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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

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Research Article
Phytochemical Screening and *in vitro* Antimicrobial Effect of Orange (*Citrus sinensis*) Ethyl Acetate Extract Silage

Ucop Haroen, Agus Budiansyah and Nelwida

Department of Nutrition and Animal Feed Science Technology, Faculty of Animal Science, Jambi University, Ma. Bulian KM 15, Mendalo Darat, Jambi, Indonesia

Abstract

Background and Objective: Secondary metabolites are complex compounds. Many citrus fruits contain such compounds in the skin, seeds and pulp that act as phytochemicals with bacterial growth-inhibiting, anti-fungal and anti-cancer activities. This study was designed to identify phytochemical compounds in ethyl acetate extracts of orange and assess their antibacterial activities. **Methodology:** An ethyl acetate extract of orange silage (EAEOS) at 250, 500, 750 and 1000 ppm was fermented for 28 days. Treatments were replicated four times. The samples were placed in a jar serving as a silo under anaerobic conditions. At the end of fermentation, phytochemical screening was performed. Data were analysed using analysis of variance under a completely randomized design. **Results:** The EAEOS contains alkaloid, flavonoid, steroid, triterpenoid, phenolic, saponin and coumarin compounds. The antibacterial activity of EAEOS was assessed using disc and MIC (minimum inhibition concentration) methods with *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Salmonella typhi* (*S. typhi*) and *Bacillus subtilis* (*B. subtilis*). The extract inhibited the growth of all test organisms, with zones of inhibition ranging from 9.75±0.00 to 16.75±0.14 mm (*E. coli*), 8.00±0.23 to 12.50±0.24 mm (*S. aureus*), 8.50±0.24 to 11.75±0.00 mm (*S. typhi*) and 7.75±0.11 to 11.75±0.12 mm (*B. subtilis*). The MICs were 38.72±0.23 to 59.54±0.23% (*E. coli*), 15.08±0.54 to 23.25±0.59% (*S. aureus*), 10.46±0.12 to 19.65±0.02% (*S. typhi*) and 9.64±0.45 to 11.28±0.44% (*B. subtilis*). **Conclusion:** The tested EAEOS compounds exhibited inhibitory activities against both gram-positive (*S. aureus*, *B. subtilis*) and gram-negative (*E. coli* and *S. typhi*) bacteria.

Key words: Ethyl acetate, inhibitory bacteria, orange, phytochemicals, silage, waste extract

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Corresponding Author: Ucop Haroen, Departement of Nutrition and Animal Feed Science Technology, Faculty of Animal Science, Jambi University, Ma. Bulian KM 15, Mendalo Darat, Jambi, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Secondary metabolites are complex compounds found in plants, occurring in the bark, leaves, roots and seeds, that can act as active phytochemicals. Moreover, phytochemicals in citrus plants show bacterial growth-inhibiting activities[1]. These compounds can exert effects against various diseases through anti-malarial, anti-cancer, anti-virus and anti-inflammatory activities[2]. Ethyl acetate extracts obtained from the waste generated from orange (*Citrus sinensis)* juice production contain complex compounds such as limonoid, alkaloids, flavonoids, steroids, triterpenoids, phenolic, saponins and coumarin[3]. The major compounds in extracts of orange (*Citrus sinensis*) juice waste are limonoids, which are derived from limonene and cause the bitter taste of citrus fruits. These compounds can act as insecticides, antifeedants for insects and inhibitors of the growth of bacteria and fungi[2]. Some researchers evaluating the biological activity of *Citrus* phytochemical compounds have found that they can improve livestock health and serve as a natural feed additive that can lower blood cholesterol[4-6]. In addition, phenolic compounds exhibit considerable antimicrobial activity that can modulate the gut ecosystem and feed efficiency[7].

Orange waste has potential as an additive for poultry feed[8-10] and dairy cattle[11]. For example, orange waste can be used at up to 20% in broiler chicken rations and orange waste extract used in drinking water up to 1000 ppm can increase broiler chicken growth and feed efficiency[12,13].Additionally, the use of sweet orange peel flour as a feed additive at 1.5% in rations can improve the growth of broiler chickens[14].

This study was conducted to investigate the possibility to minimize waste in the fruit-processing industry. Waste minimization during the production process and the recovery of valuable by-products can substantially reduce the amount of waste. Here, it is evaluated that the antibacterial potential and phytochemical compounds of orange extract silage as a source of natural feed additives for livestock.

MATERIALS AND METHODS

Plant material: The orange juice waste was obtained from a juice merchant located in Jambi city. The citrus (*C. sinensis*) fruits used are local from Jambi. After collection, the orange waste was cleaned, dried in an oven at temperature of 55°C for 3-4 days until the water content reaches 10-15% and milled into flour using a hammer mill. The material was further reduced to a powder before mixing with molasses and rice bran, wrapped in a plastic bag and stored at room temperature for 28 days. The samples were placed in a jar, which acted as an anaerobic silo.

Preparation of extracts: The powdered orange silage (up to 1000 g) was macerated in ethyl acetate (3×5 L) solvent for 3 days at room temperature and filtered through a funnel to separate the extract from the dregs. The extracts were concentrated using a rotary evaporator at 40°C and then evaporated to dryness in a water bath. The ethyl acetate extract of orange silage (EAEOS) was added to petri dishes containing the following bacteria: *Escherichia coli, Staphylococcus aureus*, *Salmonella typhi* or *Bacillus subtilis*. Coleridin (antibiotic) was used as a control. The Petri dishes were covered with glass to prevent evaporation and incubated at 28°C[15]. A completely randomized design with 5 treatments was used and the EAEOS treatments were replicated four times. The treatment groups were as follows: P0 = containing no antibiotic extract (control), P1 = 0 ppm EAEOS, P2 = 250 ppm EAEOS, P3 = 500 ppm EAEOS, P4 = 750 ppm EAEOS and P5 = 1000 ppm EAEOS. The mean zone of inhibition was calculated.

Aqueous extraction: About 20 g of orange waste silage flour was added to a test tube and macerated with ethanol with heating (in a water bath) for 15 min. The extract was filtered through Whatman No. 1 filter paper under hot conditions. Each extract was transferred to a test tube and the ethanol was evaporated[17].

Preliminary phytochemical analysis (Qualitative test[8]): The powdered material and extract were subjected to preliminary phytochemical screening following previously described methodology[9-11].

Test for alkaloids: Filtrate (2 mL) was mixed with 1% HCl and approximately six drops of Mayor's reagents. A cream-coloured or pale yellow precipitate indicated the presence of alkaloids.

Test for steroids: Acetic anhydride (2 mL) was added to 0.5 g ethanolic extract of each sample plus 2 mL of H_2SO_4. Colour change from violet to blue or green indicated the presence of steroids.

Test for flavonoids: Filtrate (2 mL) was added to concentrated HCl and magnesium ribbon. A pink-tomato red colour indicated the presence of flavonoids.

Test for saponins: A 4th test was used to detect saponins. About 2 g of fresh sample was added to a test tube and macerated with ethanol with heating in a water bath for

15 min. The mixture was filtered under hot conditions into a test tube and the ethanol was evaporated. Chloroform and distilled water were added at a ratio of 1:1 for a total of 5 mL, chloroform and aqueous layers formed. The tube was shaken to generate a foam, retention of the foam after the addition of a few drops of concentrated HCl indicated the presence of saponins[18].

Test for terpenoids: The chloroform layer was dripped onto a plate consisting of three holes and left to dry. Concentrated H_2SO_4 was added to one hole, either one drop of acetic anhydride or one drop of concentrated H_2SO_4 was added to the other holes. The formation of green or blue-green colour indicated terpenoids[19].

Test for phenolic compounds: Most of the water-methanol phase was removed with a pipette and placed in a small test tube, to which $FeCl_3$ reagent was added. The formation of blue/purple colour indicated the presence of phenolic compounds[18].

In vitro testing of extracts for antibacterial activity

Antibacterial activity assay: The antibacterial activity of EAEOS was tested via determination of inhibition zones using the disc diffusion method. Two paper discs were dipped into medium containing each concentration of EAEOS, 250, 500, 750 or 1000 ppm or coleridin (control). The discs were placed on the surface of a petri dish containing the test bacteria and incubated for 24 h at 37°C, after which the inhibition zone was measured. If the zone of inhibition formed was larger than that of the control, the sample was considered to possess antibacterial activity[20].

Determination of the minimum inhibitory concentration (MIC): The MIC of EAEOS was determined by the serial dilution method. EAEOS was serially diluted to 10, 20, 30, 40 and 50%. Bacterial suspensions (10^6 colony forming units [CFU]) were added to the tubes, which were incubated at 37°C for 24 h. The MIC was taken as the lowest concentration of EAEOS that inhibited growth after a period of 24 h.

Statistical analysis: Data were statistically analysed using one-way analysis of variance (ANOVA). If necessary Duncan's multiple range test was applied to compare differences between means[16].

RESULTS AND DISCUSSION

Table 1 presents the phytochemicals detected in EAEOS. Tests for alkaloids, flavonoids, steroids, triterpenoids and phenolics were positive. These metabolites are known to have antibacterial activity against *E. coli*, *S. aureus*, *S. typhi* and *B. subtilis*. Table 2 and 3 compare the antibacterial activity of EAEOS and coleridin, as assayed by the disc diffusion method. EAEOS showed activity against *E. coli*, *S. aureus*, *S. typhi* and *B. Subtilis* and inhibited bacterial growth at all tested concentrations. However, the antibacterial activity of EAEOS was lower than that of coleridin.

The EAEOS showed significant antibacterial activity against all tested organisms, with better antibacterial activity at 1000 ppm than at 500 and 750 ppm, no antibacterial activity was detected at 250 ppm. The greatest zone of inhibition by EAEOS (1000 ppm) was against *E. coli* (16.75 mm), followed by *S. aureus* (12.50 mm), *S. typhi* (11.75 mm) and *B. subtilis* (11.75 mm). This antibacterial activity might be due to the broad spectrum of phytochemical

Table 1: Phytochemical compounds in ethyl acetate extract of orange silage

Secondary metabolites	Reagent	Observation	Result
Alkaloids	Meyer	White precipitate formed	(+)
Flavonoids	Sianidin test	Orange solution	(+)
Steroids	Liebermann-Burchard	Blue solution	(+)
Triterpenoids	Liebermann-Burchard	Red-brown solution	(+)
Phenolic	$FeCl_3$	Solution blue/purple	(+)

Table 2: Zone of inhibition (mm) of crude ethyl acetate extract of orange silage against test bacteria on Mueller-Hinton agar using the disc diffusion method

	Zone of inhibition (mm)				
	Crude ethyl acetate extract of orange silage waste (ppm)				
Bacteria	Control (coleridin)	250	500	750	1000
E. coli	24.50^d	9.75±0.00^a	10.50±0.00^a	12.50±0.27^b	16.75±0.14^c
S. aureus	23.65^d	8.00±0.230^a	10.75±0.12^b	11.75±0.18^b	12.50±0.24^b
S. typhi	23.65^d	8.50±0.24^a	10.75±0.54^b	11.00±0.00^b	11.75±0.00^b
B. subtlis	22.67^d	7.75±0.11^a	9.50±0.00^b	11.50±0.13^c	11.75±0.12^c

Different superscripts in the same row refer to significantly different data ($p<0.05$)

Table 3: Minimum inhibition concentration of crude ethyl acetate extract of orange silage for *E. coli*, *S. aureus*, *S. typhi* and *B. subtilis*

	Percentage of minimum inhibition concentration			
	Crude ethyl acetate extract of orange silage waste (ppm)			
Bacteria	250	500	750	1000
E. coli	38.72±0.23[a]	44.54±0.00[b]	57.15±0.98[c]	59.54±0.23[c]
S. aureus	15.08±0.54[a]	17.96±0.00[b]	18.15±0.14[b]	23.25±0.59[c]
S. typhi	10.46±0.12[a]	11.28±0.27[a]	18.15±0.54[b]	19.65±0.01[b]
B. subtlis	9.64±0.45[a]	10.05±0.76[a]	10.46±0.27[a]	11.28±0.44[a]

Different superscripts in the same row refer to significantly different data (p<0.05)

compounds present in EAEOS. The EAEOS at 250 and 500 ppm showed very similar antibacterial activities against all tested organisms.

Table 1 shows the presence of various constituents in orange juice waste. No effect of silage treatment on the contents of citrus waste phytochemical compounds was observed, likely because a maceration method was employed, i.e., maceration does not alter chemical natures or structures[21]. Indeed, maceration can lead to more extracted material and avoid chemical changes to certain compounds due to heating. Factors that affect the phytochemical content of a plant are the nature of the plant itself[17] and the soil profile, harvest time, extraction method, temperature and solvent properties[22].

The results of inhibitory zone measurements caused by EAEOS are shown in Table 2 and 3. Table 2 lists the EAEOS inhibitory zones for *E. coli*, *S.aureus*, *S.typhi* and *B. subtlis*, which ranged from 38.72±0.23 to 59.54±0.23% (*E. coli*), 15.08±0.54 to23.25±0.59% (*S. aureus*), 10.46±0.12 to 19.65±0.02% (*S. typhi*) and 9.64±0.45 to 11.28±0.44% (*B. subtilis*). The ANOVA results showed that treatment with EAEOS (p<0.05) had an effect on bacterial growth, with higher inhibition against *E. coli* and *Salmonella* bacteria (3 and 7 mm)[23]. For example, inhibition by etiacetic-extracted lemon peel and orange peel against *E. coli* was reportedly 14 and 13 mm, respectively[24,25]. Variability in inhibitory power may be due to differences in the phytochemical composition of each extract and the composition of phytochemicals in plant extracts is affected by the soil profile, harvest time, extraction method, concentration, time, temperature and solvent properties of the extract[26].

The MIC of EAEOS against *E. coli*, *S. aureus*, *S. typhi* and *B. subtlis* ranged from 38.72±0.23 to 59.54±0.23% (*E. coli*), 15.08±0.54 to 23.25±0.59 (*S. typhi*) and 9.64±0.45 to 11.28±0.44% (*B. subtilis*). Duncan's test results for the minimum EAEOS inhibitory level at 1000 ppm was significantly (p<0.05) higher than that at 750, 500 and 250 ppm. This is in line with the results of the EAEOS inhibitory zone test, where the 1000 ppm EAEOS inhibition zone was larger for all tested bacteria. This is because at 1000 ppm, EAEOS contains a high concentration of phytochemical compounds, with concomitant high antibacterial activity.

CONCLUSION

Fruit waste can be recycled in various innovative ways. Here, we report the presence of multiple antibacterial compounds in *Citrus sinensis* extract silage.

SIGNIFICANCE STATEMENT

This study reveals that *Citrus sinensis* extract silage contains phytochemical compounds that might be beneficial for the livestock industry as natural feed additives.

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