Antimicrobial Activity of the Olive Oil Flavor Compounds

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A series of long-chain α,β-unsaturated aldehydes have been characterized as antimicrobial agents from the olive *Olea europaea* fruit and its oil flavor. These volatile compounds exhibited a broad antimicrobial spectrum.

Keywords: *α,β-Unsaturated aldehydes; Olea europaea; antimicrobial activity*

INTRODUCTION

The leaf of the olive, *Olea europaea* L. (Oleaceae), is known to be resistant in nature to microbe and insect attack. In a previous paper we described its multichemical defense mechanisms against microorganism invasion (Kubo and Hanke, 1985). One type of defense is the action of the bitter *seco*-iridoid glycosides, oleuropein and ligstroside, which do not show direct antimicrobial activity. They release antimicrobial compounds by a β-glucosidase (Fleming et al., 1973); the intermediate iridoid formed then breaks down spontaneously to dialdehyde (Kubo et al., 1985). The other is a preinfectional barrier of crystalline oleanolic acid that coats the leaf surface. This water insoluble triterpene may produce an *in vivo* environment highly unfavorable for fungal development as a first line of defense against invading microorganisms (Kubo et al., 1985). Interestingly, the fruit surface of the olive is not coated by this triterpenoid, although there is a large amount of the two *seco*-iridoid glycosides in the fruit. During this study we became aware that the *n*-hexane extract of the fresh fruit and leaves of *O. europaea* exhibited broad antimicrobial activity at a concentration of 2000 μg/mL. This seems to indicate the presence of another defense mechanism since none of the above-mentioned compounds themselves had antimicrobial activity.

In our continuing search for antimicrobial agents from edible plants (Kubo et al., 1991, 1992), the steam distillates of both fresh green olive and commercial olive oils were found to exhibit a broad spectrum.

MATERIALS AND METHODS

General Procedure. General procedures are the same as in previous work (Kubo and Himejima, 1991; Kubo et al., 1991; Himejima and Kubo, 1992; Himejima et al., 1992).

Plant Materials. Fresh fruit and leaves of *O. europaea* were collected on our campus (Kubo et al., 1985, 1986). Five commercial olive oils, G. Sensat and De Cecco (imported from Spain), DaVinci and Star (imported from Italy), and Vine Village (produced in California), were purchased at supermarkets in the San Francisco Bay area.

Chemicals. Hexanal (**1**), nonanal (**2**), 1-hexanol (**3**), (*E*)-2-hexenal (**5**), (*E*)-2-heptenal (**6**), (*E*)-2-octenal (**7**), (*E*)-2-nonenal (**8**), and (*E*)-2-decenal (**9**) were purchased from Aldrich Chemical Co. (Milwaukee, WI). (*E*)-2-Undecenal (**10**) and (*E,E*)-2,4-decadienal (**11**) were obtained from Wako Pure Chemical Ind. (Osaka, Japan). *N,N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, NJ).

Extraction and Identification. Fresh green olives (1.3 kg) were collected on our campus and extracted with methanol at ambient temperature (Kubo and Matsumoto, 1984a,b). The methanol was removed under reduced pressure to give a dark green crude extract (82.4 g), which was suspended in water (500 mL). The suspension was successively partitioned between *n*-hexane and water. The subsequent bioassay revealed the *n*-hexane fraction to be active. A portion of the *n*-hexane fraction (4.0 g) was steam distilled to yield a distillate (0.5 g) and residue (3.1 g). The following bioassay indicated that the distillate maintained the original broad antimicrobial spectrum. Further analysis to identify the active principles in the distillate was performed by GC–MS as described previously (Kubo and Himejima, 1991; Kubo et al., 1991; Himejima and Kubo, 1992; Himejima et al., 1992). Commercial olive oils were steam-distilled without any prepurification in order to obtain the distillates.

Microorganisms and Media. All microorganisms used for the assay were purchased from American Type Culture Collection (Rockville, MD). They are *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Staphylococcus aureus* ATCC 12598, *Streptococcus mutans* ATCC 25175, *Propionibacterium acnes* ATCC 11827, *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, *Proteus vulgaris* ATCC 13315, *Saccharomyces cerevisiae* ATCC 7754, *Candida utilis* ATCC 9226, *Pityrosporum ovale* ATCC 14521, *Penicillium chrysogenum* ATCC 10106, *Trichophyton mentagrophytes* ATCC 18748, and *Aspergillus niger* ATCC 16404.

The culture medium for the bacteria consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco), and 0.1% glucose, with the exception of *S. mutans*. For the culture of *S. mutans*, 3.7% brain heart infusion broth (Difco) was used. The culture medium for the fungi was 2.5% malt extract broth (BBL), with the exception of *P. ovale* and *T. mentagrophytes*. For the culture of *P. ovale*, 1% bactopeptone (Difco), 0.5% yeast extract, 1% glucose, and 0.1% corn oil were used, and for *T. mentagrophytes*, 1% bactopeptone and 4% glucose were utilized.

Antimicrobial Assay. The highest concentration of the samples tested was 800 μg/mL, unless otherwise specified, because of their limited solubility in water-based media. This solubility limitation in water also limits selection of assay methods for their evaluation. For example, the paper disk method is not relevant since water insoluble substances do not diffuse into the media (Himejima et al., 1992). Hence, throughout this experiment the broth dilution method was employed. The test compound was first dissolved in DMF and serial 2-fold dilutions were made using DMF. Thirty microliters of the sample solution was then added to 3.0 mL of sterile medium, resulting in 1% DMF concentration, which did not affect the growth of any of the microorganisms employed. The growth test tube was inoculated with 30 μL of a 2-day-old culture of the test organisms (5-day-old for *P. chrysogenum*, *T. mentagrophytes*, and *A. niger*) and then incubated at 30 or 37 °C depending on the microorganism used. All microorganisms were cultured stationary except the three abovementioned fungi, which were cultured with shaking. After 2 days of cultivation (3 days for *P. ovale* and 5 days for *P. chrysogenum*, *T. mentagrophytes*, and *A. niger*), the growth of the microorganisms was examined by turbidity (OD at 660

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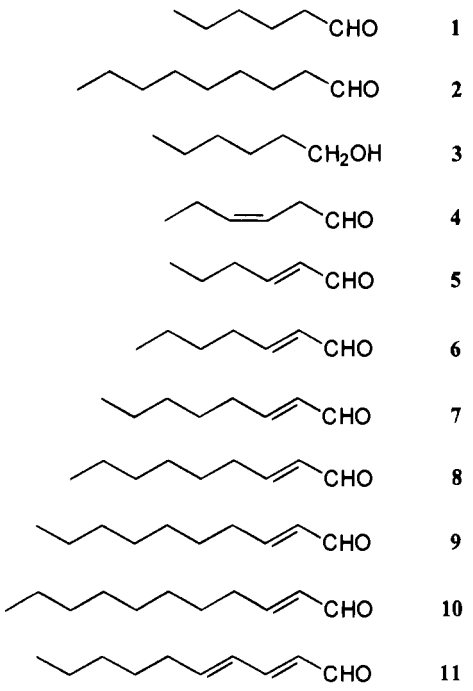
nm), except that of *P. chrysogenum*, *T. mentagrophytes*, and *A. niger*, which was examined with the naked eye. The minimum inhibitory concentration (MIC) was the lowest concentration of the test compound that completely prevented growth.

The lethal effects of some samples were examined as follows. After determining the MIC, a 30 μL aliquot was taken from each clear tube and added into 3 mL of the sample-free fresh medium. After an appropriate length of incubation, the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) was determined as the lowest concentration of the test samples in which no recovery of microorganisms was observed.

A time–kill curve method was employed to further characterize antimicrobial activity. A bactericidal kinetic assay for *E. coli* was performed in NYG broth containing sample concentrations equal to the MIC and $^1/_2$-MIC. The initial inoculum was approximately 6×10^5 CFU/mL. Culture was removed at 0, 2, 4, 8, 24, and 48 h of incubation. The number of viable cells was determined by serial 10-fold dilutions and plating onto NYG agar. The plates were incubated at 37 °C for 24 h before counting.

RESULTS AND DISCUSSION

The *n*-hexane extract of the fresh green olives was divided into distillate and residue fractions by steam distillation. Subsequent bioassay found that the distillate maintained the original broad antimicrobial activity. To identify the active principles in the distillate, chemical analysis was performed by GC–MS. As a result, the major volatile compounds were typical products of oxidative cleavage of unsaturated fatty acids, including hexanal (**1**), nonanal (**2**), 1-hexanol (**3**), (*Z*)-



3-hexenal (**4**), (*E*)-2-hexenal (**5**), (*E*)-2-heptenal (**6**), (*E*)-2-octenal (**7**), (*E*)-2-nonenal (**8**), (*E*)-2-decenal (**9**), (*E*)-2-undecenal (**10**), and (*E,E*)-2,4-decadienal (**11**). In addition to these acyclic compounds, cyclic mono- and sesquiterpene hydrocarbons, 3-carene, β-farnesene, and copaene, were also identified. Interestingly, among the 14 major compounds identified in the distillate, 9 of them were acyclic aldehyde compounds and, more specifically, 7 were a series (C_6–C_{11}) of α,β-unsaturated aldehydes. This result is in general agreement with that reported earlier (Flath et al., 1973). Most commercial olive oils analyzed are of similar composition to those found for fresh green olives, although they vary somewhat, especially in quantities. The steam distillate obtained from fresh green olives yielded lesser quantities but still had similar aldehyde composition. Hence, these acyclic compounds should be produced partly by autoxidation but mainly via enzymatic oxidation from unsaturated fatty acids in fresh green olives (Sekiya et al., 1982).

The aroma and taste of numerous fruits are determined, or at least substantially influenced, by aldehyde compounds. Thus, all kinds of aldehydes, but especially acyclic α,β-unsaturated aldehydes, have been found in apples, peas, grapes, tomatoes, raspberries, peaches, strawberries, cherries, and cranberries and in the essential oils of orange peel, grapefruit, and hops (Nursten and Williams, 1967). Among these, a high aldehyde content (~18 mg/kg) has been found in strawberries and pears (Maarse, 1991). The compounds characterized in olive oil flavor are also identified in many edible plants and are used for flavors and fragrances (Schauenstein et al., 1977; Bauer et al., 1990; Maarse, 1991). For example, (*E*)-2-hexenal (**5**) is used in perfumes to obtain a green leaf note and in fruit flavors for green nuances (Bauer et al., 1990).

The antimicrobial activity of the individual components identified in the distillate, except β-farnesene and copaene, which were present in only minute amounts, was tested against the 15 selected microorganisms. The highest concentration tested was 800 μg/mL, unless otherwise specified, because of limited solubility in the water-based media. In addition, since the MIC is determined by measuring the turbidity for most of the microorganisms, but by naked eye observation after appropriate length of incubation for *P. chrysogenum*, *T. mentagrophytes*, and *A. niger*, minimum lethal concentration (MBC for bacteria and MFC for fungi) values were also obtained for many of the microorganisms. The results are listed in Table 1. The antimicrobial activities of (*E*)-2-hexenal, hexanal, 1-hexanol, and 3-carene were previously reported (Muroi et al., 1993). Among the compounds tested, (*E*)-2-hexenal (**5**) and (*E*)-2-heptenal (**6**) showed activity against all of the microorganisms tested, while 3-carene did not exhibit any noticeable activity up to 800 μg/mL. All of the other compounds tested showed some activity against one or more microorganisms. Notably, all of the α,β-unsaturated aldehydes (**5**–**11**) exhibited broad activity.

Among the 15 microorganisms tested, fungi were the most sensitive, especially *T. mentagrophytes* and *P. chrysogenum*. Thus, eight of the compounds tested showed activity against these fungi, with MICs ranging from 1.56 to 800 μg/mL. Among them, (*E*)-2-undecenal (**10**) was the most potent, with the MIC being 1.56 μg/mL against *T. mentagrophytes* and 6.25 μg/mL against *P. chrysogenum*. α,β-Unsaturated aldehydes (**5**–**11**) also exhibited activity against all of the other fungi tested, *C. utilis*, *S. cerevisiae*, *P. ovale*, and *A. niger*, with MICs ranging from 12.5 to 200 μg/mL. The MBC to MIC ratios were no greater than 2. This relatively strong activity is a rather unique result since most plant secondary metabolites show, in general, more potent activity against Gram-positive bacteria than fungi (Mitscher et al., 1972; Taniguchi et al., 1978; Dimayuga and Garcia, 1991). These results indicate that α,β-unsaturation of the aldehyde and increasing chain length of the carbon tail seem to result in increased activity. Besides the α,β-unsaturated aldehydes, nona-

Table 1. Antimicrobial Activity of the Olive Flavor Compounds[a]

	MIC and *MBC* or *MFC*[b] (μg/mL)									
	1	**2**	**3**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Bs	>800	200	>800	400	200	100	50	25	25	50
	–[c]	–	–	*>800*	*>800*	*>800*	*>800*	*>800*	*>800*	*>800*
Ba	>800	200	>800	400	400	200	100	50	25	50
	–	–	–	*400*	*400*	*200*	*100*	*50*	*25*	*50*
Sa	>800	200	>800	400	400	200	50	25	12.5	50
	–	–	–	*400*	*400*	*200*	*50*	*25*	*12.5*	*50*
Sm	>800	>800	>800	800	400	400	100	100	50	100
	–	–	–	*800*	*800*	*400*	*100*	*100*	*50*	*100*
Pac	800	100	>800	200	100	50	25	6.25	6.25	12.5
	–	–	–	*200*	*100*	*50*	*25*	*6.25*	*6.25*	*12.5*
Pae	>800	>800	>800	400	800	>800	>800	>800	>800	>800
	–	–	–	*400*	*800*	–	–	–	–	–
Ea	>800	>800	>800	400	400	200	200	>800	>800	>800
	–	–	–	*400*	*400*	*200*	*200*	–	–	–
Ec	>800	800	>800	400	400	200	200	>800	>800	>800
	–	–	–	*400*	*400*	*200*	*200*	–	–	–
Pv	>800	100	–	200	100	50	25	12.5	12.5	25
	–	–	–	*200*	*100*	*50*	*25*	*12.5*	*12.5*	*25*
Sc	>800	100	>800	200	100	50	25	25	25	25
	–	–	–	*200*	*200*	*100*	*50*	*50*	*25*	*25*
Cu	>800	100	>800	100	100	50	25	25	25	25
	–	–	–	*100*	*100*	*100*	*50*	*25*	*25*	*25*
Po	800	100	800	50	50	25	25	12.5	12.5	12.5
	–	–	–	*50*	*50*	*25*	*25*	*12.5*	*12.5*	*12.5*
Pc	>800	200	800	50	50	50	50	12.5	6.25	12.5
	–	–	–	–	–	–	–	–	*6.25*	*12.5*
Tm	>800	100	>800	50	25	25	12.5	3.13	1.56	1.56
	–	–	–	–	–	–	–	–	*3.13*	*3.13*
An	>800	–	>800	200	200	200	200	100	100	50
	–	–	–	–	–	–	–	–	*200*	*100*

[a] *Bs*, *B. subtilis*; *Ba*, *B. ammoniagenes*; *Sa*, *S. aureus*; *Sm*, *St. mutans*; *Pac*, *P. acnes*; *Pae*, *P. aeruginosa*; *Ea*, *E. aerogenes*; *Ec*, *E. coli*; *Pv*, *P. vulgaris*; *Sc*, *S. cerevisiae*; *Cu*, *C. utilis*; *Po*, *P. ovale*; *Pc*, *P. chrysogenum*; *Tm*, *T. mentagrophytes*; *An*, *A. niger*. [b] Numbers in italic type are MBC or MFC. [c] –, not tested.

nal (**2**) exhibited some antifungal activity (Kubo et al., 1993a), while hexanal (**1**) and 1-hexanol (**3**) did not show noticeable activity.

Of specific interest is the activity of the α,β-unsaturated series against *A. niger*. *A. niger* is one of the foodborne fungi that grow on stored grains, and aside from concerns about food quality degradation, *A. niger* produces aflatoxins, potent carcinogens. The series' activity against *A. niger* is fairly good but not exceptional. It does, however, suggest new possibilities for postharvest microorganism control. In addition, it has been found that olive callus tissue extracts inhibit aflatoxin production, though not growth of the mycelium itself (Paster et al., 1988).

The same α,β-unsaturated aldehydes (**5**–**11**) also showed activity against all of the Gram-positive bacteria tested, with MICs ranging between 6.25 and 800 μg/mL, as listed in Table 1. Among them, *P. acnes* was the most sensitive and *S. mutans* the least. Their MBC to MIC ratios were no greater than 2 except against the spore-forming bacterium *B. subtilis*. Similarly, we have recently reported that long-chain alcohols did not show bactericidal activity against this spore-forming bacterium up to 800 μg/mL (Kubo et al., 1993b). Nevertheless, alcohols are known to have little effect on spores (Davidson, 1983). The current study indicates that aldehydes also possess little bactericidal activity against this spore-forming bacterium. In addition to the α,β-unsaturated aldehydes, nonanal (**2**) showed some activity against Gram-positive bacteria but the two C_6 compounds, hexanal (**1**) and 1-hexanol (**3**), did not show any noticeable activity against Gram-positive bacteria.

In the case of Gram-negative bacteria, *P. vulgaris* was the most susceptible. Thus, all of the α,β-unsaturated aldehydes tested (**5**–**11**) as well as nonanal (**2**) inhibited the growth of *P. vulgaris*, with MICs ranging from 12.5 to 200 μg/mL. In contrast, *P. aeruginosa* was the least sensitive, and (*E*)-2-hexenal (**5**) and (*E*)-2-heptenal (**6**) were found to be the only compounds active against this bacterium, with MICs of 400 and 800 μg/mL, respectively. In addition to *P. vulgaris*, the growth of *E. coli* and *E. aerogenes* was also inhibited by (*E*)-2-hexenal (**5**), (*E*)-2-heptenal (**6**), (*E*)-2-octenal (**7**), and (*E*)-2-nonenal (**8**). Importantly, the above-mentioned α,β-unsaturated aldehydes (**5**–**8**) were also found to be bactericidal. Thus, their MICs and MBCs were the same. Since few phytochemicals have been reported to exhibit activity against Gram-negative bacteria, their activity was further studied. For example, the bactericidal effect of (*E*)-2-nonenal (**8**) against *E. coli* was confirmed by the time–kill curve method. Thus, the cultures of this bacterium were exposed to two different concentrations of (*E*)-2-nonenal, the MIC and $^1/_2$-MIC. The number of viable cells was determined following different periods

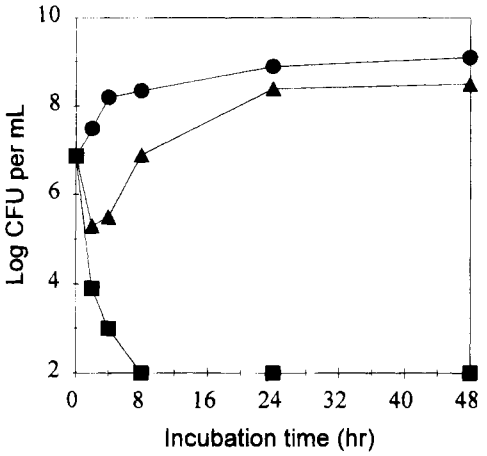


Figure 1. Effect of (*E*)-2-nonenal on the growth of *E. coli*. A 48-h culture was inoculated into NYG broth containing 200 μg/mL (■), 100 μg/mL (▲), and 0 μg/mL (*E*)-2-nonenal (control) (●).

of incubation. The results are illustrated in Figure 1. Moreover, hexanal (**1**) and nonanal (**2**), the saturated aldehydes characterized, did not show noticeable activity against Gram-negative bacteria up to 800 μg/mL. It appears that the original broad antimicrobial activity of olive extracts can be ascribed predominantly to the presence of α,β-unsaturated aldehydes. A number of phytochemicals have been characterized as antimicrobial agents. However, only a few of them showed activity against Gram-negative bacteria, especially the *Pseudomonas* species. The α,β-unsaturated aldehydes characterized in olive oil are some of the rare phytochemicals to exhibit this activity.

The C_6 and C_9 alcohols (**3**) and aldehydes (**1**, **2**, **4**, **5**, **8**) are known as "green leaf" alcohols and aldehydes, which are produced by enzymatic cleavage of unsaturated fatty acids when plants undergo microbial attack (Hatanaka et al., 1982, 1983; Sekiya et al., 1982). We recognize them by a distinct smell which we call green leaf odor (Hatanaka, 1993). On the basis of their broad antimicrobial activity as described above, especially of the α,β-unsaturated aldehydes, they may be an important defense mechanism against microbial invasion. If so, the green leaf substances may be produced enzymatically only when plants need to defend against microorganism attack and hence are classified as postinhibitins (Ingham, 1973). However, it is not yet clear whether these substances have a causal role in protecting plants from microbial attack *in vivo*. Nevertheless, at least in part, if not all, they should be valuable in the multichemical defense against microbial attack.

On the basis of our previous studies of structure–antimicrobial activity relationships with a series of long-chain alcohols (head and tail structures), we have recently reported that the maximum antimicrobial activity depends on the hydrophobic alkyl (tail) chain length from the hydrophilic hydroxyl group (head) (Kubo et al., 1993b). They can be classified as nonionic surface-active compounds (detergents) which destroy the native membrane-associated function of the integral proteins, such as ion channels or transport proteins. The balance of these two factors, the hydrophobic and hydrophilic moieties, may be expressed, for example, by the partition coefficient, log P_0 (Lien et al., 1968). Although more study needs to be done with additional appropriate compounds, the data so far obtained indicate that the effectiveness of the α,β-unsaturated aldehydes also seems to depend on the chain length from the enal group and the microorganism tested. On the basis of the similarities found for long-chain alcohols (Kubo et al., 1993b), it appears that α,β-unsaturated aldehydes greater than C_{11} may be expected to exhibit more potent activity against Gram-positive bacteria and fungi.

The antimicrobial activity of some common naturally occurring α,β-unsaturated aldehydes such as citral and citronellal has long been known. For example, citral was reported to show a strong marked bactericidal activity associated with low toxicity toward mammals and recommended for prophylaxis and therapy of potential infections (Schauenstein et al., 1977). However, the activity described above with a series of α,β-unsaturated aldehydes is more potent. This can be explained as follows. α,β-Unsaturated aldehydes are highly reactive substances that readily react with biologically important nucleophilic groups, such as sulfhydryl, amino, or hydroxyl. Because of the conjugated double bond system, the main reaction appears to be 1,4-addition under physiological conditions. Although the formation of Schiff bases is also possible, the former reaction takes place much more quickly than the latter (Schauenstein et al., 1977). In general, 1,4-addition seems more likely, and the activity of citral and citronellal is much weaker than that of the corresponding nonbranched α,β-unsaturated aldehydes because in these isoprene molecules the β-hydrogen atom in the enal group is replaced by a bulkier methyl group. This hinders nucleophilic groups from being approached.

On the basis of our previous study with long-chain alcohols (Kubo et al., 1993b), the rationale behind these compounds' antimicrobial activity is starting to become evident. All of the molecules possess the same hydrophilic portion, the α,β-unsaturated aldehyde group, and thus understanding the role of the alkyl hydrophobic portion is key. The common nature among these aldehydes is the oxygen atom, whose electronegativity may be influenced by differences in carbon tail length. As compared to long-chain alcohols, the more potent α,β-unsaturated aldehyde activity might be explained by differences in electronegativity, the aldehyde oxygen atom possessing more than that of the hydroxyl oxygen atom. The greater electronegativity of α,β-unsaturated aldehydes would cause greater incidence of intermolecular hydrogen bond formation with nucleophilic groups of the membrane, creating significant disorder in the lipid bilayer. This will be described in further detail separately.

Olive oil, one of the most important oils, is obtained from ripe fruit that is crushed in edge runner mills, and the oil is expressed in open hydraulic presses. This oil has long been widely used on a daily basis. It has been used primarily for cooking and flavoring foods, though it was also found to be a good preservative, and it is still possible to purchase certain foods, such as cheese, that are stored in olive oil.

Safety is a primary consideration for antimicrobial agents, especially those in food and cosmetic products, which may be utilized in unregulated quantities on a regular basis. Olive oil has proven its safety through many years of human use and consumption. In addition, a similar C_6–C_{11} aldehyde composition was also reported in sunflower oil (Badings, 1965) and the essential oil of grapefruit (Nursten and Williams, 1967). This further substantiates its safety. Hence, the olive

oil flavor compounds described herein may be considered as potential antimicrobial agents for food and cosmetic products.

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