

## Inhibitory Activity of Grapefruit Seed Extract Against Fungi and Its Application in Preservations of Grape and Persimmon

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**Abstract:** The grapefruit seed extract (GSE), a natural plant extracts from the edible plants used as an extremely potent broad-spectrum bactericide, and fungicide should be very effective for the fruit preservation. In this study, Grape (*Vitis vinifera* L.) and persimmon (*Diospyris kaki* L.) easily undergone deterioration, were selected as model fruits. Eight fungi familiar to the rotting cause of grape and persimmon were used to testify the antifungal activity of GSE. The results of antifungal assay by the agar diffusion method indicated that GSE can efficiently inhibit the growth of tested fungi. The values of minimal inhibitory concentration (MIC) also range from  $39.06 \times 10^{-6}$  to  $625 \times 10^{-6}$  as demonstrated by the similar results with the data taken from agar diffusion assay. In the storage application assay of both grape and persimmon, the obvious differences in sensory quality and microorganism indexes between the GSE treated fruits and control fruits support that GSE has both the strong antifungal activity and antioxidative activity. Polyphenolic compounds, rich in GSE used as the main effective antioxidative component, are capable to prevent fungal infection in storage to a large extent and to postpone ripening and aging of fruits effectively by scavenging the free radical and impacting a series of enzyme relating to postharvest physiological metabolism of fruits, particularly in the key parts liking stem and calyx. The obtained results also indicated that GSE can decrease browning and rot ratio, prolong the preservation period, ensure the quality of grape and persimmon and can be used as an effective and safe preservative.

**Key words :** grapefruit seed extract; preservation; antifungal activity; grape; persimmon

## 葡萄柚种子提取物对真菌的抑制作用及其在葡萄和柿子保鲜中的应用

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**摘 要:** 葡萄柚种子提取物是潜在的广谱性细菌、真菌杀菌剂, 应该在果蔬保鲜中具有很好的效果。本研究选取比较容易受真菌感染的葡萄和柿子作为模式保鲜材料并且测试了葡萄柚种子提取物对8种与这两种水果腐败相关的真菌的抑制效果。结果表明, 这8种真菌的最小抑制浓度分别在  $39.06 \times 10^{-6}$  至  $625 \times 10^{-6}$  之间不等。果实保鲜结果也证实了葡萄柚种子提取物对真菌具有很好的杀抑作用, 延缓了果实的成熟, 并且使保鲜的果实保留了较好的风味。这些结果可能与葡萄柚种子提取物富含多酚类物质有关, 因为多酚类物质具有很强的抗氧化活性。

**关键词:** 葡萄柚种子提取物; 保鲜; 真菌抗性; 葡萄; 柿子

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Grapefruit seed extract (GSE) is a commercial product derived from the seeds and pulp of grapefruit (*Citrus paradisi* Macf. Rutaceae). Chemical research revealed the constituents of flavonoids<sup>[1-2]</sup>, ascorbic acid, tocopherols, citric acid<sup>[3]</sup>, limonoids<sup>[4-5]</sup>, sterols and minerals<sup>[6]</sup> in the

grapefruit seeds and pulp. And GSE also contained large quantities of polyphenolic compounds such as catechins, epicatechin and epicatechin-3-O-gallate, and dimeric, trimeric and tetrameric procyanidins<sup>[7]</sup>.

GSE is an extremely potent and effective broad-spec-

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trum bactericide<sup>[8-9]</sup>, fungicide<sup>[10]</sup>, antiviral and antiparasitic<sup>[2]</sup> natural extract. GSE is environmentally safe from toxicity to man and animal within an effectively applied concentration extent. Inoescu demonstrated that GSE performed well as the other antimicrobial agents tested on 770 strains of bacteria, and 93 strains of fungus. The inhibition activity of GSE to yeast and some yeast-like fungi shows different efficacy according to the strains and is generally weaker than that of bacteria and fungi<sup>[2]</sup>. Concerning mechanism of action, the researchers propounded that GSE could disrupt the bacterial membrane and liberates the cytoplasmic content by inhibiting enzymatic activities<sup>[9]</sup>. Although the active components of GSE in relation to its antimicrobial property are still incompletely known, polyphenols compounds particularly flavonoid, are considered to play an significant role in light of the accurate positive correlation between their total contents and antimicrobial activity of GSE<sup>[11]</sup>. As antimicrobial agent, the most obvious superiority of GSE rests with its safety better than other chemical synthetic antimicrobial agent. Tests conducted by the United States Department of Agriculture (USDA) in the early 1980s confirmed that GSE is not toxic to animal and has been approved for use by USDA. Sequentially the safety of GSE was testified by several writers. Hegggers et al. testified that GSE would not be detrimental to human fibroblast skin cell in an *in vitro* culture, while still retained high antimicrobial activity. The results obtained from J. Juskiewicz et al showed that the addition of 0.1%~0.4% extract of flavonoid from grapefruit did not affect the diet intake and body weight gain of rats and slightly increased the anti-oxidative potential of serum<sup>[12]</sup>. Today consumers prefer products without any artificial chemical preservatives because of the residue and safety problems of synthetic antimicrobial agents<sup>[13-14]</sup>. Therefore GSE is of a wide application perspective in many fields including agriculture, cosmetic production, decontaminating water, treating infections and storage of fruits and vegetables as well.

Grape (*Vitis vinifera* L.) and persimmon (*Diospyris kaki* L.) are two of the most important fruits in world. But due to the soft texture and the high water content, these fruits easily undergo deterioration resulting from physiological disorder, microorganism infection or mechanical damage, so preservation is difficult. Under normal atmospheric and temperature conditions, grapes can be stored for only 2~3 days<sup>[15]</sup> and persimmon 4~5 days<sup>[16]</sup>. In order to ensure their even distribution both in time and in space, preservation of grape and persimmon is of great concern. The traditional

methods of preserving grape and persimmon are basket storage, cellar-storage, chemical storage and cold storage. Most of the storage methods are selective when kept at low temperature, but it is not easy to control temperature and humidity accurately for marketing. It is well known that variation of temperature affects respiration, metabolism and microorganism, growth and consequently, preservation efficacy. However microorganisms, particularly fungi, exert harmful effects on quality, safety and shelf life of grape and persimmon and are most responsible for yield reductions and a great many losses in the course of storage throughout the world. The use of antifungal agent is the most effective method to minimize fungi infection, although the growth of microorganisms is easily controlled by chemically synthesized preservatives, such as sorbic or benzoic acids. This preference has prompted the application of natural plant extracts to prevent rot and prolong preservation period of fruits and vegetables. The used GSE as antifungal agent from the edible plants should be superior in comparison with non-natural antifungal agents.

Our objectives were to evaluate the antifungal effectiveness of GSE for the eight fungi familiar in fruit preservation so as to determine the minimum inhibitory concentration (MIC) for these eight fungi. Specifically applied in the preservation of grape and persimmon, while results were observed and analyzed. The mechanism between antioxidant and antifungal GSE and the postharvest physiological variations of fruits were also discussed.

## 1 Materials and Methods

### 1.1 Selection of fungi and bacteria strains

The eight fungi used as test organisms are: *Botrytis cinerea* (CGMCC#3.3789), *Alternaria* sp. (CGMCC#3.1491), *Stemphylium lanuginosum* (CGMCC#3.4282), *Aspergillus niger* (CGMCC#3.310), *Pen.expansum* (CGMCC#3.5425), *Streptomyces albus* subsp. *albus* (CGMCC#4.1), *Streptomyces griseus* subsp. *griseus* (CGMCC#4.18) and *Saccharomyces cerevisiae* (CGMCC#2.0401). All of them are closely correlated to the rot of fruit and vegetable in postharvest period, especially of grape and persimmon employed in this study. The culture of each fungi was maintained on potato dextrose agar (PDA) and stored at 4℃. These fungi were obtained from the Institute of Microbiology of Chinese Academy of Sciences.

### 1.2 Antifungal assay

GSE was dissolved in the water with 0.05% (V/V) Tween-

80 as surfactant to make a 10% (V/V) stock solution filtered and sterilized.

The fungi were swabbed on solidified PDA plates. Each PDA plate was inoculated with a single identified fungal isolate. The four sterile metal cylinders  $8 \times 6 \times 10$  mm in diameter were placed on the agar and then filled with 50  $\mu$ l of 0.01%, 0.1%, 1% in 10% (V/V) GSE solution. The same volume of water containing Tween-80 was also tested as control. After 2-h diffusion at 4  $^{\circ}$ C, all the plates were incubated at 37  $^{\circ}$ C for 48 hours. At the end, inhibition zones around each metal cylinders formed in the medium with their diameters measured to determine the antifungal effectiveness of diluted GSE solutions. All the tests were made in triplicate on separate assays [17].

### 1.3 Determination of MIC

Broth microdilution assays were performed as previously described [18]. This test was performed in sterile cultivation with flat-bottomed 96-well microplates. Stock solution of GSE was diluted (5-fold) with heat-sterilized medium to give the final concentrations. 100  $\mu$ l of the GSE dilutions were inoculated into the wells with first well of each row containing the lowest concentration and last well as the control (GSE-free medium). The working suspension of the inoculum, adjusted with a spectrophotometer to give initial numbers ranging from  $10^5$  to  $10^6$  CFU/ml. Then 100  $\mu$ l inoculum suspension was added to each well. After microplates were incubated without agitation at 30 $^{\circ}$ C for 48 h, spectrophotometric readings (SP) of each well were performed with an automated plate reader (Multiskan MK3, Thermo) set at 492 nm. SP MIC endpoints were defined as the lowest GSE concentrations with OD less than or equal to 80% inhibition compared with that produced by the control tube. Experiments were performed in triplicate.

### 1.4 Application of GSE in preservation of grape and persimmon

Grape (*Vitis vinifera* L.) and persimmon (*Diospyris kaki* L.) were selected as the model plant materials in the preservation with fungi inhibiting experiments in view that they are easy to be infected by various fungi and led to spoilage. Grape and persimmon were both obtained from a local farm, and these species were most popular with consumers.

GSE was dissolved in the water with 0.05% (V/V) Tween-80 as a surfactant to make a  $100 \times 10^{-6}$  (0.01% V/V) stock solution and was filter-sterilized. Water was used as control. Fresh grape and persimmon without spoilage and browning appearance were soaked in the above solutions for 5 min

and dried in the air until solution formed a uniform film on the surfaces of the fruits. Treated grape and persimmon were then put into preservative bag with permeability to air in which the fruits were capable to maintain natural respiration. After drying, the treated grape and persimmon were stored at  $27 \pm 2$   $^{\circ}$ C and 65% RH for different lengths of time. At regular intervals the fruits were removed and assayed. The various sensory indexes, microorganism indexes and physiological indexes of both treated grape and persimmon and control were assayed and recorded.

## 2 Results and Discussion

### 2.1 Antifungal activity of GSE with related active compounds

The results of antifungal activity assays showed that GSE had the inhibitory effects on the growth of all the tested fungi strains while GSE antifungal activity differs slightly with respect to the different strains in each specific concentration of GSE (Table 1). However with the increase of GSE concentrations, inhibitory effects were clearer and inhibitory zones became larger gradually. At highest concentration of GSE, the growth of all fungi strains was reduced significantly and some strains were even inhibited completely. This proved that the eight tested fungi were susceptible to GSE in different degree of concentrations. The response of *Saccharomyces cerevisiae* is the strongest, while the susceptibility of *Streptomyces griseus* subsp. *griseus* is slightly lower.

Table 1 Mean inhibition zone sizes (mm) of GSE against 8 fungi strains

Test fungi	Average diameter(mm)			
	0.01%	0.10%	1%	10%
<i>Botrytis cinerea</i>	9	12	26	35
<i>Alternaria</i> sp.	10	19	30	> 40
<i>Stemphylium lanuginosum</i>	13	19	33	> 40
<i>Aspergillus niger</i>	12	20	35	> 40
<i>Pen.expansum</i>	8	15	32	> 40
<i>Streptomyces albus</i> subsp. <i>albus</i>	4	10	24	38
<i>Streptomyces griseus</i> subsp. <i>griseus</i>	5	11	20	33
<i>Saccharomyces cerevisiae</i>	13	21	38	> 40

The minimum inhibitory concentration (MIC) is an important index for application of GSE in postharvest treatment. Because the fundamental of dose is toxicity, although some antimicrobial agents at high concentration exhibit the strong inhibitory effect against a wide range of organism, these concentrations always have the harmful effects to the fruit itself and health of consumers. So antimicro-

bial agent with high MIC is not suitable to be used in postharvest treatment of fruit because of problem of cost and safety. The MIC of GSE to the eight fungi is showed in Fig 1.

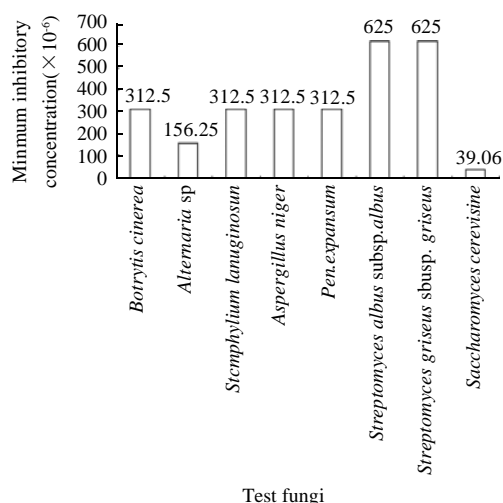


Fig.1 Mean minimum inhibitory concentration (MIC) of GSE against 8 fungal species

In conclusion, the GSE showed antifungal activities against 8 fungal species at or above 0.01% concentration. The values of minimal inhibitory concentration (MIC) demonstrated the similar results with the data from agar diffusion assay. And the MIC of GSE to 8 fungal species is lower than some chemical antifungal agent such as Nisin (data not shown). Thereby, it was suggested that the GSE may be used as antifungal agent to protect fruits against fungal diseases.

## 2.2 Antifungal mechanism of GSE

Microorganisms are inactivated when they are exposed to factors that substantially alter their cellular structure or physiological functions. Structural damage includes DNA strand breakage, cell membrane rupture or mechanical damage to cell envelope. Cell functions are altered when key enzymes are inactivated or membrane is selectively disabled. In contrast to bacterial cell and yeast cell, who often exist in the style of dissociative unicellular state or have no cell wall, the fungal cells developing from a spore, particularly mold, form an organic whole and cling close to the surface of fruit. Therefore the antimicrobial factors including GSE show the different effectiveness on bacteria, fungi and yeast.

Antifungal action of GSE largely lies on its effect on the membrane of fungi. Concretely it comes mainly from ability of antifungal compounds in GSE to act as surfactants (physical disruption of the membrane). They have an effect on the fluidity and permeability of the membrane lipids by disrupting and disorganizing the lipid bilayer-protein inter-

face unspecifically. Thus compartmentalization structures in the fungal cell depending on biomembrane system and some membrane-bound enzymes and receptors have been impacted to a large extent. So the growth of fungi will be inhibited even completely.

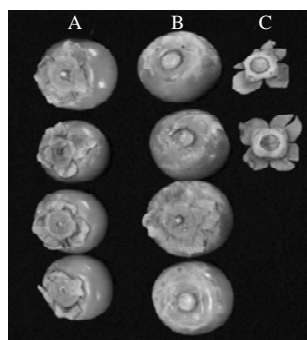
Compared with other chemical synthetic antifungal agents, GSE showed the better inhibitory effect on the growth of test fungi in view of synergistic effect of various antifungal compounds such as limonoids, polygodial and sesquiterpene dialdehyde. The synergistic effect is one of the most important characteristics exhibited by natural extracts, increasing their efficacy in contrast to those which could be obtained with the equivalent amount of the active constituents alone.

Microorganisms have different intrinsic or natural resistance to a disinfectant, but resistance can also be acquired by genetic transfer and mutation or by exposure to sublethal concentrations of the antimicrobial agent. The latter property can be reversible and is often termed adaptation. For GSE, it may hinder the development of resistant mechanisms in microorganisms because of its synergistic effect.

## 2.3 Application effects of GSE in preservations of grape and persimmon

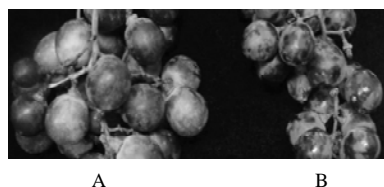
The sensory evaluation of grape and persimmon treated with GSE revealed significant different in color, texture, flavor and taste compared with control grape and persimmon. Fruits treated with GSE had maximum freshness, surface color, texture, hardness and taste and were best even after 15-day storage. There were not fungal infection, rot phenomena and pathological changes among fruits treated with GSE. But the control fruits were on the other hand, calyxes of most persimmons had fall from the fruit and wound was infected by fungi obviously, and pulp tissues exhibited a very soft and collapsed texture. Almost all control persimmons lost the edibleness. Calyxes is very important for the preservation of persimmon. Wound from dropping of calyxes will arouse wound respiration, accelerate ripening and aging of fruit and cause infection by fungi. The storage time and quality of persimmon mainly depends on whether calyxes drop or not (Fig.2). For the grape, the stem of grape dehydrated badly and browned. Some berry in a bunch of grapes dropped because of rot in calyxes. Most grapes blackened and soften due to over-ripening and fungal infection (Fig.3). Some hormones, especially ABA, regulate the formation of abscission layer in the position of calyxes in many plants. The variation of hormones content also is important to the

preservation of grape and persimmon <sup>[12]</sup>.



A.Persimmon treated with GSE; B.Control persimmon without any treatment; C.Calyxes dropping from control persimmon.

Fig.2 Grape treated with GSE and control persimmon after storage of 5 days at room temperature

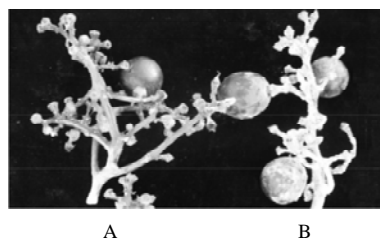


A.Grape treated with GSE; B.Control grape without any treatment.

Fig.3 Grape treated with GSE and control grape after storage of 7 days at room temperature

Worth mentioning especially, stem is physiological active parts with larger respiration intensity than berry and is the key point of grape storage to the grape <sup>[19]</sup>. In the Fig. 4, stem of grape treated with GSE and stem of control grape are shown when they were store at room temperature for 25 days and most berries had been picked off in order to highlight stem. The white hypha bestrewed on the surface of stem and berry of control grape and the stem had browned due to fearful fungal infection. The Fig.5 is the close-up view of the control. In contrast to berry that is non-climacteric fruit, grape's stem has the obvious climacteric. In the course of storage, although berry has not yet produced ETH mutation, ETH signal from stem could be passed to berry and lead to variation of respiration intensity in berry. So the stem is the key point in the storage of grape. The evident difference in grape stem between treated and control grape exhibited strong antimicrobial and antioxidant effect of GSE.

The experiment indicated that GSE could decrease browning and rot ratio, prolong the preservation period and ensure the quality of grape and persimmon. The application prospect of GSE in the preservation of grape and persimmon is very bright, for it has good antimicrobial effectiveness and



A.Grape stem treated with GSE; B.Control grape stem.

Fig.4 Grape stem treated with GSE and its control after storage for 27 days at room temperature



Fig.5 Close-up of stem and berry of control grape after storage for 27 days at room temperature

is extracted from natural plant.

#### 2.4 Effects of GSE on postharvest physiological variations of grape and persimmon

GSE showed a good antimicrobial activity in the above experiment. For the antimicrobial activity of GSE, firstly GSE solution was able to form a semi-permeable coat, which can provide protection against fungal infections. Physically, the coat prevents the osculation between the surface of fruit and fungi and spore existing in the storage environment. Chemically, the antimicrobial compounds in GSE inhibit the growth of fungi and spore in the surface of fruit. This coat also can reduce the respiration and decrease the loss of water from the surface and adjust the postharvest physiological metabolism to some extent.

GSE contains a great many of phenolic compounds, which are the main active compounds for its antioxidant activity. Abu-Amsha et al. had suggested that the higher the total polyphenolic content, the greater is the antioxidant capacity <sup>[20]</sup>. Once these phenolic compounds diffuse from coat into the fruit, they will affect activity of enzymes relating to postharvest physiological metabolism by scavenging of free radical that leads to the oxidation of biomolecules and ripening and aging of fruit. And the decrease of free radical will delay activity peak of polyphenol oxidase (PPO) and peroxidase (POD). Sequentially phenolic and pectic compounds in

the fruit will not degrade when enzyme activity has been inhibited. Reflected in sense, fruits maintain former firmness, freshness, colour and texture without browning and softening.

### 3 Conclusion

Because of strong antifungal activity and antioxidant activity, GSE can decrease browning and rot ratio, prolong the preservation period and ensure the quality of grape and persimmon. So it can be used as an effective and preservative.

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