

EFFICACY OF LEMON GRASS (*CYMBOPOGON CITRATUS* STAPF.) ESSENTIAL OIL AS A NATURAL PRESERVATIVE IN READY-TO-DRINK MORINGA (*MORINGA OLEIFERA* LAM.) BEVERAGE

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ABSTRACT

Essential oil from a variety of sources has been proven to be effective antimicrobial for the past decades. However, its efficacy when applied to the food system is a major consideration. This study aimed to evaluate the antimycotic property of lemon grass (*Cymbopogon citratus* Stapf.) essential oil (LGEO) and its effect on the microbial stability and quality of ready-to-drink (RTD) moringa (*Moringa oleifera* Lam.) beverage. Agar well diffusion assay was used to screen the antimycotic activity of the essential oil against common fungi that may grow in ready-to-drink beverages. The largest zone of inhibition (10.67 ± 1.15 mm) was observed against the yeast *Debaryomyces hansenii*. It was followed by *Hansenula anomala* and *Torulopsis candida* with zones of inhibition of 5.67 ± 0.58 mm and 3.67 ± 0.58 mm, respectively. For molds, *Penicillium roqueforti* and *Mucor rouxii* were reported to have 2.33 ± 0.58 mm and 2.00 ± 1.00 mm zones of inhibition, respectively. Agar dilution method was performed to determine the minimum inhibitory concentration (MIC) against each microorganism. *D. hansenii* was the most susceptible yeast which was inhibited by 1.5 uL/mL of LGEO. It was followed by *H. anomala* and *T. candida* which were inhibited at minimum concentrations of 3 uL/mL and 5 uL/mL, respectively. Microbial count monitoring of moringa beverage containing different concentrations of LGEO was performed within 10 days and 10 uL/mL of LGEO was found to be the most effective concentration in maintaining the stability of the beverage. The addition of LGEO combined with heat treatment exhibited a synergistic effect in the preservation of RTD moringa beverage. Sensory evaluation results indicated that addition of LGEO did not negatively affect the organoleptic properties of the drink. Results showed that LGEO is an effective antimycotic agent and a potential natural preservative in RTD moringa beverage.

Keywords: natural preservative, lemon grass essential oil, antimycotic agent, moringa beverage

INTRODUCTION

Ready-to-drink beverages and juices usually have an acidic pH range, and with this condition, the common microorganisms that cause their spoilage are yeast and molds. Molds can produce mycotoxins in juice such as patulin, which is produced by several species of *Penicillium*, and ochratoxin A and citrinin produced by other genus of molds (Vasavada, 2003). The most common preservation technique applied in juices is pasteurization. Nevertheless, there are still heat resistant species of mycelia fungi such as several species of *Byssoschlamys*, *Monascus*, *Paecilomyces* and *Aspergillus* which are reported to spoil juices, and at some extent, mycotoxins are produced (Keller and Miller, 2006). Additional techniques include the use of antimicrobial agents which preserve the food by inhibiting the growth of microorganisms and prevent subsequent spoilage by serving as fungistat, mold and rope inhibitor (Jiang, 2009).

Consequently, there has been an increasing demand for processed foods that contain fewer or no synthetic additives. Food manufacturers responded to what the consumers require for the 'natural' food thus, many researches for possible preservatives derived from natural sources were established (Russell and Gould, 1996). The use of natural food preservative is in line with the growing demand in green consumerism. Green consumerism is a set of practices which are primarily focused on sustainable consumption, alleviating the social and environmental effects of consumption, reducing the harmful effects of different commodities on people by consuming green products which are natural rather than those which are synthetically produced (Mansvelt, 2011).

Natural antimicrobials are derived from different sources like plants, animals and microorganisms. They can exhibit antimicrobial activity in the foods by adding them as ingredients or as additives. Chitosan, lysozyme, and lactoperoxidases are

examples of antimicrobials from animals. Plant antimicrobials include essential oils, aldehydes, esters, herbs and spices. Nisin, on the other hand, is of microbial origin (Jiang, 2009). Among these natural origins, plants have been considered to be one of the most abundant sources consisting a vast range of compounds with a broad spectrum of biological activities. Such components include alkaloids, dienes, flavonols, flavones, glycosides, lactones, organic acids, phenolic compounds, and protein among others. These substances have high potential to be utilized as food preservatives (Bhat, 2012).

Lemon grass (*Cymbopogon citratus* Stapf.) or locally known in the Philippines as 'tanglad' is commonly utilized as a food spice and flavoring, herbal medicine, and even as scent in cosmetic products. The distinct odor of lemon grass is due to its essential oil. Essential oils are complex mixture of acyclic and/or cyclic terpenoids. These are monoterpenes, lipophilic, volatile substances which gives the characteristic odor of many plants (Croteau, 1987). Lemon grass essential oil was also known to exhibit antimicrobial activity, and its use as a natural preservative in foods is worth to be investigated.

Moringa oleifera, locally known as 'malunggay', on the other hand, is known to contain substantial amount of nutrients essential to health like β -carotene, ascorbic acid, folic acid and minerals such as iron, calcium, phosphorus and zinc (Simopoulos, 2003). It is commonly utilized as an ingredient in local cooking. Because of the known nutritional value of moringa, it has been incorporated as an ingredient in food supplements and is processed into beverages.

In line with green consumerism and the explorations of new preservation method to further increase the microbial stability of juices, processors tend to find more natural ways in preserving food. Essential oil is of plant origin and is considered as Generally Recognized as Safe (GRAS) for its intended use (USFDA, 2019). In the present study, the application of lemon grass essential oil to an arising

beverage, ready-to-drink moringa juice, as a natural antimycotic agent was investigated.

MATERIALS AND METHODS

Plant material

Fresh lemon grass (*Cymbopogon citratus* Stapf.) was procured from Nagcarlan, Laguna, Philippines. The roots and soil particles were removed, and the plant material was washed thoroughly, air dried and kept in dark at 25 °C until use.

Extraction of essential oil

Dried lemon grass leaves were crushed using Osterizer blender (Oster Manufacturing, Florida, USA) and mixed with acetic acid-sodium acetate buffer at pH 4.5 with 1:3 (lemon grass : buffer) ratio. After mixing, 1% (v/w) of the enzyme cellulase obtained from the National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños was added in the mixture. Enzyme-aided extraction was performed by allowing it to stand at 30°C for 24 hours. The resulting mixture was filtered and added with 5 mL of petroleum ether per 250 mL. The solvent was evaporated from the organic layer and the extract was concentrated using rotary evaporator. The lemon grass essential oil (LGEO) was filtered over anhydrous Na₂SO₄, collected and stored in dark at 4°C until use.

Microbial cultures

Pure cultures of representative microorganisms that may proliferate in the acidic environment of juices and beverages such as *Debaryomyces hansenii*, *Hansenula anomala*, *Torulopsis candida*, *Penicillium roqueforti* and *Mucor rouxii* were obtained from the Food Microbiology Laboratory, Institute of Food Science and Technology, University of the Philippines Los Baños. All cultures were maintained in Potato Dextrose Agar (PDA) slants and kept at 4°C until needed.

Agar well diffusion assay

In order to determine the antimycotic property of the extracted LGEO, agar well diffusion assay was performed based on the method of Helal *et al.*, (2006) against the test organisms. Different amounts of essential oil (20, 40 and 60 uL) were placed in holes and sterile water containing 0.5% Tween 80 was used as the control. After the complete diffusion of essential oil in the agar, the plates were incubated at 30°C for 3 and 5 days for yeast and molds, respectively, and the diameter of the zones of inhibition was measured using a Vernier caliper (Mitutoyo Corporation, Japan) and was expressed in mm.

Determination of minimum inhibitory concentration (MIC)

Modified procedure based on the method of Tyagi & Malik (2010) was used in the determination of MIC. PDA with different concentrations of essential oil (10, 5, 4, 3, 2, 1.5 and 1 uL/mL) were prepared. Each tube, which contained 500 uL of 0.5% Tween 80 and 65 uL of 10% tartaric acid, was inoculated with 150 uL of 10⁶ cfu/mL cell suspension. The test tubes were incubated at 30°C for 3 and 5 days for yeast and molds, respectively. The lowest concentration of essential oil where no colony was observed after incubation was recorded as the minimum inhibitory concentration.

Food application

Moringa beverage formulation

The formulation of the ready-to-drink beverage included moringa leaves extract, 'calamansi' extract as acidifier and refined sugar. The mixture was added with 1% of soybean lecithin to serve as emulsifier. Moringa beverage with lemon grass essential oil as preservative at a concentration of 5 and 10 uL per mL beverage, was prepared. The pH and total soluble solids were adjusted to 4.5 and 6 °Brix, respectively, and the ready-to-drink beverage was pasteurized at 80°C for 15 minutes.

Microbial analysis

Yeasts and molds in the normal flora of the packaged ready-to-drink beverage were monitored within 10 days of storage at ambient condition. Fungal counts of different treatments namely pasteurized beverage containing LGEO at 5 and 10 uL per mL of beverage and unpasteurized beverage containing the same amount of LGEO, were determined using spread plating technique. Pasteurized and unpasteurized beverages without the LGEO were used as the control. The microbial count was calculated using the formula,

$$\frac{CFU}{mL} = \frac{\Sigma C}{[(n1x1) + (n2x0.1)]xd}$$

where, CFU/mL= colony forming unit per mL of sample; C= number of colonies in the counted plates; n1 and n2 are the number of plates counted for two consecutive dilutions with valid counts; and d = dilution from which n1 was obtained. The valid count ranges considered were 25-250 for molds and 35-350 for yeasts and the microbial load was expressed as log of CFU/mL.

Sensory evaluation

To determine whether the addition of lemon grass essential oil could affect the sensory characteristics of the beverage, sensory evaluation was performed (Watts *et al.*, 1989). The intensity of sensory characteristics namely color, aroma, sweetness, sourness, flavor, as well as the general acceptability of the moringa beverage with the different concentrations of essential oil were assessed by 20 laboratory panelists using quality scoring in a 6-in line scale. Samples were evaluated at room temperature (25°C) under white lighting condition in separate sensory evaluation booths.

Statistical analysis

All analyses were carried out in triplicates. Randomized complete block design was employed for agar well diffusion experiment and determination of minimum inhibitory concentration. For the experiments on the juice application of essential oil, two factorial experimental design was used. The data gathered from the experiments were subjected to the Analysis of Variance (ANOVA) and analyzed using SAS General Linear Models (GLM) procedure with SAS software 9.1.3. Least Significant Difference (LSD) test was performed to determine if there are significant differences among treatments. For the sensory evaluation results, Duncan's New Multiple Range Test (DNMRT) was used to locate which treatments are significantly different from each other. Difference was defined at p<0.05 level of significance.

RESULTS AND DISCUSSION

Antimycotic activity of lemon grass essential oil

The antifungal activity of lemon grass essential oil (LGEO) was assessed by the presence or absence of inhibition zones against the selected yeasts and molds. Three different amounts of essential oil were used (20, 40 and 60 uL) with 0.5% v/v Tween 80 as control. Inhibition at 60 uL for *Torulopsis candida* was the highest (3.67±0.58 mm). For *Debaryomyces hansenii*, all treatments showed significant differences with each other. The highest inhibition was observed at 60 uL (10.67±1.15 mm) followed by 40 uL (7.33±0.58mm) and 20 uL (4.67±0.58 mm). There was no significant difference in the zones of inhibition exhibited by 60 uL and 40 uL of LGEO against *Hansenula anomala* which measured 5.67±0.58 mm and 5.00±0.00 mm, respectively. For molds, no significant differences in the zones of inhibition were observed in all amounts of LGEO against *Mucor rouxii*. Small clearings of inhibition were measured, 2.00±1.00 mm at 60 uL, 1.67±0.58 mm at 40 uL and 1.33±0.58 mm at 20 uL. Similar result was observed in *Penicillium roqueforti* which registered no significant differences among amounts of LGEO. Measure of the zones of inhibitions were 2.33±0.58 mm at 60 uL, 2.00±1.00 mm at 40 uL and 1.67±0.58 mm at 20 uL. In general, the zone of inhibition increased in a dose-dependent manner. Results also showed that yeasts exhibited more susceptibility than molds as presented on table 1.

Table 1 Zone of inhibition by different concentrations of lemon grass essential oil against selected microorganism

Microorganism	Zone of inhibition, mm ¹			
	Control (0.5% v/v Tween 80)	20 uL	40 uL	60 uL
<i>Torulopsis candida</i>	0.00 ± 0.00 ^c	0.67 ± 0.58 ^{bc}	1.00 ± 0.00 ^b	3.67 ± 0.58 ^a
<i>Debaryomyces hansenii</i>	0.00 ± 0.00 ^d	4.67 ± 0.58 ^c	7.33 ± 0.58 ^b	10.67 ± 1.15 ^a
<i>Hansenula anomala</i>	0.00 ± 0.00 ^c	2.67 ± 0.58 ^b	5.00 ± 0.58 ^a	5.67 ± 0.58 ^a
<i>Mucor rouxii</i>	0.00 ± 0.00 ^b	1.33 ± 0.58 ^{ab}	1.67 ± 0.58 ^a	2.00 ± 1.00 ^a
<i>Penicillium roqueforti</i>	0.00 ± 0.00 ^b	1.67 ± 0.58 ^a	2.00 ± 1.00 ^a	2.33 ± 0.58 ^a

¹(N=3) Means followed by the same letter within each row are not significantly different from each other at p<0.05 using LSD.

Monoterpenoids and oxygenated terpenes were the most dominant components of LGEO (Bassole et al., 2011). Among these compounds, citral, a major constituent in essential oil of *Cymbopogon* species, was reported to have a strong antimicrobial activity against plant and human pathogens Redov et al. (1995). Other identified major compounds in lemongrass essential oil which could have contributed to its potential antimicrobial activity are limonene, geranyl acetate and β-caryophyllen. The presence of these substances effectively inhibited certain foodborne pathogens such as *Escherichia coli*, *Salmonella typhimurium albicans*, *Bacillus cereus* and *Staphylococcus aureus* at a relatively low MIC of 0.5 µL/mL (Vaziriana et al., 2012). Most studies have described the greater susceptibility of bacteria to LGEO and its components in comparison to molds and yeasts (Helal, 2006). On the contrary, the findings of Singh et al., (2011) have shown the relative sensitivity of certain yeasts, particularly *Candida albicans*, to LGEO at a MIC of 1.0 µL/mL. Furthermore, geraniol, a lemon grass essential oil constituent, attacks the yeast cells causing an excessive K⁺ ion leakage. This induces alterations in cell membrane composition by increasing the proportion of saturated fatty acid and decreasing the unsaturated one as exemplified by the same study on *Saccharomyces cerevisiae* (Ganjewala & Gupta, 2013).

The effect of LGEO on molds is primarily based on its ability to alter its morphological characteristics. In a study by Yousef (2013), *Aspergillus niger*, as a representative, had shown some morphological changes after the treatment of LGEO. These changes included decrease in sporulation, less pigmentation and reduction and distortion of conidiophores. It should also be noted that the effects of LGEO on fungal morphology causing its inhibition varies from species to species, and specific study is needed to determine the effect on particular test organisms.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of LGEO against the different test organisms with initial count of 10⁶ cfu/mL was determined using the agar dilution method. In this case, the lowest concentration of essential oil, in which no colony formation was observed on the agar media, was considered the MIC. *M. rouxii* and *P. roqueforti* had the least susceptibility to LGEO, with MIC of >10uL/mL, among test organisms. This observation was parallel to its zone of inhibition with a low value implying that a higher amount of EO is needed to inhibit the microorganism. On the other hand, yeasts had a lower MIC compared to molds. Among the yeasts used in this experiment, *T. candida* was the least susceptible with MIC of 5uL/mL, followed by *H. anomala* with MIC of 3uL/mL; while *D. hansenii* was inhibited by LGEO at MIC of 1.5uL/mL. It was noted that *D. hansenii* was the most susceptible to LGEO as identified through its zone of inhibition, implying that low concentration of LGEO is needed to inhibit the microorganism. Results are shown on table 2.

Table 2 Minimum inhibitory concentration of lemon grass essential oil against the test organisms.

Test organism	MIC, uL/mL
<i>T. candida</i>	5 ± 0.08
<i>D. hansenii</i>	1.5 ± 0.07
<i>H. anomala</i>	3 ± 0.12
<i>P. roquefortii</i>	>10
<i>M. rouxii</i>	>10

These results are in accordance to the studies on the minimum inhibitory concentration of several essential oils against specific test organisms as reported by Krisch et al. (2011). Specific yeasts are inhibited at lower concentration of essential oil as compared with molds. For instance, MIC values of cumin, cassia, allspice and thyme against the tested yeasts *Candida* species and *S. cerevisiae* were in the range of 0.04 µl/ml and 1.25 µl/ml (Kamble and Patil, 2008). Meanwhile, molds such as *Aspergillus flavus* and *A. niger* were inhibited at MIC of ≥20,000 µl/ml (Carmo et al., 2008).

Food application

Microbial stability of moringa juice with lemon grass essential oil

In order to evaluate the effect of addition of LGEO on the growth of inherent microorganisms in moringa juice, unpasteurized juice with 5 and 10 uL/mL LGEO was prepared. As shown in figure 1A, yeast and mold count of moringa juice without LGEO increased from 1.8 log cfu/mL to 23.2 log cfu/mL after the completion of the sampling period. Moringa beverage containing 5 uL/mL LGEO exhibited only 1.0 log cfu/mL increase in the yeast count at the end of the sampling period. Meanwhile, the moringa beverage with 10 uL/mL of EO exhibited a decrease in the number of yeast cells from 1.6 log cfu/mL to 1.1 log cfu/mL.

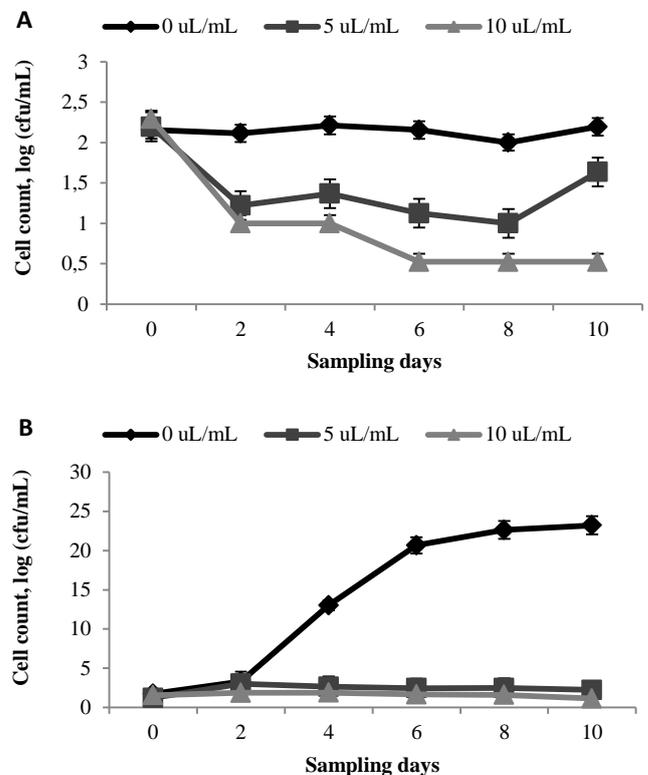


Figure 1 Yeast and mold count of (A) unpasteurized and (B) pasteurized moringa juice during the sampling period.

The effect of LGEO as a natural antimycotic agent in pasteurized moringa juice was also determined. There was no significant change observed in the yeast and mold count of the control (juice without LGEO) throughout the sampling period (Figure 2B). A reduction from 2.2 log cfu/mL to 1.6 log cfu/mL was observed in the juice with 5 uL/mL of LGEO. The microbial count of the juice with 10 uL/mL of LGEO was reduced from 2.3 log cfu/mL to 0.5 log cfu/mL.

The result of this experiment showed that lemon grass essential oil can prevent the proliferation of yeast and molds that may cause spoilage of the moringa juice and could help prolong its shelf stability even without heat treatment. The findings also signified that addition of LGEO caused a significant reduction on the number of yeast and mold count in the pasteurized RTD moringa juice.

According to Krisch et al. (2011), the antifungal activity of essential oil is often influenced by external factors specifically water activity and temperature. Higher water activity results in greater inhibition of yeast and mold. Also, the effect of temperature on the target microorganism is more pronounced at higher water activity. In the study conducted by Beletti et al. (2007), the combination of linalool and β-pinene, together with a mild (55 °C) heat treatment resulted in the

lower spoilage probability in citrus based non-carbonated beverages. In this experiment, it was also demonstrated that lemon grass essential oil can act synergistically with heat treatment in prolonging the microbial stability of the RTD moringa beverage.

Sensory evaluation of moringa beverage with lemon grass essential oil as natural preservative

Sensory attributes namely color, aroma, sweetness, sourness, and flavor, as well as the general acceptability of moringa juice with 5 and 10 uL/mL LGEO were assessed and results are presented on table 3.

Table 3 Sensory characteristics of moringa beverage with lemon grass essential oil

Attribute	Mean Sensory Scores ¹		
	Control	5 uL/mL	10 uL/mL
Color ^{ns}	5.00	5.01	4.93
Aroma	1.00 ^b	2.01 ^a	2.33 ^a
Sourness ^{ns}	2.33	2.53	2.60
Sweetness ^{ns}	3.33	3.47	3.27
Flavor	1.40 ^c	2.67 ^b	3.33 ^a
General Acceptability ^{ns}	3.13	3.07	3.27

¹(N=20) Means followed by the same letter within each row are not significantly different from each other at p<0.05 using DNMRT.

Range of scores: Color: 0=light, 6=dark; Aroma: 0=weak, 6=strong; Sourness: 0=weakly perceptible, 6=strongly perceptible; Sweetness: 0=not sweet, 6=very sweet; Flavor: 0=weak, 6=strong; General acceptability: 0=not acceptable, 6=very acceptable

The evaluated sensory attributes were the common characteristics perceived by most consumers on products like juices and alteration in such attributes may affect the acceptability of the product. Specifically, the flavor, taste and aroma are usually affected by the addition of ingredients such as essential oil due to characteristic odor that this component may impart to the food product. In the conducted sensory evaluation, the laboratory panelists did not perceive significant differences (p<0.05) in color, sweetness and sourness of the ready-to-drink moringa beverage. On the other hand, in terms of flavor, samples with LGEO significantly differed from each other as well as from the control. The lemon grass aroma was also detected in the samples. The juice sample with 5 uL/mL of EO did not significantly differ from the sample with 10 uL/mL in terms of lemon grass aroma. Consequently, the addition of LGEO did not significantly affect the general acceptability of the RTD moringa beverage. Hence, addition of a potent concentration of LGEO in the RTD moringa juice as natural antimicrobial did not negatively affect its sensory characteristics as perceived by the panelists.

In the research conducted by Tserennadmid *et al.* (2011), lemon oil was used as an antimicrobial in apple juice to extend its 'open' shelf life. It was found that the growth of the fermentative yeasts *Saccharomyces cerevisiae* and *S. pombe* was significantly halted, particularly in clear apple juice, while the lemon oil harmoniously blended with the flavor of the apple juice and added to its refreshing taste as perceived by the sensory panelists. Based on the study of Beletti *et al.* (2011) as cited by Krisch *et al.* (2011), the addition of essential oil components at concentrations 40 and 60 µL had no negative effect on the flavor of the beverages and the mild thermal treatment employed which was below the usual temperature range (65-75 °C) reduced the energy costs in the production of the beverages. Meanwhile, addition of 500 ppm thyme essential oil in tomato paste reduced the growth of *A. flavus* by 87% while the taste was still acceptable to the consumer panel (Omidbeygi *et al.*, 2007).

Assessment of the effect of the incorporation of essential oils, to serve as preservative, on the organoleptic properties of actual food products is essential as these components may have significant impact on the sensory attributes as well as the overall acceptability. It is an important consideration to maintain the overall quality of the food product in terms of sensory properties while keeping it safe for consumption.

CONCLUSION

The results of this study highlighted the effectiveness of lemon grass essential oil as an antimycotic agent against target microorganisms that may commonly survive in a food system specifically juice and beverages. The lemon grass essential oil was found to be specifically effective against the yeasts which were tested in the experiments. Also, minimum concentrations of 1.5-3.0 uL/ml were potent enough to inhibit the growth of the targeted yeasts. Upon application of the lemon grass essential oil in the formulated ready-to-drink moringa beverage, the findings showed its efficacy as a natural preservative as it repressed the proliferation of the yeasts and molds present in the juice without causing a negative impact on the organoleptic properties as well as the general acceptability of the beverage. This study also revealed the potential application of lemon grass essential oil in combination with other preservation technologies such as thermal

treatment or as a natural preservative in cold-pressed or unpasteurized juices to prolong their shelf-life.

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