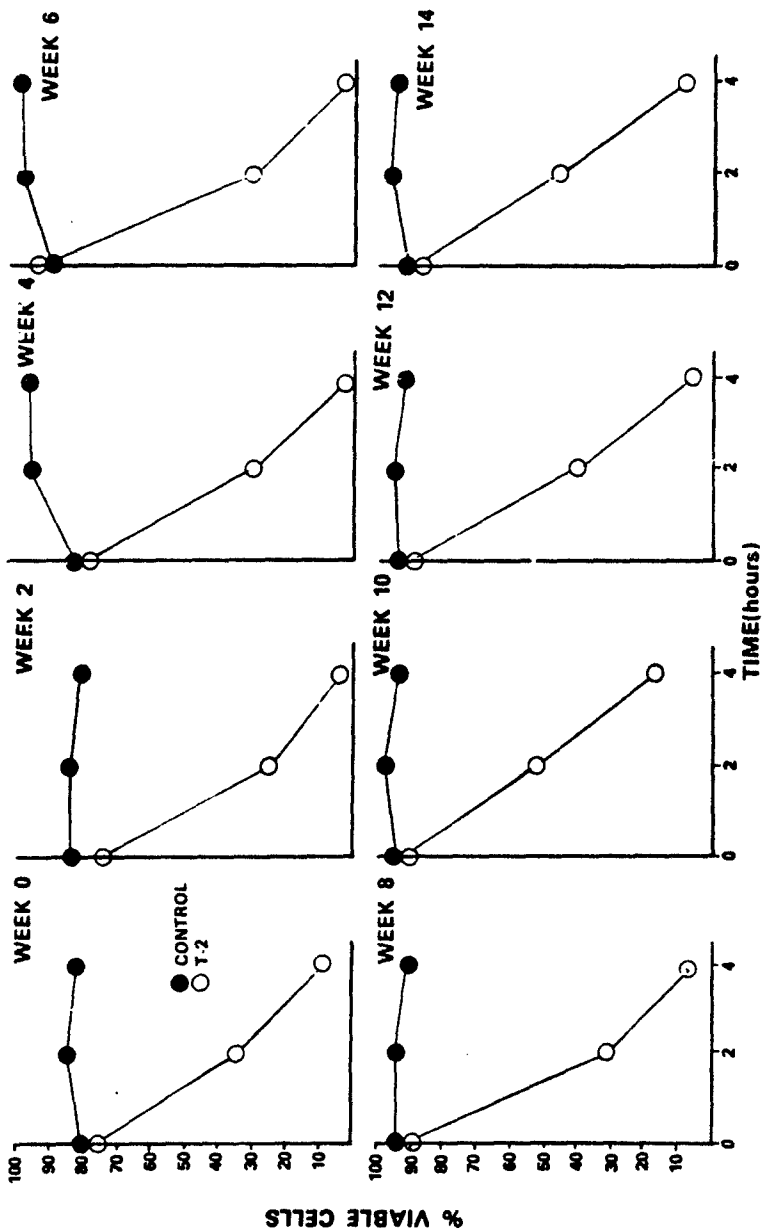



**Figure 1**

**Longitudinal studies of the cytotoxicity of T-2 toxin on murine thymocytes. ● control cells incubated with DMSO; ○ cells incubated with 200 μg/mL T-2 toxin. Thymus cells from two mice were used in each experiment. Mean values ± 1 standard deviation for control cells and T-2 treated cells at 0, 2 and 4 hour are 89.4 ± 5, 93.0 ± 5 91.0 ± 6 and 84.9 ± 36.3 ± 10 and 6.6 ± 4 respectively.**

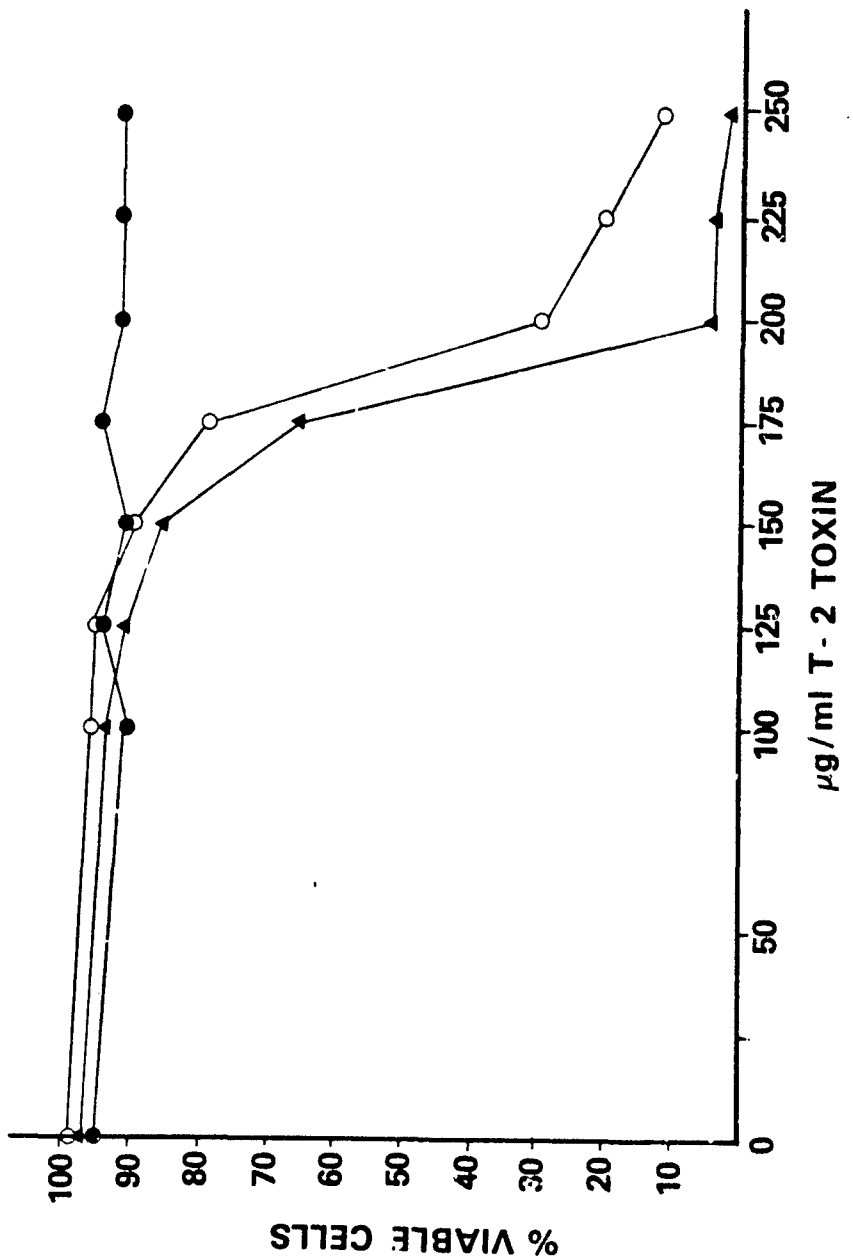



Figure 2

Effect of concentration on the cytotoxicity of T-2 toxin on murine thymocytes. ● cytotoxicity measured at 1 hr; ○ cytotoxicity measured at 2 hr; ▲ cytotoxicity measured at 5 hr. Cells representing a pool of 3 mice were treated with different amounts of T-2 toxin.

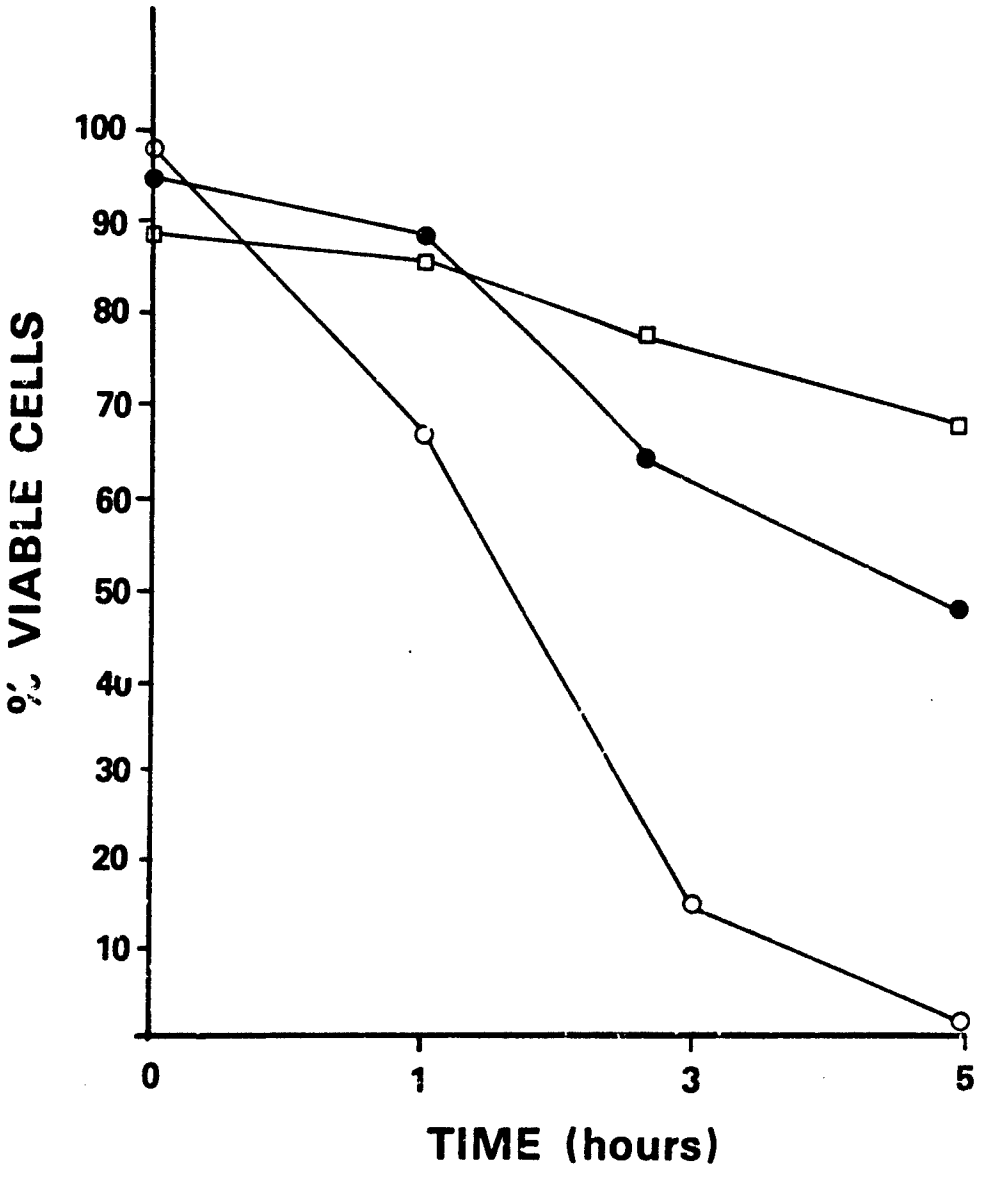



**Figure 3**

**Effect of cell number on the cytotoxicity of T-2 toxin on murine thymocytes. ○ 2 x $10^7$ cells/mL; ● 4 x $10^7$ cells/mL; □ 6 x $10^7$ cells/mL. All cell preparations were incubated with 200 μg/mL T-2 toxin. Cells representing a pool of 3 mice were used.**

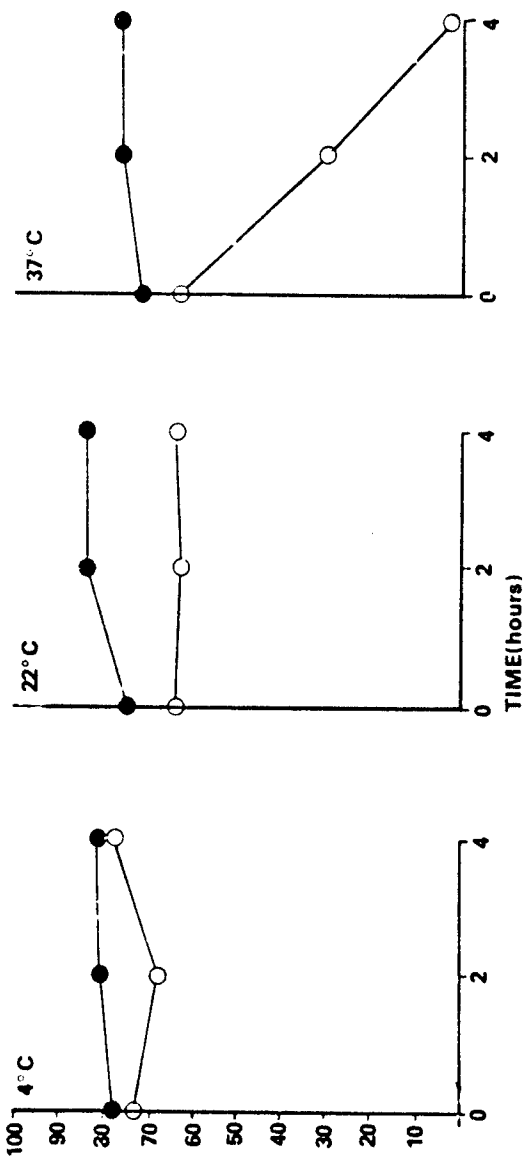



Figure 4

Effect of temperature on the cytotoxicity of T-2 toxin on murine thymocytes. ● control cells incubated with DMSO; ○ cells incubated with 200 μg/mL T-2 toxin. Cells representing a pool of 3 mice were incubated at the different temperatures.

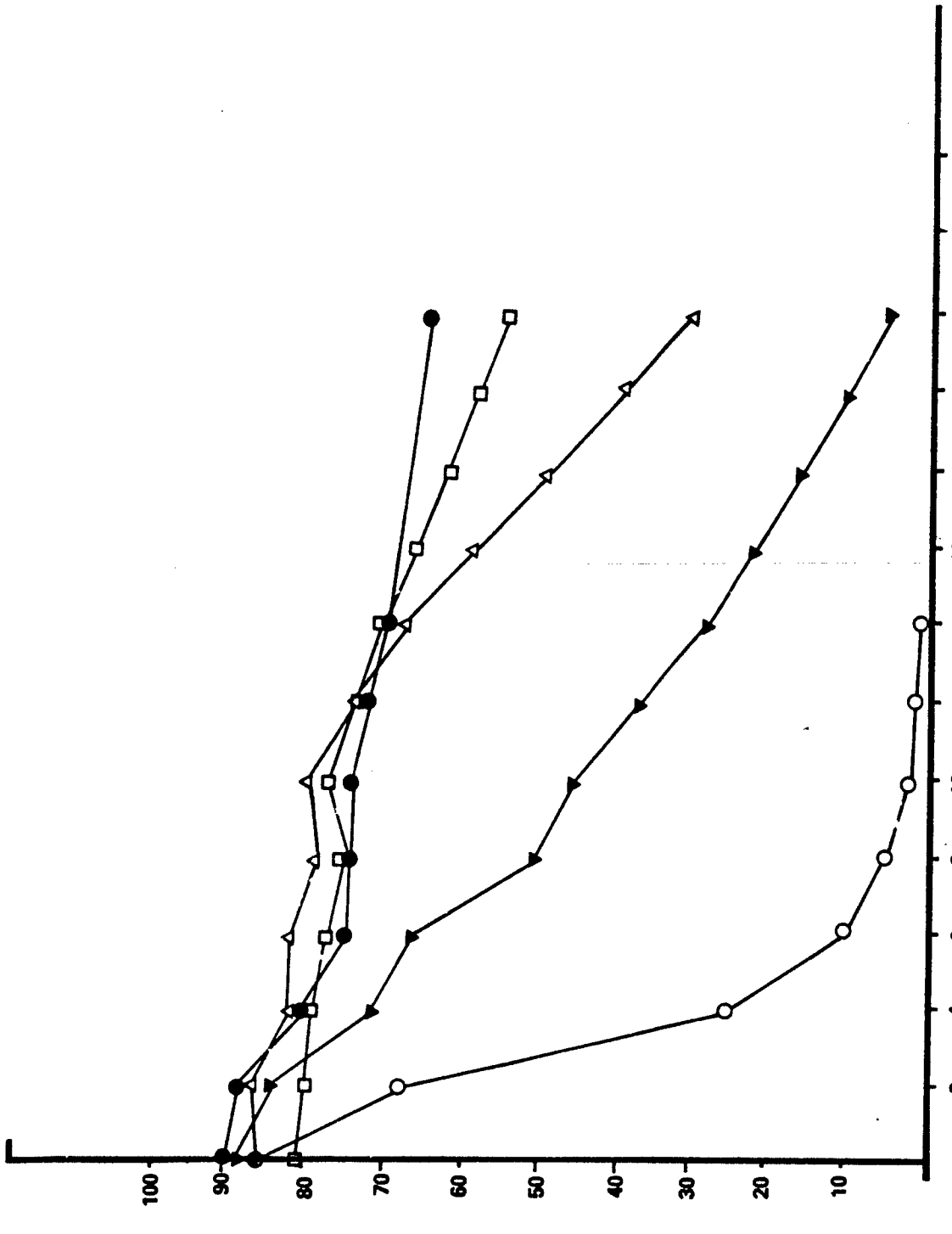
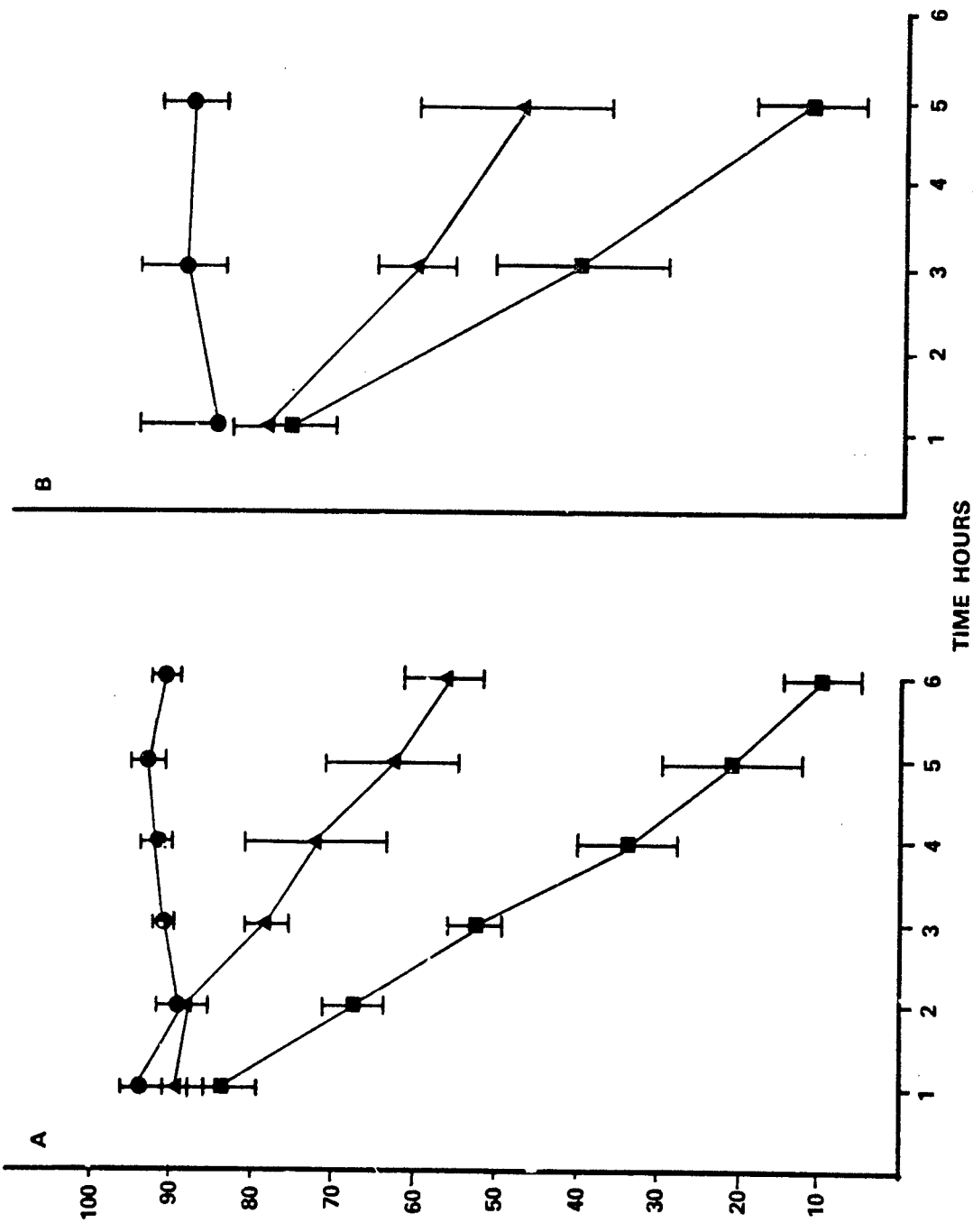



Figure 5

Comparative cytotoxicity of various mycotoxins on murine thymocytes. ● control cells incubated with DMSO; ○ cells incubated with T-2; ▼ HT-2; △ T-2 triol; □ T-2 tetrol. Cells representing a pool of 3 mice were incubated with 400 μg/mL of each of the compounds




A. Protective effect of quercetin on cytotoxicity of T-2 toxin on murine thymocytes. ● control cells incubated with DMSO; ■ cells incubated with 400 μg/mL T-2 toxin; ▲ cells incubated with 400 μg/mL T-2 toxin and 10 μg/mL quercetin. Data represents mean ± 1 standard deviation of at least 4 separate trials.

B. Protective effect of quercetin on cytotoxicity of T-2 toxin on murine thymocytes. ● control cells incubated with DMSO; ■ cells incubated with 200 μg/mL T-2 toxin; ▲ cells incubated with 200 μg/mL T-2 toxin and 10 μg/mL quercetin. Data represents mean ± 1 standard deviation of at least 4 separate trials.

Figure 6

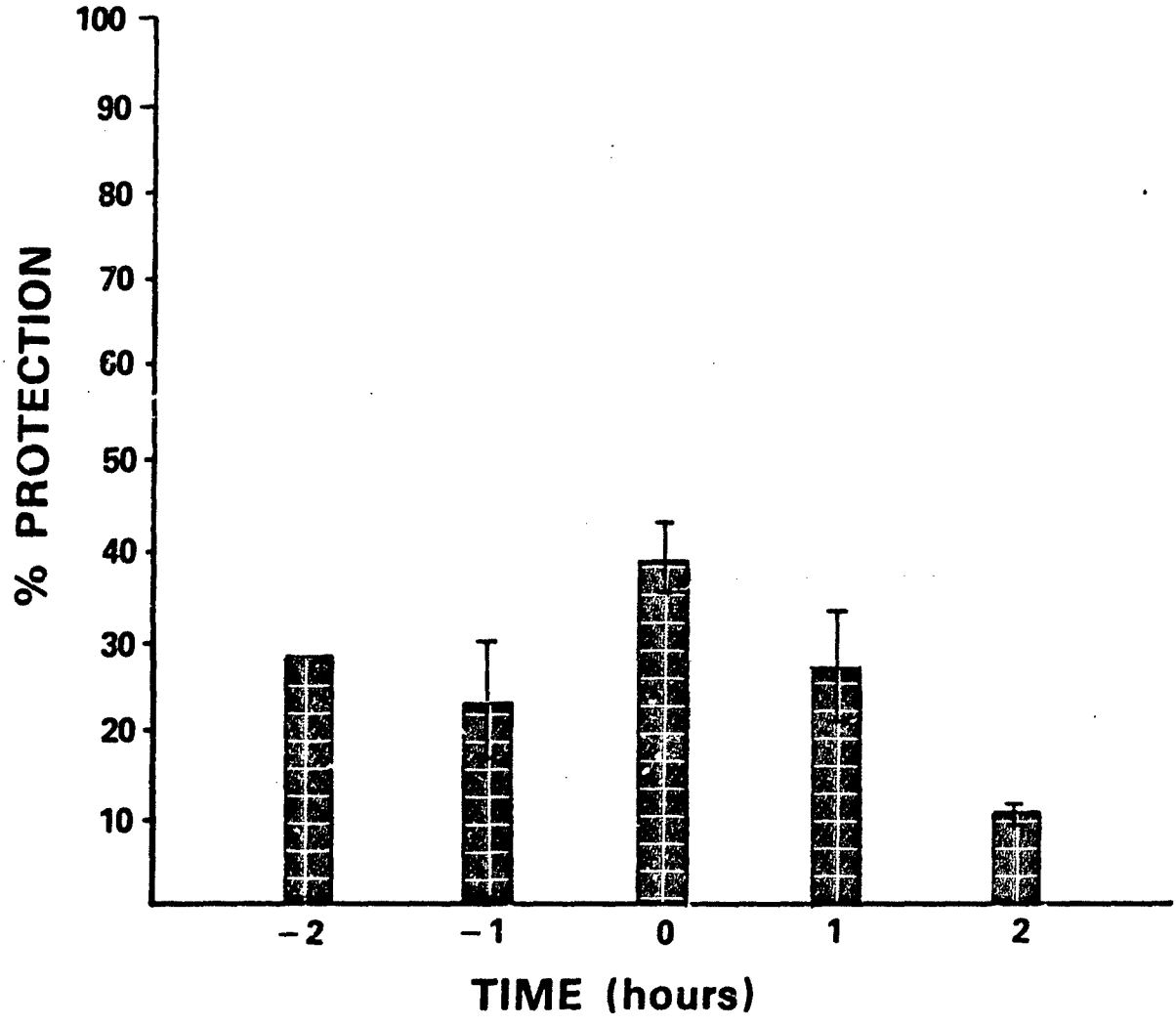



**Figure 7**

**The effect of time of addition of quercetin on protection from cytotoxicity of T-2 toxin on murine thymocytes. Cells were mixed with 200 μg/mL T-2 toxin at 0 hr. 10 μg/mL quercetin was added at times indicated. Cytotoxicity Assay was performed 5 hours after addition of T-2 toxin. Except for −2 hour, which was a single trial, data represents the mean ± 1 standard deviation of at least 3 separate trials.**

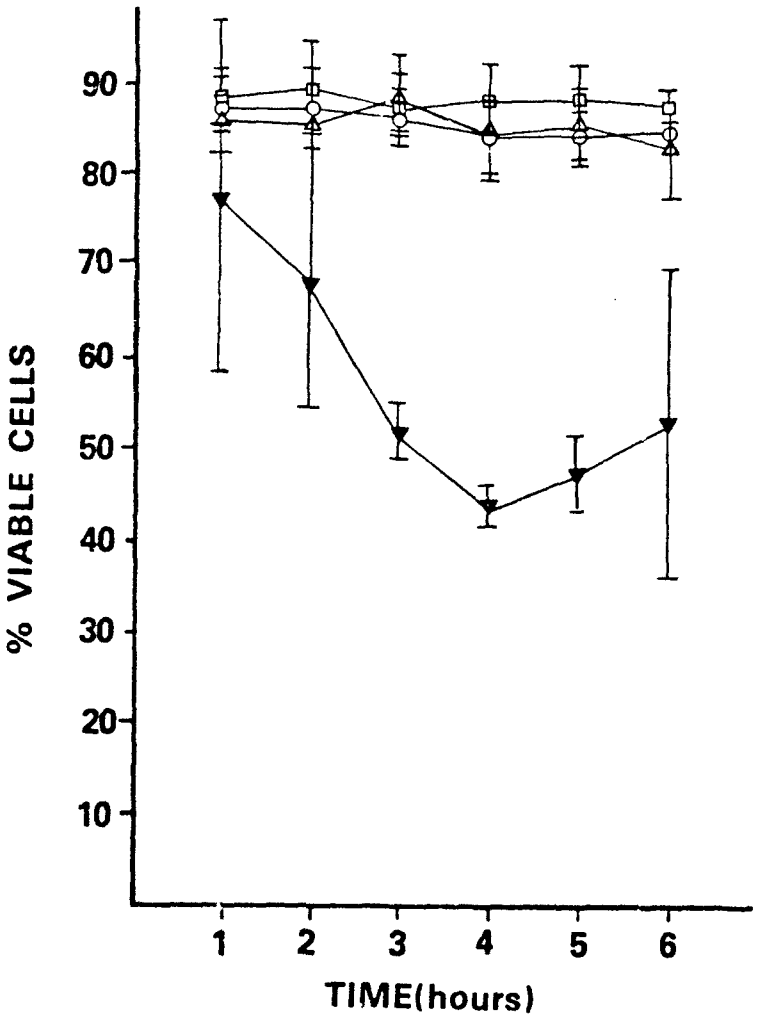



**Figure 8**

**Effect of quercetin on murine thymocytes. ○ control cells; cells incubated with □ 5 μg/mL; △ 10 μg/mL; ▼ 50 μg/mL quercetin. Data represents the mean ± 1 standard deviation of at least 3 separate trials.**

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13. ABSTRACT

Aspects of the in vitro cytotoxic effects of T-2 mycotoxin on murine thymocytes were investigated. Cytotoxicity was found to be consistent when tested bi-weekly for a four month period. Cytotoxicity was found to be consistent when over a narrow range of doses and was dependent on temperature (37°C optimum) and number of cells in the reaction mixtures. Quecertin, a flavonoid compound was able to decrease the effect of T-2 toxin when the drug was added within an hour of mixing of T-2 toxin with the thymocytes.

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Quecertin
Flavonoids

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