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Allelopathic Effects of Volatile Substances from *Chamaecyparis obtusa*

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ABSTRACT: The allelopathic effects of volatile substances from *Chamaecyparis obtusa* (S. et Z.) Endl. were examined on the germination and seedling growth of some plant species, and on the population growth of some microorganisms. The germination and seedling growth of the receptor plants were suppressed more severely by leaf and fruit essential oils than by those of other parts. Colonial growth of fungi was severely inhibited by essential oils extracted from leaves and fruits. The development of root hairs of the receptor plants was also severely inhibited by the essential oils. The cortical cells at the root tips of *Lactuca sativa* L. treated with essential oils showed contraction of the cytoplasm, resulting in plasma membranes becoming detached from the cell walls and the cells metamorphosing irregularly. Accumulation of lipid granules inside the contracted cytoplasm and degeneration of mitochondrial cristae were also observed.

Key Words: Allelopathic effects, *Chamaecyparis obtusa*, Essential oil, Root hair, Root tip

INTRODUCTION

Allelopathy is expressed through the release of allelochemicals by the donor plant in the vicinity of a receptor plant species. Aside from their many roles in allelopathy - influencing soil microbial ecology, nutrient dynamics, and other abiotic and biotic factors - allelochemicals play key roles in structuring of other trophic levels, especially affecting predators and pests and mediating competitive circumstances (Dakshini *et al.* 1999).

Terpenes are as widespread in the plant kingdom as phenolic compounds. They have potential as allelopathic agents because they volatilize readily from intact leaves and can be phytotoxic at concentrations as low as 3×10^{-6}M (Asplund 1968). Terpenoids can affect physiological activities such as cell division, mineral uptake, enzyme activity, etc. (Robinson 1983).

Essential oil emitted from *Chamaecyparis obtusa* (Cupressaceae) has a strong odor, that drives some insects away, so *C. obtusa* wood is useful for building structures because the foul smell repels harmful insects. The essential oil is used widely in aromatic essences, insecticides, and medicine. *C. obtusa* trees were introduced from Japan and planted in hillocks in Korea. Chemical analysis of *C. obtusa* essential oil has been performed by Hayashi *et al.* (1964) and by Shieh *et al.* (1981). but the allelopathic effects of *C. obtusa* was not studied in depth yet.

The objectives of the present experiments are to test 1) germination and seedling growth of some plants, 2) growth of fungal colonies, and 3) development of root hair and root tip morphology by allelochemicals collected from *C. obtusa.*

METHODS AND MATERIALS

Experimental plants

Volatile substances were collected from *C. obtusa*, and the seeds were collected from the following selected bioassay plants: *Metaplexis japonica* (Tunb.) Makino, *Plantago asiatica* L., *Oxalis corniculata* L., *Amaranthus mangostanus* L., *Achyranthes japonica* (Mig.) Nakai, *Geum japonicum* Thunb., *Lactuca sativa* L., and *Brassica campestris* subsp. *napus* var. *pekinensis* Makino. Filamentous fungi *Aspergillus nidulans* and *A. niger*, and pathogenic fungi *Alternaria mali* and *Fusarium oxysporum* were used for bioassays of fungicidal activity.

Extraction of essential oil

The essential oil of *C. obtusa* was obtained by Yun's method employing the Karlsruker's apparatus (Stahl 1973).

Bioassay for germination and seedling growth

The leaves, branches, bark, fallen leaves, fruits, and roots of *C. obtusa* were prepared by crushing. Then, these six materials were put into 1,700 mL glass containers in amounts of 30, 40, and 70 grams. Fifty milliliters of distilled water in 100 mL beakers were placed in each container to compensate for vapor loss. An empty glass container was used for the control. Two sheets of filter paper were put on the bottom of each container and moistened with 10 mL distilled water. Fifty seeds of the bioassay species were sown in a 12 cm Petri dish and placed in each glass container. After sowing, the Petri dishes were sealed with vinyl wrap. Ten days later germinated seeds were counted and seedling lengths were measured.

To ascertain whether the volatile substances of *C. obtusa* leaves could be hydrated by water, 30, 50, and 70 grams of *C. obtusa* leaves were placed in 1,700 mL containers with 100 mL beakers half full of distilled water and sealed with vinyl wrap. After three days, the distilled water from the beakers was used to moisten the filter paper for a germination test as previously described. Controls were included.

Alternate germination testing was performed in which two sheets of filter paper covering the bottom of a Petri dish were moistened with 8 mL distilled water and 50 seeds of *Amaranthus mangostanus* were sown. A small aluminum vial (diameter 5 mm, height 5 mm) was put in the center of the Petri dish containing either 5 μL, 10 μL, 15 μL, or 20 μL of the essential oil of *C. obtusa* leaves. These were sealed with parafilm. An empty aluminum vial was used as the control. Six days after sowing, seed germination and seedling length of test plants were evaluated.

To test fungal growth, 2.5, 12.5, 25, and 50 μL of the essential oils of fallen leaves, fresh leaves, tree bark and fruit of *C. obtusa* were used to make complete media of *Aspergillus nidulans* (Harsani *et al.* 1976). The complete media were inoculated with *Alternaria mali*, *Aspergillus nidulans*, *A. niger*, and *Fusarium oxysporum* and then sealed with parafilm. *A. mali* and *F. oxysporum* were cultured at 30℃ while *A. nidulans* and *A. niger* were cultured at 37℃ in the dark. Ninety-six hours after inoculation, the diameters of colonial growth were measured.

Development of root hairs and root tip test

From the germinated seedlings of *Lactuca sativa* and *Brassica campestris* subsp. *napus* var. *pekinensis*, five radicles of about the same length were transferred onto filter paper in Petri dishes with the essential oils using the same procedures as described above for the germination and growth tests. After 24 hours, the development of root hairs was examined under a binocular microscope.

Lettuce seedlings were cut and prefixed in 5% glutaraldehyde (0.1M phosphate buffer, pH 7.0) at 4℃ for 4 hours, then washed with buffer solution. Post fixation was done with 1% osmium tetroxide using the same buffer solution and then washed again. Fixed specimens were dehydrated in ethanol, metathesized with propylene oxide and then embedded in Epon mixture. Embedded materials were cut into 2μ m slices by rotary ultramicrotome. These thin slices were dyed with 0.5% toluidine blue. Then 80 mm silver sections were made using an LKB-V type ultramicrotome. Silver sections were collected on a copper grid (100 mesh) and double-dyed with uranyl acetate for 30 minutes and lead citrate for 10 minutes then examined by transmission electron microscope (Hitachi H-600, 75 KV).

RESULTS

Influence on germination and growth

The effects of the volatile substances from leaf, root and fruit of the *C. obtusa* plant on germination and seedling growth in the bioassays are shown in Table 1. Seed germination of *Oxalis corniculata* and *Achyranthes japonica* was inhibited by the volatile substances from leaves of *C. obtusa*, but that of *Metaplexis japonica* and *Geum japonicum* was rather stimulated by root and fruit extracts. Germination of *Plantago asiatica* was slightly inhibited at all

Table 1. Effect of volatile substances from different parts of *Chamaecyparis obtusa* on germination of receptor plants

Receptor species	Leaf (g/l)				Root (g/l)				Fruit (g/l)			
	Cont.	15	30	40	Cont.	15	30	40	Cont.	15	30	40
Metaplexis japonica	25a	25a	5b	3b	28a	30a	28a	30c	28a	29a	31a	30a
Plantago asiatica	33a	13b	1c	2c	38a	33ab	31bc	30a	40a	38a	38a	37a
Oxalis corniculata	21a	1b	1b	1b	11a	8b	9ab	8b	26a	23a	23a	23a
Amaranthus mangostanus	32a	28ab	25b	26ab	25a	25a	26a	23a	28a	29a	22a	27ab
Achyranthes japonica	26a	5b	1c	1c	25a	20a	21a	20a	15a	11ab	11ab	9c
Geum japonicum	41a	39a	7b	3b	35a	42b	40b	41b	6a	13b	9b	5a

Means followed by the same letters in a row are not significantly different at the 5% level by Duncan's multiple range test.

Table 2. Effect of volatile substances from different parts of *Chamaecyparis obtusa* on seedling growth of receptor plants

Receptor species	Leaf (g/ l)				Root (g/ l)				Fruit (g/ l)			
	Cont.	15	30	40	Cont.	15	30	40	Cont.	15	30	40
Metaplexis japonica	100a	42b	17c	23c	100a	77a	75a	73b	100a	100a	100a	97a
Plantago asiatica	100a	61b	3c	3c	100a	70b	69b	66b	100a	95a	93a	98a
Oxalis corniculata	100a	2b	2b	2b	100a	58b	52b	52b	100a	93a	93a	87ac
Amaranthus mangostanus	100a	64b	56b	50b	100a	41b	42b	41b	100a	81b	88c	92ac
Achyranthes japonica	100a	77b	29c	12d	100a	99a	98a	97a	100a	81a	80a	72a
Geum japonicum	100a	67b	43c	30c	100a	101a	101a	100a	100a	79a	80a	82a

Means followed by the same letters in a row are not significantly different at the 5% level by Duncan's multiple range test.

Table 3. Effect of essential oils (ppm) from different parts of *Chamaecyparis obtusa* on germination and seedling elongation of *Amaranthus mangostanus*

Essential oil	Germination					Seedling elongation				
	Cont.	25	50	75	100	Cont.	25	50	75	100
Fallen leaf	100a	97a	98a	87a	60b	100a	52b	20c	14c	17c
Fresh leaf	100a	103a	88a	74a	69b	100a	47b	37bc	34c	24c
Branch	100a	88a	90a	89a	90a	100a	68b	58c	59c	48c
Bark	100a	92a	101a	92a	81a	100a	87ab	77b	61c	55c
Root	100a	105a	110a	97a	95a	100a	70b	70b	71c	53c
Fruit	100a	100a	63b	17c	0c	100a	42b	27c	16c	0c

Means followed by the same letters in a row are not significantly different at the 5% level by Duncan's multiple range test.

plots. *A. japonica* proved to be the most susceptible species among the receptor plants tested with *C. obtusa* volatiles (Table 1).

Seedling growth of all receptor species except *M. japonica* was inhibited severely by treatment with volatile substances from *C. obtusa* leaf, while that of all species with root and fruit from *C. obtusa* was not much influenced by them (Table 2).

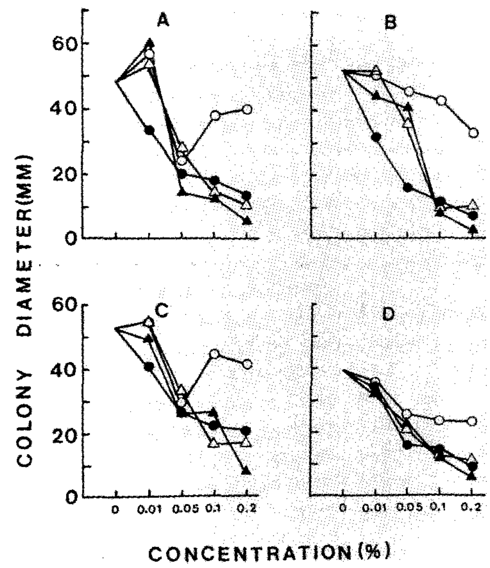
Seed germination and seedling growth of *A. mangostanus* was tested at different concentrations (ppm) with the essential oil from *C. obtusa* (Table 3). At 25 ppm essential oil of fallen and fresh leaf, germination of *A. mangostanus* was less than that of the controls and from 75 ppm of fruit essential oil, the inhibitory effect was remarkable, while at 100 ppm there was no germination. However, the seeds of *A. mangostanus* germinated well in the essential oils from branches, bark and root of *C. obtusa*. Elongation growth of *A. mangostanus* was gradually suppressed with increasing concentration of essential oil, except in the 25 ppm plot. Among the different parts from *C. obtusa*, for example, fresh leaves, bark, and fruit, fruit was the most toxic against germination and seedling growth of *A. mangostanus*.

Growths of four fungi, *Alternaria mali*, *Aspergillus nidulans*, *A. niger*, and *Fusarium oxysporum*, were tested with essential oil from fallen leaves, fresh leaves, bark and fruit of *C. obtusa* (Fig. 1). By increasing concentrations of the essential oil from fallen leaves, fresh leaves, and fruit of *C. obtusa*, the growth of the four fungal species was severely inhibited. In treatments with bark essential oil, the colony growth of *Alternaria mali* and *Aspergillus niger* was somewhat lower than control, but colonies of *Aspergillus nidulans* and *Fusarium oxysporum* could grow at a low rate by increasing concentrations of essential oil. Colony growth of *Alternaria mali* was promoted in tests with essential oil from bark, fallen leaves and fresh leaves of *C. obtusa*. At 0.2% concentration of essential oil, antifungal activity relative to controls was 12.5% in *A. mali*, 5.8% in *A. nidulans*, 15.4% in *A. niger*, and 15.4% in *F. oxysporum*.

Effects on the development of root hair and root tip

The morphology of healthy root hairs of *Lactuca sativa* is characteristically tangles (Fig. 2). When treated with fruit essential oil of *C. obtusa*, root hairs of *L. sativa* were sharply reduced at 10 μL and 15 μL, and were nearly non-existent at 20 μL.

At 10 μL treatment, the root hairs developed



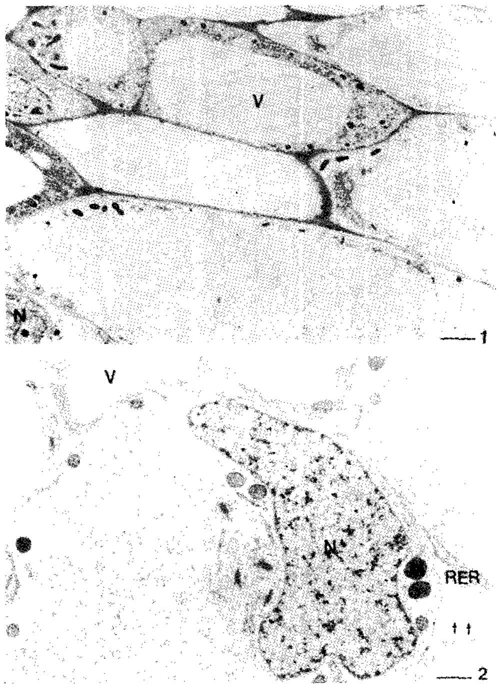


Fig. 1. Effect of essential oils from fallen leaf, fresh leaf, bark and fruit of *Chamaecyparis obtusa* on the growth of 4 fungi incubated for 96 hours.

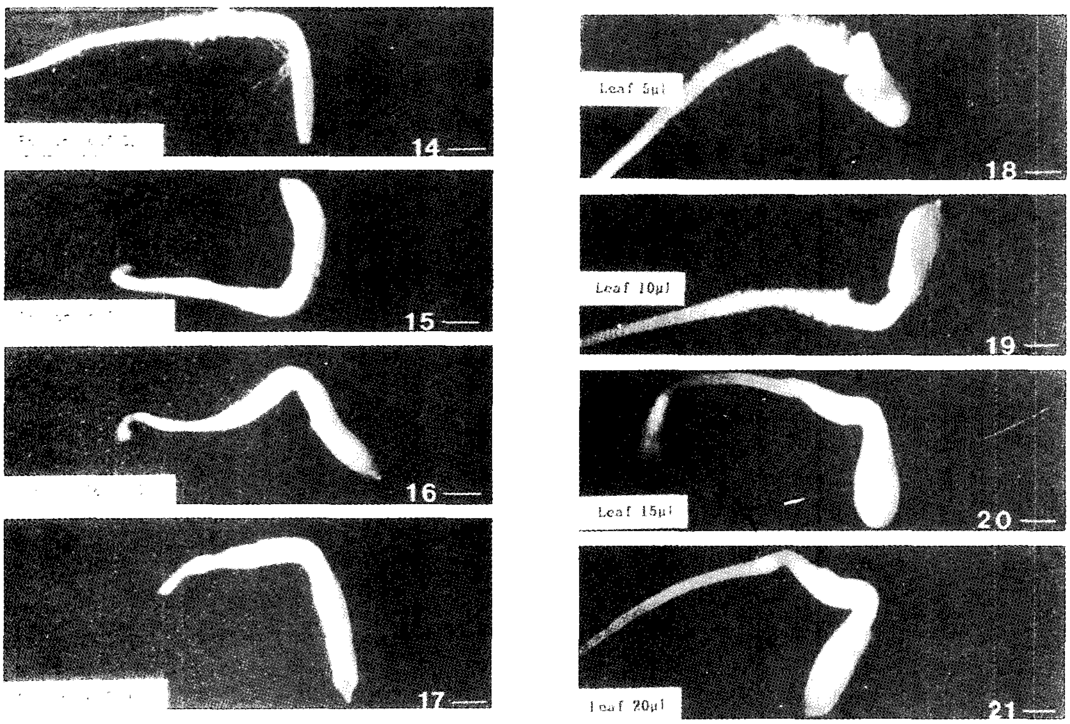


Fig. 2. Effect of essential oils from different parts of *Chamaecyparis obtusa* on development of *Lactuca sativa* root hairs.

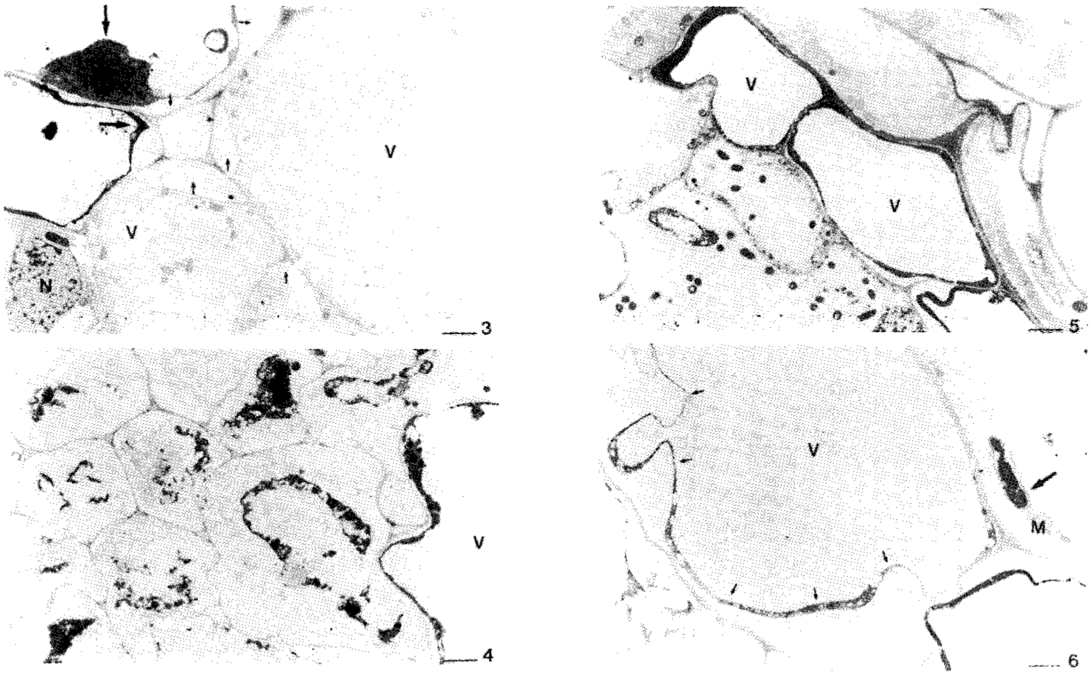


Fig. 3. Transmission electron micrographs showing the sections of cortical cells of *Lactuca sativa* root tips treated with fruit essential oil of *C. obtusa*. No. 1, 2, control showing plasma membranes attached to the cell walls closely. No. 3, 5μL treatment; detachments of the plasma membranes from the cell walls are often observed (small arrows), and contraction of the cytoplasm is also visible (large arrows). No. 4, 10μL treatment; both degeneration of cytoplasm and metamorphosis of cells are severe. No. 5, 15μL treatment; degenerated cytoplasms are like slim belts. No. 6, 20μL treatment; both slim belt-like (small arrows) and severely collapsed (large arrow) cytoplasms are visible. V; Vacuole, N; Nucleus, M; Mitochondrion, RER; Rough surfaced endoplasmic reticulum. Bar; 2.5μm

very little. At 5μL, root hairs were short but comparatively well developed and very few root hairs occurred in either the 15 or 20μL treatments. Essential oil from bark showed relatively good development of root hairs from the 5μL to 20μL treatments, but their length was reduced. These results are similar to those of the essential oils from bark and root of *C. obtusa*. The worst development of root hairs was from the essential oil of fruit. It thoroughly disrupted root hair development in all the tests, and moreover, hairs did not develop at all in the 15μL and 20μL treatments (Fig. 2). The strongest inhibitor of root hair development of *L. sativa* was the essential oil of fruit.

The cortex cells of the root tips of *L. sativa* challenged with 5, 10, 15, and 20μL were examined by transmission electron microscopy. The cells of the control revealed normal shaped cell walls, protoplasmic membranes, and mitochondria of globular or elliptical shapes. Treated cells have a separation between the cell walls and the protoplasmic membranes due to shrinking of the cytoplasm. Moreover there was an accumulation of granular material of high electron density in the vacuoles (Fig. 3).

At 10μL, the cytoplasm was severely shrunken with deformity of cells and many accumulated granule bodies, including lipid in the cytoplasm of the *L. sativa* root tips (Fig. 3. No. 4). The treatments with 15μL and 20μL of fruit essential oil affected a shrunken morphology of whole root tips and cells were further deformed into very fine belt shapes with mitochondria of retrogressed or reduced cristae (Fig. 3. No. 5, 6).

DISCUSSION

The experiments on the effect of volatile substances from different parts of *C. obtusa* were shown the strongest germination inhibition from leaves, while the weakest effect came from fruit (Table 1). To find the relationship between inhibition and quantity of essential oil, a Karlsruker apparatus was employed for the extraction of essential oils. Seventy grams of *C. obtusa* and 300 mL of water were subjected to 50 volts for 3 hrs. The obtained oil quantities were in the following ratio. Fallen leaves : fresh leaves : branches : bark : roots : fruits = 165 :

215 : 1 : 5 : 4.5 : 15. These ratios corresponded to the degree of inhibition to seedlings.

Essential oils obtained from the 6 parts were bioassayed on the germination and seedling growth of *A. mangostanus*. The most inhibitory effect was caused by fruit oil treatments (Table 3). These results suggested that the kinds and amounts of allelochemicals included in the essential oil from the six parts of *C. obtusa* may differ from one another.

Colony growth of the four fungi was clearly suppressed by the essential oils of fruit, fallen leaves, fresh leaves and bark (Fig. 1). It has been proved that not only is the antifungal effect of essential oils useful against food decay fungi or human pathogenic fungi (Espinosa-Garcia *et al.* 1991), but can also counter fungal diseases in crops and stored foods (Arora and Pandey 1977). Essential oil components had selective susceptibility of conifer plants against fungal pathogens (Rockwood 1973), and retain their protective response after attacking pathogenic organisms (Miller *et al.* 1986). Several kinds of essential oils from different plants had proven antifungal activities. Such plants as *Artemisia princeps* var. *orientalis* (Yun 1991), *Palma rosa* (Dikshit *et al.* 1981), *Citrus medica, Trachyspermum ammi, Nepata hindostana, Amomum subulatum*, and *Hyptis suaveolens* (Yun *et al.* 1992) produced active metabolites.

Development of root hairs of *L. sativa* was severely inhibited by the essential oil of fallen leaves, fresh leaves and fruit and extension of roots was also less than control and was proportional to the concentration of the essential oil (Fig. 2). Subsequent inhibitory development of root hairs indicates a decreased uptake of water and nutrients, leading to suppression of plant growth.

Root tip cells of *L. sativa* treated with fruit essential oil were examined by transmission electron microscope. Abnormal morphology observed in this experiment was shrunken cytoplasm, plasmolysis from the cell wall, malformation or irregular cell shape, accumulation of lipid granules, and retrogression of mitochondrial cristae (Fig. 3). Rho (1992) and Kim (1993) have reported deformity of root tip cells of receptor plants by aqueous extracts. These findings were similar to those of the present study where the greater the supply of essential oil was, the worse the abnormalities were. These facts suggest that the irregular cells are related to retrogression of the cytoplasm and separation of the plasma membrane from the cell wall. Retrogression of mitochondrial cristae in this study was similar to that observed and agreed with previous studies with *Cucumis sativus* L. cells treated with volatile substances from *Salvia leucophylla* (Lorber and Muller 1980). This fact indicates a breakdown of the mitochondrial inner membrane. Therefore, it is suggested that growth inhibition was due to disruption of the normal respiration of *L. sativus* when treated with extracts of fallen leaves and fruit essential oils.

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