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Determination Of Antifungal Activity and Phenolic Compounds Of Endemic Muscari aucheri (Boiss.) Baker Extract

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Abstract: Antifungal activity and phenolic compounds of the methanol extract (flower + peduncle) derived from *Muscari aucheri* (Boiss.) Baker that grows endemically in Turkey were identified against five different plant pathogens [*Fusarium oxysporum f. sp. cucumerinum, Alternaria solani, Verticillium dahliane, Rhizoctonia solani, Botrytis cinerea*]. Methanol extract of *M. aucheri* was obtained from the aerial parts (flower + peduncle). Antifungal activity studies were performed by examining the obtained extract against plant pathogens at the final concentrations of 2.5, 5, 10 and 20 mg/ml doses using the agar plate method. Mycelial growth inhibition and LD_{50} doses of the extract to the pathoges were determined. Significant levels of antifungal activities were observed at all the doses used in the extracts. At the same time, 100% inhibition were observed at 10 and 20 mg/mL doses used against the pathogens. Individual phenolic compound rutin (693,2 ug/g) was found in the extract at its highest level. Total phenolic compound and monomeric anthocyanin amounts were determined. Results from these findings suggest that phenolic compounds in natural antifungal agents may offer positive results in the control of plant pathogens.

Keywords: *Muscari aucheri*, phenolic compounds, antifungal activity, plant pathogens

Endemik *Muscari aucheri* (Boiss.) Baker. Ekstraktının Antifungal Aktivitesi ve Fenolik Bileşiklerinin Belirlenmesi

Öz: Türkiye'de endemik olarak yetişen *Muscari aucheri* (Boiss.) Baker bitkisinden elde edilen methanol ekstraktının (Çiçek + çiçek sapı) beş farklı bitki patojenine [*Fusarium oxysporum f. sp. cucumerinum*, *Alternaria solani*, *Verticillium dahliae*, *Rhizoctonia solani*, *Botrytis cinerea*] karşı antifungal aktivitesi ve bazı bireysel fenolik bileşikleri belirlenmiştir. *M. aucheri*'nin methanol ekstraktı toprak üstü aksamından elde edilmiştir. Elde edilen ekstraktın son konsantrasyonu 2.5, 5, 10 ve 20 mg/mL dozlarında agar petri yöntemi kullanılarak bitki patojenlerine karşı antifungal aktivite çalışmaları yürütülmüştür. Ekstraktın patojenlere olan miselyum gelişim engellemeleri ve LD_{50} dozları belirlenmiştir. Kullanılan bütün dozlar ve ekstraktda önemli düzeyde antifungal etkiler gözlenmiştir. Patojenlere karşı kullanılan 10 ve 20 mg/mL dozunda %100 engellemeler gözlenmiştir. Ekstraktda en yüksek bireysel fenolik bileşik rutin (693,2 µg/g) olarak bulunmuştur. Toplam fenolik bileşik ve monomerik antosiyanin miktarlarları belirlenmiştir. Bu sonuçlara göre, doğal antifungal maddelerin içerdiği fenolik bileşiklerin bitki patojenlerinin kontrolünde ümitvar sonuçlar ortaya çıkaracağı belirlenmiştir.

Keywords:*Muscari aucheri*, fenolik bileşikler, antifungal aktivite, bitki patojenleri

1. Introduction

All plants produce many numbers of phenolic components in their metabolisms as seconder metabolites. Therefore, plants contain a wide range of phenolic in different quality and amount (Rauha et al., 2000; Proestos et al., 2006; Xia et al., 2011).

The usage of plants as bio-pesticides due to the rich antifungal metabolites they contain dates back to the start of human history. Antifungal effects of plants and plant based products become clearly evident every passing day. Antifungal metabolites obtained from plants present unique benefits with generally no side effects. Nowadays, it has emerged that side effects of pesticides used

against plant pathogens are harmful to the environment and people. Therefore, researchers for alternative management methods that will reduce the usage of pesticides to minimum come in to prominence. Following the researches, compounds and essential oils that plants contain are discovered to show antifungal (Gökce et al., 2006; Kordali et al., 2013; Bucchini et al., 2015; Elansary et al., 2016), antibacterial (Deans and Ritchie 1987; Hammer et al., 1999; Xia et al., 2011), herbicidal (Kordali et al., 2009; Verdeguer et al., 2011; Yilar et al., 2013, nematicidal (Elbadri et al., 2008; Kepenekci and Saglam 2015) and antiviral (Abad et al., 1999; Vijayan et al., 2004) activities.

Muscari aucheri (Boiss.) Baker. is a perennial bulbous plant is known as Grape Hyacinth. It is an endemic plant originally from Turkey (Uranbey, 2010). *M. aucheri* is usually less than 10 centimetres tall. There are two or three leaves per bulb, peduncle and a flower. The flowers are arranged in a raceme (Mathew, 1987). *Fusarium oxysporum* (Schlechtend.:Fr.) *f. sp. cucumerinum* (Owen) Snyder & Hansen [FOC], *Alternaria solani* Sorauer, *Verticillium dahliae* Kleb., *Rhizoctonia solani* J.G. Kühn and *Botrytis cinerea* Pers., cause damages in cucumber, tomato, potato and strawberry plants in the world respectively. FOC is the causal agent for fusarium wilt disease in cucumbers in many parts of the world. It causes serious economic damages in cucumbers. This pathogen is particular to cucumber plant (Jenkins and Wehner., 1983). The factor of the disease called early blight that can widely be found on tomato plants is *A. solani* (Agrios, 1988). *V. dahliae* is a soil born pathogen that causes *verticillium* wilt disease in tomato plants (Diwan et al., 1999). *R. solani* is the effects of the disease called soft rottenness in potatoes roots and tubers and causes serious crop losses in potatoes (Carling et al 1989). *B. cinerea* is the effect that causes grey mold disease in strawberries around the world where strawberry production is made (Grabke et al 2014).

This study aims that to show antifungal activities of individual phenolic compounds obtained from methanol extract of *M. aucheri* plant that grows endemically in Turkey and to identify their effects to control diseases caused by FOC, *A. solani, V. dahliae, R. solani* and *B. cinerea* pathogens.

2. Methodology

2.1. Plant Material

Muscari aucheri plants were collected from Tokat, Turkey in April, 2016. Aerial parts of plant (flower and peduncle) were used for the experiment. Plants were washed by sterile water and then dried in room temperatures under shadow. Dried plants materials were passed through a mill to separate small pieces.

2.2. Fungi Cultures

Plant pathogens (Table 1) were obtained from stock cultures at Department of Plant Protection Gaziosmanpaşa University, Fungi cultures were developed at 20 mL potato dextrose agar (PDA) on petri dish (90 mm) and kept at 22±2 ° C during 7 days and these fungi were used for the experiment. The abbreviations used in the article of plant pathogens are given in Table 1.

2.3. Plant Extracts

The aerial parts of *M. aucheri* (100 g) were weighted and put 1 lt jar. The methanol (Merck, Germany, 1.06007.2500) was added to cover the plant materials. These were incubated on an orbital shaker (Lab. Corporation Group, Model SI-300) (120 rpm) at 30°C for 3 days. The methanol was evaporated to dryness in the rotary evaporator (Heildolph Group, Model Hei-Vap-Presicion) at 40°C. These dried plant materials were reconstituted solution by 5% dimethly sulfoxide (DMSO) (Onaran and Yılar 2012).

2.4. *In vitro* Antifungal Activity

The antifungal activities were determined by using agar plate methods (Nwosu and Okafor, 1995). PDA [95mL (w/v)] was autoclaved and kept at 40°C. Dried plant extracts were weighted 250, 500, 1000 and 2000 mg and reconstituted solution by 5% dimethly sulfoxide (DMSO), and then added to PDA media (95 mL). The final

concentrations were arranged to 2.5, 5, 10 and 20 mg/mL and transferred to PDA petri plate (60 mm) (~10mL plate^{-1}). The mycelium disc (5 mm in diameter) from 7-day-olds fungi cultures were transferred to petri plates. Then incubated at 22±2 ^{o}C (Memmert Group, Model-High Precision Incubator) during 7 days and were recorded growth of fungi daily (Onaran and Yılar, 2012). The recommended dose of commercial fungicide (80% Thiram) was used as a positive control. DMSO (%5) was used as a negative control. The experiment was set up 3 replicate and twice.

Table 1. The list of plant pathogens, their abbreviations and the name of plants which pathogens were isolated

Çizelge 1. *Patojenlerin izole edildiği bitkilerin isimleri ve kısaltmaları, bitki patojenlerinin listesi*

Plant Pathogens	Abbreviations	Isolated
Fusarium oxysporum f. sp. cucumerinum **J.H. Owen**	Foc	Cucumber
Alternaria solani **(Ell. And G. Martin)**	As	Tomatoes
Verticillium dahliae **(Kleb.)**	Vd	Tomatoes
Rhizoctonia solani **Kühn**	Rs	Potatoes
Botrytis cinerea pers.:Fr	Bc	Strawberry

The percentage of mycelial growth inhibition was calculated accordingly the formula mentioned by Pandey et al. (1982):

I=100×(dc-dt)/dc

I: Mycelial growth inhibition

dc: the mycelial growth in control

dt: the mycelial growth in treatment

2.5. Determination of Some Individual Phenolic Compounds

The gallic acid, 4 hydroxybenzoic acid, vanillic acid, cafeic acid, ferulic acid, rutin, cisic acid, rozmarinic acid, quercetin, gentisic acid were purchased from Sigma-Aldrich Chemical Co. (United States). The analysis was carried out by LC 20AT pump (Shimadzu), SPD-M20A model DAD detector (280 nm) and HPLC system with CTO-20AC model column oven; for seperation, Dionex, (150x4.60 mm, 3 µm) C16 reversed phase filler column were used. The solvent A (2,5% methanol with formic acid), solvent B (2,5% deionized water with formic acid) and solvent C (acetonitrile) were used for mobile phase at HPLC. 10 mL extract samples which used for analysis were filtered by 0,45 µm membrane filter. The flow rate was 1 mL/min, injection volume was 20 µl and the column temperature was set at 30 °C. The gradient elution program was given in Table 2.

2.6. Determination of Total Phenolic Compounds

Total phenolic compounds were determined by using Folin-Ciocalteu method (Singleton and Rossi, 1965). Results were expressed as mg gallic acid equivalents (GAE) per litre of sample extracts (µg GAE/g).

Table 2. The gradient elution program for determination of phenolic compounds

Çizelge 2. *Gradient elüsyon programı ile fenolik bileşiklerin belirlenmesi*

Time (min.)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	5	95	0
9	10	90	0
27	15	85	0
29	0	85	15
40	0	70	30
50	0	45	55
55	0	0	100

2.7. Determination of Total Monomeric Anthocyanin

Total anthocyanin content of extracts were evaluated by pH-differential method described by Giusti and Wrolstad (2001). By this method, buffer of 0.025 M KCl (pH 1.0) and buffer of 0.4 M CH_3COONa(pH 4.5) dilutions were equilibrated for 15 min. The absorbance of each solution was measured at 520 nm and 700 nm.

Results were calculated by the following formula and expressed as µg malvidin 3-glycoside equivalent per gram.

$A = (A\lambda_{520} - A\,\lambda_{700})pH\ 1.0 - (A\lambda_{520} - A\,\lambda_{700})pH\ 4.5$

TA (mg/kg) = A x MA x SF x 1000 / ε x 1

A: absorbance, Malvidin-3-O-glycoside molecular weight (MA): 493.5 gmol/L;

The dilution factor (DF);

ε, molar absorptivity (28.000)

2.8. Statistical Analysis

Data were analysed by using One Way procedure of ANOVA (Windows version of SPSS, release 15.00). Differences among concentrations were compared with using DUNCAN Multiple Range Test of $p<0.05$. The probit analysis of the data derived in consequence of the tests was performed through POLO Plus 1.0 computer program and the values of LD_{50} were calculated.

3. Results and Discussion

3.1. Some Individual Phenolic Compounds, Total Phenolic Compounds, Total Monomeric Anthocyanin Content

HPLC chromotogram of the standart (280 nm) and the extract (280 nm and 330 nm) were shown in figure 1. The amount of individual phenolic compounds were shown Table 3. The highest individual phenolic compound was found to rutin (693,2 µg/g) from *Muscari aucheri*. This was followed by ferulic acid (377,9 µg/g), rozmarinic acid (127,9 µg/g) respectively. Phenolic compounds from the aerial part (flower + peduncle) of *M. aucheri* were determined first time with this study. Gallic acid, vanillic acid and cisic acid were not determined from *M. aucheri* plant extract (Table 3). In a study on the antioxidant capacity of this plant extract was showed that the amount of antioxidant capacity was found to be 3,164 (bulb) to 1,145 (leaf) mmol Trolox Equiv/L (Yıldırım 2013).

The total phenolic compound content was determined 1882,13 µg GAE/g from *M.aucheri* extract. In addition, the amount of total monomeric anthocyanin was calculated as 52,34 µg/g malvidin-3-O-glycoside equivalent. Phenolic compounds in plants consist of phenolic acids, flavonoids with small molecules and mostly essential compounds. Antifungal and antibacterial (Aziz et al., 1998; Rauha et al., 2000) and antiviral (Cline et al; 1969; Özcelik et al., 2011) activities of phenolic compounds of plants were reported from many studies. Phenolic acid such as rozmarinic acid and polyphenols have synergistic effects (Choi et al., 2002). The extracts of *M.aucheri* have high level of phenolic acids had antifungal activity.

Table 3. The amount of individual phenolic compound from extract

Çizelge 3. *Ekstratın bireysel fenolik madde miktarı*

Retention Time	R^2 value	Phenolic Compounds	Amount (µg/g)
5.607	0,999	Gallic Acid	nd*
12.068	0,998	4hydroxybenzoic Acid	83,0
13.719	0,995	Gentisic Acid	99,4
14.901	0,999	Vanillic Acid	nd
17.432	0,997	Cafeic Acid	39,8
26.902	0,996	Ferulic Acid	377,9
34.952	0,998	Rutin	693,2
37.119	0,999	Cisic Acid	nd
38.575	0,999	Rozmarinic Acid	127,9
44.980	0,999	Quercetin	8,8

*nd: not detected

3.2. *In vitro* Antifungal Activity

Antifungal activities were found differently rate for each plant pathogens. All doses of plant extract were showed antifungal activity noticeably. This activity has also increased with the rise of the amount of doses. The highest dose (20 mg/mL) of plant extract was observed 100% mycelium inhibition rate on *Alternaria solani*, *Verticillium dahliae*, *Rhizoctonia solani* and *Botrytis cinerea*. This was followed by 88% of inhibition on *Fusarium oxysporum f. sp. cucumerinum* (Table 4). These results were compared with positive control, similar inhibition rates were found. In the study, LD_{50} values of plant extract was calculated against plant pathogens (Figure 2). According the value of the LD_{50}, *V. dahliae* was found most susceptible pathogen against plant extract. This was followed

by *A. solani*, *R. solani*, *B. cinerea* ve *Fusarium oxysporum f. sp. cucumerinum*. All doses of plant extract compare with negative control was showed antifungal effect on plant pathogens (Figure 3). Mycelium growth was found to stop completely dependent on the doses increases. Even though lowest dose was showed limited mycelium growth, it was stopped after a certain period time. According the difference of fungi species, there have observed that differential such as aerial mycelium growth and inhibition of sporulation formation (Figure 3). One of the similar study reported by Yıldırım (2013), the leaf and bulb of *M. aucheri* was collected from Munzur valley of Tunceli.

The leaves and bulbs were used for obtaining methanol, n- hexane, ethanol and water extracts against identifying antifungal effect. The dose of 2.5% (SDA media) of extracts were used against *Coriolus versicolor* and found low antifungal activities.

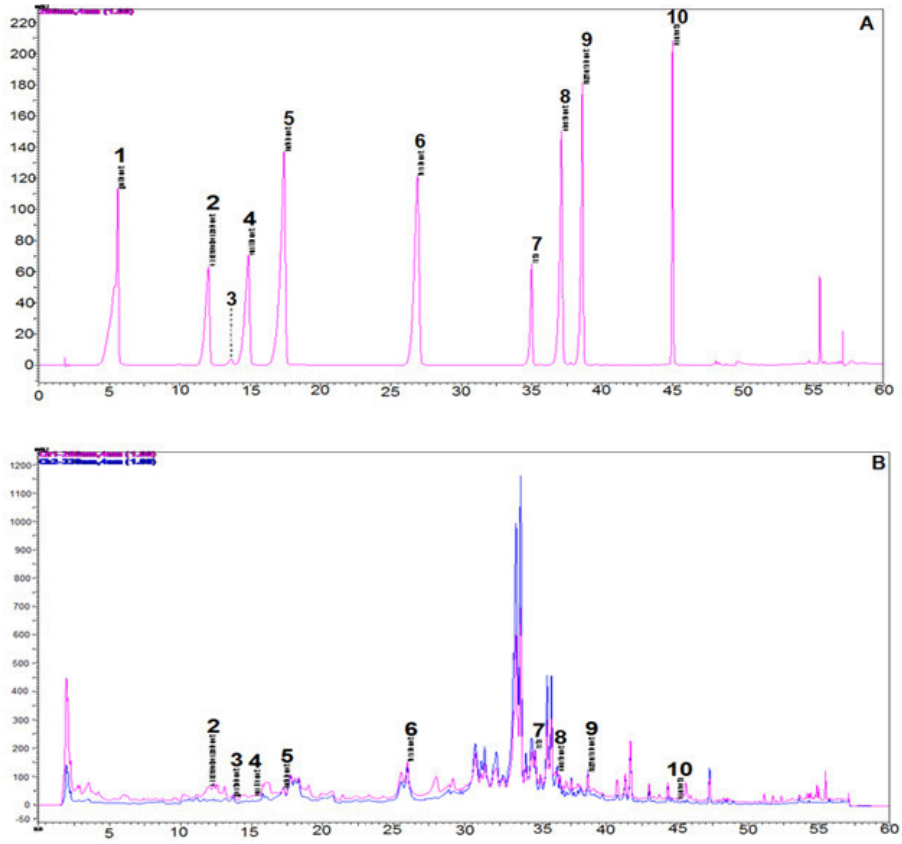

Figure 1. HPLC chromatogram of the phenolic standards at 280 nm **(A);** HPLC chromatogram of the extract at 280 and 330 nm **(B);** Gallic acid (1), 4 hydroxybenzoic acid (2), Gentisic acid (3), Vanillic acid (4), Cafeic Acid (5), Ferulic Acid (6), Rutin (7), Cisic acid (8), Rozmarinic acid (9), Quercetin (10)
Şekil 1. *Fenolik standartların 280 nm'de HPLC kromatogramı* ***(A);*** *Ekstratın 280 ve 330 nm'de HPLC kromatogramı* ***(B);*** *Gallik asit (1), 4 hidroksibenzik asit (2), Gentisik asit (3), Vanillik asit (4), Kafeik asit (5), Ferulik asit (6), Rutin (7), Sisik asit (8), Rozmarinic asit (9), Kuersetin (10)*

Methanol and n-hexane extracts had not showed any antifungal activities but the leaf of ethanol extracts was found mycelium inhibition at 44.40% the leaf of water extract, 28.80% the leaf of water extract, 10.40 % the bulb of ethanol extract and 12,80 % the bulb water extracts respectively. According to this finding, different parts of extracts of *M. aucheri* against different plant pathogens have shown antifungal activities. This difference may be due to the fact that the same plant species was grown in different areas.

In similar studies, the test organisms which used our study have been tested different plant extracts for identifying antifungal activities by several researchers such as *Fusarium oxysporium* (De Rodriguez et al., 2005; Çakır et al., 2005; Onaran and Yılar, 2012), *Alternaria solani* (Çakır et al., 2005;Yanar et al.,2011) *Verticillium dahliae* (Arslan ve Derviş 2010; Onaran and Yılar, 2012) *Rhizoctonia solani* (Alkhail, 2005, De Rodriguez et al., 2005, Onaran and Yılar 2012) *Botrytis cinerea* (Alkhail, 2005; Soylu et al., 2010). In this study, the methanol extracts of flower and peduncle parts of endemic *M. aucheri* has been studied first time identifying antifungal activities according to literatures. This aspect increases the originality of the study.

Table 4. Mycelial growth inhibition of fungi at different doses of *Muscari aucheri* (%)
Çizelge 4. *Muscari aucheri'nin farklı dozlarında fungusların miselyum gelişim engellemeleri (%)*

Doses (mg/mL)	Foc	As	Vd	Rs	Bc
C-	0	0	0	0	0
2,5	20	48	50	28	22
5	56	66	75	52	58
10	85	95	100	88	84
20	88	100	100	100	100
C+	100	100	100	100	100

C-: Negative control; C+: Positive control

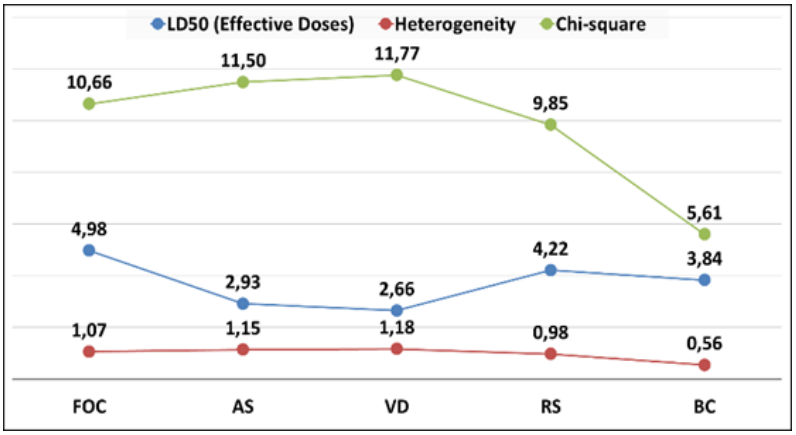

Figure 2. LD_{50} values of plant extract against used plant pathogens
Şekil 2. *Kullanılan bitki patojenlerine karşı bitki ekstraktlarının LD_{50} değerleri*

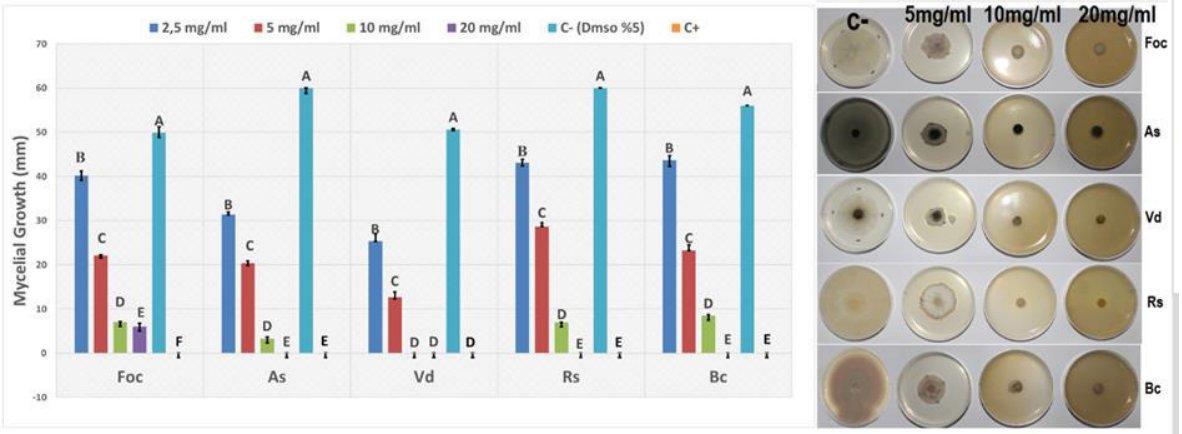

Figure 3. The growth of mycelium of pathogens against the extract of *Muscari aucheri* (The photo was taken at the end of 7th day).
Şekil 3. *Muscari aucheri ekstratının patojenlerin miselyum gelişimi üzerine etkisi(Fotoğraf 7. Günün sonunda çekilmiştir.)*

4. Conclusions

Plants have antifungal compounds such as flavonoids and phenolic compounds therefore plants have always been of interest the researchers. Each day, new compounds find from plants are identified and used in scientific studies. As a result, new identified compounds of plant structures may replace the synthetic chemicals which heavily used in industry, agricultural practice and food industry. Thus, this allows to find natural substances that are less harmful or harmless to human, environment and non-target organisms. With this study, the flower and peduncle of endemic *Muscari aucheri* plant in Turkey were done to collected and identified of phenolic compounds and antifungal activities. According to result, this compounds and extracts give promising result to control test pathogens so they can be replaced the synthetic chemicals that are used to control them.

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