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ANTIMICROBIAL ACTIVITY OF *THYMUS VULGARIS* EXTRACT, *SYZYGIUM AROMATICUM* EXTRACT, AND *ZINGIBER OFFICINALE* EXTRACT ON *CRONOBACTER* SPP. AS COMPARED WITH COMMON PRESERVATIVES

Abu-Ghazaleh, Bayan M.

Department of Biology and Biotechnology [Hashemite University] Zarqa, Jordan

bay.ghaz@yahoo.com

Abstract

Cronobacter spp. has been implicated in spoilage of various types of food, and in infection of infants and adults, especially immunocompromised persons. Therefore, application of compounds to eliminate these organisms in food may extend the shelf life of food and improve their quality. This study investigated the antimicrobial effect of *Thymus vulgaris* extract, *Syzygium aromaticum* extract, and *Zingiber officinale* extract on strains of *Cronobacter* spp.; and compared it with the antimicrobial effects of some common preservatives (e g potassium sorbate, ascorbic acid, citric acid, and sodium chloride). *Thymus vulgaris* extract (0.3%) alone reduced the growth rate ($p < 0.05$) of the tested strains, the percentage of inhibition after 24h incubation was about 48%. *Syzygium aromaticum* extract (1%) alone or *Zingiber officinale* extract (1%) alone greatly reduced growth of the tested strains, the percentages of inhibition after 24h incubation in nutrient broth containing these extracts were about 71% and 52%, respectively. Ascorbic acid (0.2%) alone greatly reduced the growth (the percentage of inhibition of growth was about 80%). Potassium sorbate (0.05%) slightly inhibited growth ($p < 0.05$). Ascorbic acid (0.1%) or citric acid (0.03%) alone did not affect growth of the strains, but a lag occurred before increase in number could be observed. Combination of potassium sorbate (0.05%) and citric acid (0.03%) together or potassium sorbate (0.05%) and ascorbic acid (0.1%) together completely inhibited growth of *Cronobacter* spp. tested. *Thymus vulgaris* extract or ascorbic acid had the lowest minimum inhibitory concentration (MIC: 1%) followed by *Syzygium aromaticum* extract and *Zingiber officinale* extract (MIC: 2% and 3%, respectively). Sodium chloride had the highest minimum inhibitory concentration (MIC: 20%).

Keywords: *Cronobacter spp., inhibition, chemicals, plant extracts*

Introduction

Cronobacter spp. (formerly *Enterobacter sakazaki*) are Gram-negative rods that belong to the family Enterobacteriaceae. They are isolated at high frequency from foods of plant origin such as teas, chocolate, and vegetables [1]. Also, they may be present in instant powdered food products (e g instant soups, instant tea) [2]. *Cronobacter* spp. can also be found on meat, poultry and fish [3]; and in milk, dairy products and reconstituted powdered infant formula [4, 5]. The presence of these microorganisms in food is reflective of the original environment (such as water and soil) and/or the processing environment. Treated wastewater, for example, is reused for irrigation and other purposes in many countries [6]. *Cronobacter* spp. are one of the prevalent species in the influent and effluent of wastewater treatment plants; therefore, vegetables, fishes and other foods in contact with this water may be contaminated. Also, *Cronobacter* spp. can be found in dust in factories, restaurants, and houses [2] and this may introduce the microorganisms to foods during processing.

Cronobacter spp. can cause disease in adults, especially elderly and immunocompromised persons; and can infect infants causing life-threatening disease [7]. Also, *Cronobacter* spp. have been implicated in spoilage of many types of food. For example, milk can be spoiled by *Cronobacter* spp., which can multiply even at low temperatures [8]. Cook [9] reported that fecal coliforms, such as *Enterobacter*, are found in oysters and are capable of multiplying in them, and eventually causing spoilage when stored above 10°C. Also, Kim and Beuchat [10] reported that *Enterobacter sakazakii* can grow at 12°C on fresh cut fruits and vegetables and in juices. Similarly, Narasihma and Sreenivasamurthy [11] studied the shelf life and microbial spoilage of sheep meat carcass under commercial conditions, and found that *Enterobacter* may form a major part of the final flora of spoiled sheep carcass meat at ambient temperature. Also, Soriano et al. [12] reported that *Enterobacter cloacae* was isolated from Spanish potato omelette samples and from cooked meat samples.

Nowadays there is increased use of plant extracts to treat diseases and/ or preserve and extend the shelf life of food and pharmaceutical products. There is little information on the effect of *Thymus vulgaris* extract, *Syzygium aromaticum* extract, *Zingiber officinale* extract on *Cronobacter* spp.. Therefore, this study was conducted to investigate the activity of these extracts on *Cronobacter* spp. and compare it with common antimicrobial chemicals (e g potassium sorbate, ascorbic acid, citric acid and sodium chloride).

Methods

Bacterial strains

Strains of *Cronobacter* spp. used in this study were previously isolated by the author. The strains were isolated from the effleunts of large rural and large urban wastewater treatment plants. The organisms were maintained on nutrient agar slants at 4°C. To prepare the inoculum, nutrient broth (20 ml) was inoculated with appropriate cultures and incubated at 31°C for 20h.

Media and chemicals

Crude extracts of *Thymus vulgaris* (thyme) , *Syzygium aromaticum* (clove), *Zingiber officinale* (ginger) (extracted by hydrodistillation) were obtained from Systema Co. Ltd. (Amman, Jordan). Stock solutions of potassium sorbate, citric acid and ascorbic acid were freshly prepared before each use and sterilized by filtration through membrane filters (0.45 um, Micron Separation Inc., Philadelphia, Pa., USA).

Growth conditions

Flasks containing 30 ml of nutrient broth (pH7.4), nutrient broth plus crude extract of *Thymus vulgaris* (0.3% v/v), nutrient broth plus crude extract of *Syzygium aromaticum* (0.3% or 1% v/v), nutrient broth plus crude extract of *Zingiber officinale* (0.3% or 1% v/v), nutrient broth plus citric acid (0.03% w/v), nutrient broth plus ascorbic acid (0.1% or 0.2% w/v), nutrient broth plus NaCl (3% or 4% w/v), or nutrient broth plus various combinations of these compounds were inoculated with 0.5 ml of overnight grown bacterial culture. For experiments containing potassium sorbate (0.05%), the pH of nutrient broth was adjusted to 5.77 before addition of the tested compounds. Then, the tested compounds were added to flasks containing 30 ml of nutrient broth (pH 5.77) and treated as previously described. After inoculation, the flasks were incubated static at 31°C for 24h. Samples were withdrawn from each flask at suitable intervals and growth was monitored by measuring optical density at 560 nm spectrophotometrically (Spectronic,

Cheshire, UK). During incubation, at least five readings were obtained from each flask.

All experiments in this study were performed five times, and the optical density readings presented are the mean values. Student's *t*-test was used to determine the significant differences ($p < 0.05$) among the different compounds tested.

Minimum inhibitory concentration test

The test was done according to the method of Finegold and Martin [13].

Results

Isolated strains were examined; the results shown are of representative strain from effleunt of large rural treatment plant (*Cronobacter* spp. 33) and of representative strain from large urban wastewater treatment plant (*Cronobacter* spp. 13).

Thyme extract effect

The presence of thyme extract (0.3%) alone in the growth medium reduced growth ($p < 0.05$) of the tested strains. The percentage of inhibition after 24h incubation was ~ 48% for both strains (Table 1). Also, there was a long lag (more than 4 h) before increase in number could be detected. Addition of thyme extract (0.3%) and potassium sorbate (0.05%) together, or thyme extract (0.3%) and ascorbic acid (0.1%) together to the growth medium caused enhanced inhibition of growth of both strains. The percentages of inhibition after 24h incubation were 50%, and 52%, respectively for *Cronobacter* spp. 33 (Table 1A). For *Cronobacter* spp. 13, the percentages of inhibition were 52%, and 53%, respectively (Table 1B).

Clove and ginger extracts effect

The presence of clove extract (0.3%) or ginger extract (0.3%) alone in the growth medium did not significantly reduce growth of *Cronobacter* spp. 33 ($p > 0.05$) (Table 2). However, presence of clove extract (1%) or ginger extract (1%) alone in the growth medium greatly reduced the growth. The percentages of inhibition after 24h incubation were 71%, and 52% for clove or ginger extracts, respectively (Table 2). Similar results were obtained for *Cronobacter* spp. 13 (data not shown).

Sodium chloride, ascorbic acid, and citric acid effect

The OD_{560} readings of *Cronobacter* spp. 33 and *Cronobacter* spp. 13 subjected to sodium chloride alone are presented in Tables 3 and 4. Addition of NaCl (3%) to the growth medium slightly inhibited growth of the tested strains ($p < 0.05$). The percentage of inhibition after 24h incubation was about 26% for both strains. Exposure of the strains to 4% NaCl caused enhanced inhibition of growth, where the percentages of inhibition of *Cronobacter* spp. 33 and *Cronobacter* spp. 13 were 34% and 39%, respectively. Also, there was a lag before increase in number could be detected, and the lag was longer for strains grown in broth containing 4% NaCl (Tables 3& 4).

The presence of ascorbic acid (0.1%) or citric acid (0.03%) alone in the growth medium did not inhibit growth of the tested strains (Tables 3 & 4). The presence of ascorbic acid (0.2%) in the growth medium greatly reduced growth of the strains ($p < 0.05$), where the percentages of inhibition of *Cronobacter* spp. 33 and *Cronobacter* spp. 13 were 80% and 83%, respectively (Tables 3& 4) .

Addition of citric acid (0.03%) and NaCl (3%) together to the growth medium did not significantly reduce growth ($p > 0.05$) of *Cronobacter* spp. tested (Tables 3 & 4). Addition of ascorbic acid (0.1%) and NaCl (3%) together to the growth medium only slightly inhibited growth of both strains ($p > 0.05$). However, very long lag was observed (more than 4h) before growth could be detected in broth containing these compounds (Tables 3& 4).

Potassium sorbate effect

The results of exposure of *Cronobacter* spp.33 to potassium sorbate are presented in Table 5. Addition of 0.05% potassium sorbate alone to the growth medium slightly reduced growth rate ($p < 0.05$), but the strain grew after a lag of ~ 4h. The presence of potassium sorbate (0.05%) and NaCl (3%) together in the growth medium caused enhanced inhibition of the tested strain, where the percentage of inhibition after 24h incubation was 47%. Exposure of *Cronobacter* spp. 33 to potassium sorbate (0.05%) and citric acid (0.03%) together or to potassium sorbate (0.05%) and ascorbic acid (0.1%) together completely inhibited growth. For *Cronobacter* spp. 13, similar results were obtained for growth in broth containing these compounds (data not shown).

Minimum inhibitory concentrations

Minimum inhibitory concentrations of the tested extracts and chemicals against *Cronobacter* spp. 33 are presented in table 6. Thyme extract or ascorbic acid had the lowest minimum inhibitory

concentration (1%), followed by clove extract and ginger extract (2% and 3%, respectively). Combination of clove extract and ascorbic acid (0.1%), or ginger extract and ascorbic acid (0.1%) greatly reduced the minimum inhibitory concentration of clove extract or ginger extract against *Cronobacter* spp. 33 (Table 6). Similar results were obtained for *Cronobacter* spp. 13.

Discussion

Cronobacter spp. are widely distributed in the environment such as soil and vegetation. They can cause diseases in humans and can spoil many types of food including cheese, meat, fish and vegetables [8]. Therefore, addition of chemicals before storage to control growth of these microorganisms is intended to extend the shelf life of food. In this study, the antimicrobial activity of *T. vulgaris* extract, *S. aromaticum* extract, and *Z. officinale* extract on *Cronobacter* spp. was investigated; and compared with the effects of common preservatives (i e citric acid, ascorbic acid, sodium chloride and potassium sorbate). Thyme extract and clove extract effectively reduced the growth of *Cronobacter* spp. tested. Other studies reported that ethanolic and methanolic extracts of clove had good inhibitory activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *E. coli* [14]. Saeed and Tariq [15] reported that clove oil was effective against *Salmonella typhi* and *Shigella dysentriae.*

Ginger extract (1%) reduced the growth of *Cronobacter* spp. tested, but its effect was less than that of thyme extract or clove extract. Other studies reported that oils of thyme or clove have bacteriostatic concentrations less than oil of ginger against *E. coli* and *Salmonella enteritidis* [16]. Akintobi et al [17] reported that ethanolic extract of ginger was ineffective against *E. coli* and *Bacillus subtilis*, and slightly inhibited *S. aureus* and *Salmonella typhi.*

In this study, ascorbic acid (0.1%) did not show an inhibitory effect on the tested strains, but ascorbic acid (0.2%) greatly reduced the growth. Other studies have reported that ascorbic acid at low concentrations is not as effective as other preservatives. Fletcher et al. [18] observed that ascorbic acid had some inhibitory properties toward *Campylobacter jejuni.* Also, it has been reported that ascorbic acid (0.1%) showed only a slight inhibitory effect on *Aeromonas caviae* and *Aeromonas sobria* [19]. Citric acid (0.03%) did not demonstrate antimicrobial activity against *Cronobacter* spp. tested. It is possible that the strains had utilized this compound as source of carbon.

In this study, the antimicrobial effect of potassium sorbate (0.05%) on *Cronobacter* spp. was slight. Other studies reported similar results. Potassium sorbate (0.1%) had a minimal effect on enterohemorrhagic *E. coli* O157 :H7 [20]. On the other hand, application of higher concentrations of potassium sorbate increased the shelf life of seafood and poultry [21, 22].

Interactions between the tested compounds were investigated in this study for possible synergism against *Cronobacter* spp. Minimum inhibitory concentration of thyme extract, clove extract and ginger extract decreased significantly in presence of 0.1% ascorbic acid in the growth medium. Also, potassium sorbate (0.05%) activity against *Cronobacter* spp. greatly increased in presence of citric acid (0.03%) or ascorbic acid (0.1%) in the growth medium. Presence of citric acid (0.03%) or ascorbic acid (0.1%) in the growth medium lowers the pH of the medium, and *Cronobacter* spp. may not tolerate this pH. Smittle and Flowers [23] reported that citric and lactic acids potentiate the antimicrobial action of sorbate. Also, Efiuvwevwere and Ajiboye [24] reported that a combination of 0.4% potassium sorbate and smoking caused significant reduction in microbial population of catfish.

In conclusion, this study showed that thyme extract, clove extract, or ascorbic acid alone greatly reduced the growth rate of *Cronobacter* spp. tested. Also, various combinations of the tested compounds prevented growth of the strains tested.

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Conflict of interest: no conflict of interest is present

Table 1: Antimicrobial effect of thyme extract on *Cronobacter* spp. 33 (A) and *Cronobacter* spp.13 (B).

(A)

Thyme extract (%)	Potassium sorbate (%)	Ascorbic acid (%)	Growth after 2h	4h	24h	Reduction in growth (%)[a]
0	0	0	0.05	0.15	0.58	-
0.3	0	0	0	0	0.3*	48
0.3	0.05	0	0	0	0.29*	50
0.3	0	0.1	0	0	0.28*	52

(B)

Thyme extract (%)	Potassium sorbate (%)	Ascorbic acid (%)	Growth after 2h	4h	24h	Reduction in growth (%)[a]
0	0	0	0.048	0.16	0.61	-
0.3	0	0	0	0	0.31*	49
0.3	0.05	0	0	0	0.29*	52
0.3	0	0.1	0	0	0.284*	53

a: Reduction in growth (%) =
[Growth in broth without chemicals – growth in broth with chemical (s) / Growth in broth without chemicals] X100

* Means are significantly different ($p < 0.05$) from control (compound(s) is not added to growth medium)

Table 2: Antimicrobial effect of clove extract and ginger extract on *Cronobacter* spp. 33

Clove extract (%)	Ginger extract (%)	Growth after 4h	Growth after 24h	Reduction in growth (%)[a]
0	0	0.16	0.62	-
0.3	0	0.06	0.58	6
1	0	0.03	0.18*	71
0	0.3	0.08	0.6	3
0	1	0.03	0.3*	52

a: Reduction in growth (%) =
[Growth in broth without chemicals – growth in broth with chemical (s) / Growth in broth without chemicals] X100

* Means are significantly different ($p < 0.05$) from control (compound is not added to growth medium)

Table 3: Inhibition of growth of *Cronobacter* spp. 33 in nutrient broth by combination of various compounds

NaCl (%)	Ascorbic acid (%)	Citric acid (%)	Growth after 2h	4h	24h	Reduction in growth (%)[a]
0	0	0	0.052	0.17	0.61	-
3	0	0	0.017	0.06	0.45*	26
4	0	0	0	0.01	0.4*	34
0	0.1	0	0	0.03	0.8	0
0	0.2	0	0	0	0.12*	80
3	0.1	0	0	0	0.58	4.9
0	0	0.03	0.035	0.09	0.67	0
3	0	0.03	0	0	0.59	3

a: Reduction in growth (%) =
[Growth in broth without chemicals – growth in broth with chemical (s) / Growth in broth without chemicals] X100

***** Means are significantly different ($p < 0.05$) from control (compound(s) is not added to growth medium)

Table 4: Inhibition of growth of *Cronobacter* spp. 13 in nutrient broth by combination of various compounds

NaCl (%)	Ascorbic acid (%)	Citric acid (%)	Growth after 2h	4h	24h	Reduction in growth (%)[a]
0	0	0	0.053	0.18	0.64	-
3	0	0	0.016	0.062	0.47*	27
4	0	0	0	0.01	0.39*	39
0	0.1	0	0	0.04	0.7	0
0	0.2	0	0	0	0.11*	83
3	0.1	0	0	0	0.6	6
0	0	0.03	0.04	0.1	0.68	0
3	0	0.03	0	0	0.6	6

a: Reduction in growth (%) =
[Growth in broth without chemicals – growth in broth with chemical (s) / Growth in broth without chemicals] X100

* Means are significantly different ($p < 0.05$) from control (compound(s) is not added to growth medium)

Table 5: Effect of potassium sorbate and combinations of potassium sorbate with various compounds on growth of *Cronobacter* spp. 33.

Potassium sorbate (%)	NaCl (%)	Citric acid (%)	Ascorbic acid (%)	Growth after 2h	4h	24h	Reduction in growth(%)[a]
0	0	0	0	0.05[b]	0.16[b]	0.7[b]	-
0.05	0	0	0	0.01	0.05	0.6*	14
0.05	3	0	0	0.01	0.03	0.37*	47
0.05	0	0.03	0	0	0	0*	100
0.05	0	0	0.1	0	0	0*	100

a: Reduction in growth (%) =
[Growth in broth without chemicals – growth in broth with chemical (s) / Growth in broth without chemicals] X100

b: The values represent growth in nutrient broth (pH 5.77)

***** Means are significantly different ($p < 0.05$) from control (compound(s) is not added to growth medium)

Table 6: Minimum inhibitory concentrations of the tested compounds on *Cronobacter* spp. 33

Clove extract	Ginger extract	Thyme extract	Ascorbic acid	Sodium chloride	Clove extract in NB plus 0.1% asc	Ginger extract in NB plus 0.1% asc	Thyme extract in NB plus 0.1% asc
2%	3%	1%	1%	20%	1%	1%	0.75%

NB: nutrient broth, asc: ascorbic acid