Phytotoxicity of the Volatile Monoterpene Citronellal against Some Weeds

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A study was undertaken to assess the phytotoxicity of citronellal, an oxygenated monoterpenoid with an aldehyde group, towards some weedy species [*Ageratum conyzoides* L., *Chenopodium album* L., *Parthenium hysterophorus* L., *Malvastrum coromandelianum* (L.), Garcke, *Cassia occidentalis* L. and *Phalaris minor* Retz.]. A significant effect on weed emergence and early seedling growth was observed in a dose-response based laboratory bioassay in a sand culture. Emergence of all test weeds was completely inhibited at 100 μg/g sand content of citronellal. Seeds of *A. conyzoides* and *P. hysterophorus* failed to emerge even at 50 μg/g content. Root length was inhibited more compared to shoot length. The failure of root growth was attributed to the effect of citronellal on the mitotic activity of growing root tips cells as ascertained by the onion root tip bioassay. At 2.5 mM treatment of citronellal, mitosis was completely suppressed and at higher concentrations cells showed various degrees of distortion and were even enucleated. The post-emergent application of citronellal also caused visible injury in the form of chlorosis and necrosis, leading to wilting and even death of test weeds. Among the test weeds, the effect was severe on *C. album* and *P. hysterophorus*. There was loss of chlorophyll pigment and reduction in cellular respiration upon citronellal treatment indicating the impairment of photosynthetic and respiratory metabolism. Scanning electron microscopic studies in *C. occidentalis* leaves upon treatment of citronellal revealed disruption of cuticular wax, clogging of stomata and shrinkage of epidermal cells at many places. There was a rapid electrolyte leakage in the leaf tissue upon exposure to citronellal during the initial few hours. In *P. minor* electrolyte leakage in response to 2 mM citronellal was closer to the maximum leakage that was obtained upon boiling the tissue. The rapid ion leakage is indicative of the severe effect of citronellal on the membrane structure and loss of membrane integrity. In all, the study concludes that citronellal causes a severe phytotoxicity on the weeds.

Key words: Post-Emergent Herbicidal Activity, Mitotic Inhibitor, Membrane Integrity

Introduction

Monoterpenoids, the constituents of volatile essential oil, are the simplest chemical molecules of the terpenoid family with a multitude of biological and ecological functions. Because of their natural aroma they find an extensive use in food and fragrance industry and are used in aromatherapy. In the natural ecosystems they play an important role in plant-plant interactions, defense mechanism (as plant protectants), herbivory and as pollinator attractants and thus help in maintaining delicate balance in ecosystems (Swain, 1977; Fischer, 1991; Vokou, 1999; Paiva, 2000). Being potent inhibitors of seed germination monoterpenes are involved in allelopathic interactions among plants and thus play an important role in structuring and patterning of plant communities (Muller, 1965; Asplund, 1968; Abrahim *et al.*, 2000; Singh *et al.*, 2002a, b; Weidenhamer *et al.*, 1993; Vokou, 1999). This property of monoterpenoids coupled with their biodegradable nature and little toxicity against mammals and other non-target species makes them chemicals of immense interest and potential for agroindustry (Isman, 2000; Beuchat, 2001). Some studies have already reported their potential use for weed and pest management in sustainable agriculture (Isman, 2000; Romagni *et al.*, 2000; Singh *et al.*, 2002a, b). Monoterpenes like citronellal, citronellol, linalool and cineole have been found to inhibit germination and initial seedling growth of weeds such as *Cassia occidentalis*, *Amaranthus viridis, Echinochloa crus-galli*, and *Bidens pilosa* under *in vitro* conditions (Singh *et al.*, 2002b, 2004). These studies have concluded that citronellal, an oxygenated monoterpene with an

aldehyde group, is the most potent germination inhibitor and deleteriously affects early seedling growth and dry weight. Citronellal also known as rhodinal or 3,7-dimethyl-6-octen-1-al ($C_{10}H_{18}O$; M = 154.3 g/mol) is a major constituent of essential oil with lemon-scent from a number of plant species such as *Cymbopogon* spp., *Citrus* spp., *Eucalyptus citriodora* and *Melissa officinalis*. In spite of abundant availability and potent phytotoxicity, the herbicidal potential of citronellal remains to be determined. It is thus imperative to explore its potential activity against a wide range of weeds – both grassy and broad-leaved – in terms of early plant growth, injury levels, impact on metabolic processes such as photosynthesis and respiration, effect on membrane integrity and mitotic cell division.

Material and Methods

Plant material and chemical

For the present study seeds of six weeds *viz.* billy goat weed (*Ageratum conyzoides* L.), common lambsquarters (*Chenopodium album* L.), ragweed parthenium (*Parthenium hysterophorus* L.), prickly malvastrum [*Malvastrum coromandelianum* (L.) Garcke], coffee weed (*Cassia occidentalis* L.) and littleseed canarygrass (*Phalaris minor* Retz.) were collected locally from the agricultural fields on the outskirts of Chandigarh, India. (±)–Citronellal used for the present work was of technical grade (94% purity) and procured from Alfa Aesar Co., Massachusetts, USA.

Dose-response studies

Dose-response experiments were conducted under controlled laboratory conditions by the method of Romagni *et al.* (2000) to determine the effect of different contents of citronellal on emergence and early seedling growth of test weeds. Petri dishes (15 cm diameter) were lined with a circle of Whatman no. 1 filter paper. These were treated with different amounts of citronellal so as to get 5, 10, 25, 50 and 100 μg citronellal/g sand. After treatment, nearly 400 g sand was placed on the top of a filter circle, sown with weed seeds (25 of *A. conyzoides*, *C. album*, *P. hysterophorus, P. minor*; 15 of *M. coromandelianum*, *C. occidentalis*) and covered with lids. 100 ml of water were added to moisten the sand. Afterwards, the Petri dishes were sealed with cello tape and parafilm to avoid loss of citronellal upon volatilization. Treatment without citronellal served as control for each of the weed types. Five replications were maintained per treatment and for each weed type in a completely randomized manner in a growth chamber set at (25 ± 2) °C temperature, 16 h/8 h light/dark period of approx. 150 μmol/m^2/s photosynthetic photon flux density and a relative humidity of (75 ± 2)%. After 2 weeks, number of seedlings emerged and their root and shoot length were determined.

Effect on mitotic activity

The squash technique was used to study the impact of citronellal on the mitotic activity as per the method of Armbruster *et al.* (1991) with slight modifications. Onion root tips were used for this purpose as these are the standard bioassay material for determining the impact on mitotic cell division. Onion bulbs were placed on the test tubes filled with water to raise roots. On the 5th day roots were excised and subjected to treatment with 2.5 and 5.0 mM of citronellal or distilled water (as control) for 24 h. These were then fixed in ethyl alcohol/glacial acetic acid (3:1, v/v) for 24 h. Next day roots were rinsed with distilled water thrice followed by dipping in 70% ethyl alcohol for another 24 h. Then, these were hydrolyzed with 1 N HCl for 1 min at 25 °C followed by staining with Schiff's reagent for half an hour. After staining, 2 root tips were macerated in one drop of 40% glacial acetic acid on a glass slide, covered with a cover slip and sealed with a clear nail polish. The slides were then observed under a bright field laboratory microscope (Getner, India, model 66475). At least five replicates were maintained per treatment.

Determination of post-emergent activity

It was determined under greenhouse conditions by raising the weed plants under controlled conditions and spray treating them with citronellal.

Raising of weeds

Plants of all the six test weeds were raised from collected seeds in 12 cm diameter pots under greenhouse conditions with a 14 h/10 h light/dark photoperiod of approx. 170 μmol/m^2/s photon flux density, day/night temperature of (25 ± 2) °C/(14 ± 2) °C, and a relative humidity of around

75%. For this, 1 kg of garden soil (soil : sand 3:1 w/w) was taken in each pot and seven seeds of each weed species were sown per pot. One week after emergence, the plants were thinned to three plants per pot. Plants were watered every alternate day and after 3 weeks they were flushed with half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950). When the weed plants were six weeks old, they were used for citronellal treatment.

Spray treatment

To determine the post-emergent activity of citronellal, six-week-old weed plants were spray-treated with 7.5, 15, 30 and 60 mg/ml solution of citronellal or distilled water (to serve as control) at the rate of 100 ml/m^2. Five replicates were maintained for each weed species in a completely randomized manner. 1 d after spraying, the treated plants were observed for visible injury levels in terms of percent chlorotic or necrotic areas. In addition, the leaves were sampled from all the treatments for the determination of total chlorophyll content and cellular respiration.

Scanning electron microscopy (SEM) studies

To visualize the changes in the leaf structural morphology due to citronellal treatment leaves of *C. occidentalis* plucked from the citronellal-treated plants were subjected to scanning electron microscopy (SEM) studies. Leaf segments (5 mm size) were rinsed with distilled water twice and fixed in glutaraldehyde (4%, v/v, in 0.2 M phosphate buffer, pH 7) for 1 h and then dipped in 0.2 M phosphate buffer (pH 7) for another hour. They were then subjected to dehydration in a series of solvents including 50%, 70%, and 80% acetone for 15 min each, 90% acetone for 20 min, 100% acetone for 1 h, acetone/amyl acetate (1:1 v/v) for 15 min, and finally in pure amyl acetate for another 15 min. It was followed by critical point drying (CPD) and mounting on a SEM stub using a double adhesive tape. Samples were then sputtered with a gold film using an ion-beam sputter coater (JEOL, JFC 1100) and finally examined at different acceleration voltages in a JEOL, JSM 6100 scanning electron microscope. Secondary electron images were taken with a Pentax K 1000 camera at different magnifications and the changes in the leaf surface morphology were observed in both control and citronellal-treated leaves.

Estimation of chlorophyll content

Chlorophyll was extracted from 25 mg leaves in 4 ml of dimethyl sulphoxide following the method of Hiscox and Israelstam (1979). Its concentration was determined spectrophotometrically using the equation of Arnon (1949) and expressed in terms of tissue dry weight as suggested by Rani and Kohli (1991).

Determination of cellular respiration

Respiratory values were determined from the fresh plant tissue indirectly using 2,3,5-triphenyl tetrazolium chloride following the method of Steponkus and Lanphear (1967). This is an indirect measurement of cell respiration whereby formation of red formazan occurs due to trapping of the oxygen molecules released through the respiratory chain. The values of treated samples were expressed as % respiration with respect to control.

Effect of citronellal on membrane integrity

Since the citronellal spray-treated plants were wilted in appearance indicating severe electrolyte leakage and thus loss of membrane integrity in response to citronellal treatment, the possible effect on electrolyte leakage vis-à-vis membrane permeability was studied on two weeds – one grassy (*Phalaris minor*) and one broad-leaved (*Ageratum conyzoides*) – as per the method of Duke and Kenyon (1993). Fresh leaves (100 mg) from 6-week-old weed plants were dipped in 5 ml of 1 mM MES [2-(*N*-morpholino)ethanesulfonic acid sodium salt] buffer containing 2% sucrose and citronellal (2 mM) dissolved in Tween 80. A parallel control was also run with everything except monoterpenes. The electrical conductivity of the bathing medium containing plant tissue and volatile monoterpenes was measured in darkness at regular intervals for 20 h followed by exposure to light for 10 h. Conductivity of the boiled leaf samples boiled for 5 min was measured to express the maximum electrolyte leakage. Five replicates were kept for each weed species and each treatment and the experiment was repeated.

Statistical analysis

All the experiments were performed in a completely randomized block design with at least five replications. Data were subjected to one-way analysis of variance followed by separation of means.

Different treatments were compared with control using the Dunnett's test at $p < 0.01$ and 0.05.

Results and Discussion

Emergence of test weeds was significantly reduced when sown in soil treated with different contents of citronellal. None of the seeds of any weed type could emerge in response to 100 μg citronellal/g sand. Among the weed species, *A. conyzoides* was affected the most followed by *P. hysterophorus* and in both of them no emergence was observed even at 50 μg citronellal/g sand treatment. On the other hand, *P. minor* (a grassy weed) was relatively less affected compared to other weeds. Not only the emergence even the seedling growth (both root and shoot length) were severely reduced in response to citronellal (Table I). The inhibitory effect increased with increasing amount of citronellal treatment and it was species-specific. The inhibition of seedling growth indicates that citronellal affects the division of meristematic cells thereby causing growth inhibition. The effect was more pronounced on root growth compared to shoot length indicating that citronellal severely affects the root division. The precise reasons for such an observation remain obscure. However, the available literature point that monoterpenes, in general, inhibit the mitotic activity of growing cells and this in turn adversely affects the root elongation (Lorber and Muller, 1976; Vaughn, 1991; Vaughan and Spencer, 1993; Romagni *et al.*, 2000). In the present study, it was observed that citronel-

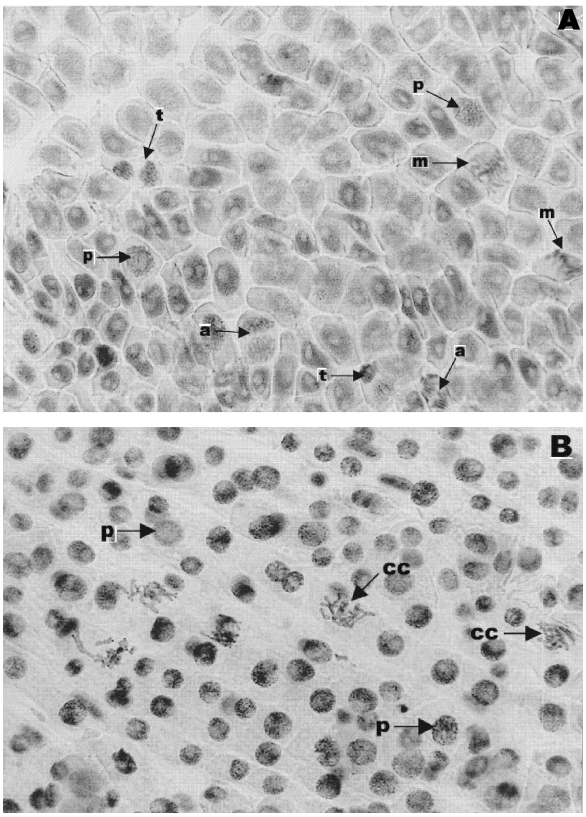

Fig. 1. Photomicrographs of (A) control and (B) 2.5 mM of citronellal on the mitotic activity in onion root tip cells. Arrows indicate different stages of mitosis; p, prophase; m, metaphase; a, anaphase; t, telophase; cc, condensed chromosomes.

Table I. Effect of citronellal on the root (mm) and shoot length (mm) of selected weeds. Values in parenthesis represent standard deviation.

Citronellal content [μg/g sand]	*A. conyzoides*		*C. album*		*P. minor*		*M. coromandelianum*		*P. hysterophorus*		*C. occidentalis*	
	RL	SL	RL	SL	RL	SL	RL	SL	RL	SL	RL	SL
0	23.7 (0.82)	41.2 (1.37)	30.1 (1.97)	60.1 (2.02)	31.5 (2.12)	59.7 (2.09)	32.4 (1.39)	57.2 (1.94)	22.4 (2.42)	42.1 (2.19)	47.5 (2.49)	61.5 (2.42)
5 (0.13 mM)	20.3* (1.14)	34.8* (1.50)	24.7* (1.58)	54.2* (1.59)	26.7* (1.67)	46.1* (1.3)	29.7* (1.46)	43.8** (2.34)	19.7* (1.32)	37.9* (2.38)	35.8* (2.19)	54.5* (1.97)
10 (0.26 mM)	16.1** (1.27)	28.2** (1.23)	20.1** (1.39)	42.9** (2.37)	20.1** (1.38)	37.9** (2.76)	21.6** (0.83)	37.6** (2.03)	13.4* (0.79)	30.6** (1.69)	31.9** (2.19)	49.5** (2.67)
25 (0.65 mM)	6.2** (0.45)	12.1** (0.36)	13.5** (1.82)	24.9** (1.34)	14.8** (0.84)	36.4** (2.19)	13.7** (1.21)	30.7** (1.35)	6.9** (0.24)	15.9** (1.28)	20.4** (1.32)	42.1** (2.38)
50 (1.30 mM)	–	–	4.2** (0.37)	17.6** (0.87)	6.7** (0.37)	20.8** (1.31)	5.9** (0.39)	18.6** (1.19)	–	–	17.2** (1.07)	30.1* (1.67)
100 (2.60 mM)	–	–	–	–	–	–	–	–	–	–	–	–

RL, root length; SL, shoot length.
* and ** represent significance from control at $p < 0.05$ and 0.01, respectively, applying Dunnett's test.

lal severely inhibits the mitotic activity in the growing cells of onion root tips (Fig. 1). In untreated control root tips most of the cells were in dividing state and showing different mitotic stages (Fig. 1A). Even at 2.5 mM citronellal concentration mitosis appeared to have been affected and most of the cells were at the prophase stage with only a few cells showing condensed chromosomes (Fig. 1B). However, at higher concentrations (5 mM) of citronellal, none of the cells was at the dividing stage but they were rather distorted in appearance and even enucleated probably owing to the rupturing of the nuclei (not presented). Such an observation is not surprising as volatile terpenes from *Salvia leucophylla* have been reported to bring a structural breakdown of cells by damaging organelle (mitochondrial) membranes (Lorber and Muller, 1976). The experiment indicates that citronellal suppresses the mitotic activity of the growing root tip cells and this in turn results in poor seedling growth.

Not only early growth, even the mature plants of test weeds were severally damaged upon postemergent application of citronellal. It caused severe visible injuries in test weeds resulting in complete wilting of weed plants. In general, application of citronellal caused symptoms like chlorotic and necrotic spots and a varying level of injuries was observed. At lower concentrations (7.5 and 15 mg/ml) injuries were less severe and reversible, whereas at higher concentrations of 60 mg/ml, they were very severe and irreversible followed by complete wilting and even shedding of leaves. Weeds like *C. album*, *P. hysterophorus* and *P. minor* did not survive at 60 mg/ml treatment of citronellal.

Based on the symptomology of chlorosis and necrosis, it was speculated that citronellal application might have caused severe damages since the plant comes in direct contact with the monoterpene. To assess this leaf surface morphology of *C. occidentalis* was examined through SEM studies. In control, cells were fully turgid, covered with prominent cuticular wax and open stomata. In contrast, in response to citronellal treatment, an apparent disintegration of cuticular wax, distortion of epidermal cells, stomatal closure and even clogging of stomatal pore were observed (pictures not presented). Probably this clogging of stomatal pore was caused by the intrusion of the dissolved cuticular wax into the pore. At many places cells appeared to have loosened and shrunken. All these changes on the leaf surface of citronellal-treated plants are indicative of a detrimental effect of citronellal on the leaf surface morphology which in turn results in altered cell physiology.

The content of chlorophyll pigment was drastically reduced in the citronellal-treated weed plants (Table II). The reduction was evident even at the lowest concentration (7.5 mg/ml) of citronellal and with increasing concentration a greater reduction in chlorophyll content was observed. With the application of 60 mg/ml citronellal, chlorophyll content was reduced by over 70% in all the test weeds. In general, the effect was more on broad-leaved weeds with maximum in *C. album*, and least on *P. minor* – a grassy weed (Table II). These observations suggest that citronellal interferes with the chlorophyll pigment resulting in bleaching of the tissue. Loss of the chlorophyll pigment due to volatile monoterpenes (cineole, citronellol, limonene and linalool) has in fact been reported by

Table II. Effect of citronellal spray treatment on the total chlorophyll content (μg/mg dry weight) in six-week-old plants of test weed species measured one day after spray. Data are presented as means ± SD and figures in parenthesis represent percent decrease over control.

Concentration [mg/ml]	*A. conyzoides*	*C. album*	*P. minor*	*M. coromandelianum*	*P. hysterophorus*	*C. occidentalis*
0	6.97 ± 0.31	18.25 ± 1.75	14.35 ± 0.78	13.05 ± 0.54	10.84 ± 0.75	13.21 ± 0.76
7.5	5.12 ± 0.23* (26.54)	14.25 ± 0.45* (21.92)	12.48 ± 0.96* (10.03)	11.25 ± 0.23* (13.80)	8.39 ± 0.47* (22.05)	10.85 ± 0.89* (17.87)
15	4.86 ± 0.51* (30.17)	11.16 ± 0.36** (38.85)	8.80 ± 1.01** (38.63)	10.37 ± 0.58* (20.54)	6.08 ± 0.34** (46.04)	7.36 ± 0.37** (44.32)
30	3.86 ± 0.33** (44.62)	3.77 ± 0.71** (79.34)	5.21 ± 0.89** (63.67)	7.67 ± 0.29** (41.23)	5.70 ± 0.50** (49.37)	5.91 ± 0.23** (55.27)
60	1.70 ± 0.14** (74.63)	2.08 ± 0.29** (88.60)	4.10 ± 0.68** (71.41)	3.39 ± 0.16** (74.02)	2.08 ± 0.16** (81.53)	3.62 ± 0.86** (72.63)

* and ** represent significance from control at $p < 0.05$ and 0.01, respectively, applying Dunnett's test.

Table III. Effect of citronellal spray treatment on the percent respiratory activity in six-week-old plants of test weed species determined one day after spray. Data are presented as means ± SE and with respect to control.

Concentration [mg/ml]	*A. conyzoides*	*C. album*	*P. minor*	*M. coromandelianum*	*P. hysterophorus*	*C. occidentalis*
7.5	86.17 ± 3.12*	74.08 ± 1.64*	91.60 ± 1.28	91.67 ± 1.84	78.91 ± 2.59*	58.04 ± 1.64**
15	63.19 ± 2.81**	33.36 ± 1.51**	81.14 ± 2.35*	82.28 ± 2.23*	58.95 ± 1.23**	37.17 ± 1.40**
30	29.37 ± 1.74**	13.08 ± 0.84**	60.34 ± 1.88**	64.26 ± 3.42**	43.04 ± 1.61**	30.88 ± 1.32**
60	11.76 ± 1.31**	7.51 ± 1.25**	30.26 ± 2.31**	21.44 ± 1.30**	13.21 ± 1.23**	12.15 ± 1.41**

* and ** represent significance from control at $p < 0.05$ and 0.01, respectively, applying Dunnett's test.

many workers (Romagni *et al.*, 2000; Singh *et al.*, 2002a, b; Ibrahim *et al.*, 2004). However, whether the loss is due to inhibition of *de novo* chlorophyll synthesis or enhancing of chlorophyll degradative pathways is unknown. In any case, it is likely to interfere with the photosynthetic machinery of the weed plants and affect their overall growth.

Further, a significant reduction in the activity of cellular respiration, measured indirectly by TTC reduction, was also observed (Table III). Reduction in respiratory activity was at maximum in *C. album* and at minimum in *P. minor* (Table III). A decrease in respiratory activity points that citronellal affects the energy metabolism of the plants by interfering with the electron transport chain. A number of monoterpenes such as α-pinene, limonene and eugenol have been reported to act as uncouplers of oxidative phosphorylation and suppress respiration (Peñuelas *et al.*, 1996; Abrahim *et al.*, 2000).

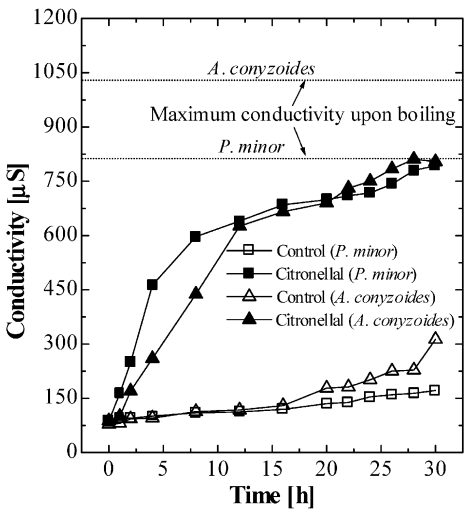

Fig. 2. Effect of citronellal (2 mM) on the electrolyte leakage in *P. minor* and *A. conyzoides*.

Since citronellal caused severe wilting in the test weed plants, the leaves of one dicot (*A. conyzoides*) and one monocot (*P. minor*) were analyzed for the possible electrolyte leakage. A rapid loss of electrolyte (ion) leakage was observed in response to citronellal treatment as indicated by increased conductivity of the bathing medium (Fig. 2). The ion loss increased with the time and was irrespective of the light. The impact on electrolyte leakage in *P. minor* after 30 h was similar to that observed after boiling the leaves for 30 min indicating that nearly complete electrolyte loss occurred after 30 h (Fig. 2). A rapid loss of electrolyte leakage indicates that citronellal disrupts the membrane integrity.

Thus, based on the present study it can be concluded that citronellal causes a severe phytotoxicity against the test weeds through various growth and physiological processes and possesses herbicidal activity that is worth to exploite.

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