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Potential Synergistic Antimicrobial Activity of Extract from Peelings of Common Local Fruits: An In-Vitro Analysis

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ABSTRACT
The objective of this study is to investigate the potential synergistic antimicrobial activity of methanolic extract from peelings of Philippines' common local fruits, namely, orange, mango, and papaya. Peelings from these local fruits were properly washed, dried and powdered. Extracts from the peelings were obtained using methanol as the solvent, concentrated in a rotary evaporator set and were finally reconstituted in 20% dimethylsulphoxide (DMSO).The preliminary determination of antimicrobial effect of the individual fruit peelings was tested using plate count method. A two-fold dilution of the individual formulated extracts were done and combined, after which the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using *Staphylococcus aureus* and *Escherichia coli* isolates were determined. MIC results of the mango extract showed best antimicrobial effect, which worked at a minimum concentration of 6.25mg/ml on both isolates**.** This was confirmed by the MBC, which showed the absence of growth of viable microorganisms after 24-hour incubation on nutrient agar. Orange and papaya were found effective against *S. aureus* at a minimum concentration of 50mg/mL. However, these extracts did not demonstrate individual antibacterial activity against *E. coli*. The combination of mango and papaya was the most effective among the three sets of extracts used in the synergistic antibacterial activity. It was found to be effective against *E. coli* at a minimum concentration of 6.25mg/mL and against *S. aureus* at 25-100mg/mL. Other combination of extracts such as orange-mango and orange-papaya exhibited synergistic antibacterial activity at higher concentrations. Moreover, orange and mango combination had a better synergistic activity against *E. coli* than *S.aureus* while orange and papaya if combined has greater synergistic activity against *S. aureus* than *E. coli.* From this study, it can be concluded that the combination of the extracts of mango and papaya has a more potential synergistic antimicrobial activity against *S. aureus* and *E. coli.*

Keywords
Orange; mango; papaya; synergistic activity; antimicrobial.

1. INTRODUCTION

Infectious diseases caused by bacteria affect a lot of people worldwide and they are now one of the world's leading causes of death. The discovery of antibiotics was an essential part in combating bacterial infections that once ravaged humankind. Different antibiotics exercise their inhibitory activity on different pathogenic organisms. Thus, the development and spread of resistance to currently available antibiotics is a worldwide concern [1].

Recently, multiple drug resistance in human pathogenic microorganisms has necessitated a search for new antimicrobial compounds and for this reason, researchers are increasingly

turning their attention to herbal products, looking for new leads to develop better drugs against pathogenic microbial strains. The emergence of antibiotic resistance is further complicated by the fact that bacteria and their resistant genes are traveling faster and further. Mankind is facing not only epidemics but also pandemics of antibiotic resistance. Existing antibiotics are losing their effect at an alarming rate, but development of new antibiotics is declining [2]. There is a tremendous need for novel antimicrobial agents from different sources. Screening of plants with validated methods can lead to identify potentially useful molecules against infectious diseases.

The antimicrobial properties of different parts of plant like leaves, fruits, roots, seeds, stems, stalks and flowers have been extensively investigated worldwide. For instance, pasteurized milk supplemented with sappan wood extract had shown strong antibacterial activities against *Escherichia coli, Shigella flexneri, Salmonella thypimurium, Staphylococcus aureus,* and *Listeria monocytogenes* [3]. According to Sreevaniet al. [4], chloroform extracts from *C. argentea* stem and root showed to be more effective against the *S. aureus, E. coli, C. albicans* and *A. niger*. Leaf extracts of mango were found effective as bacteriostatic agents when tested against *Salmonella typhi, Escherichia coli, Staphylococcus aureus and Bacillus cereus*[5]. Besides, lavender essential oils also showed high activity against bacteria (*B. subtilis, S. aureus, E. coli, P. aeruginosa*), yeast and filamentous fungi (*Candida sp., A. niger, P. expansum*), inhibiting their growth at concentrations ranging from 0.4 to 4.5 µg/mL [6].Vu, Scarlett and Vuong [7] reported that phenolic within banana peels have been found to possess potent antioxidant and antimicrobial properties which are beneficial to health.An in-vitro study of the antimicrobial activity of pomegranate peelings also exhibited significant antibacterial activity [8].

Studies on the synergistic antimicrobial activities of natural plants and antibiotics emerged also in order to overcome multiple drug resistance that is widely developing due to indiscriminate use of antibiotics [9]. Combination of antibiotics and non-antibiotic drugs was proven to enhance antimicrobial efficacy [10]. The in-vitro antibacterial activities against *S. aureus* were confirmed and verified among clove, guava and lemongrass extracts with high synergism rate [11].

Besides its outer cover protecting the edible portion of fruits, the peelings indeed hold some of health benefiting constituents and antimicrobial properties. However, to date, very few studies have been conducted on the synergistic antimicrobial activity of combined fruit peelings [12]. Thus, fruit peelings of common local fruits in Philippines such as oranges, mangoes, and papayas were investigated for the presence of antimicrobial properties in this study.

Looking at the importance of herbal products and with an idea of antimicrobial nature of the common local fruits' peelings, the present study was designed in order to decipher potential synergistic antimicrobial properties of orange, mango and papayawith the following objectives: (1) to determine the presence of microbial contamination from the peelings of the selected fruits; (2) to perform two-fold serial dilution from the formulated methanolic extract; (3) to evaluate the antimicrobial activity of each of the diluted extract; and, (4) to determine the presence of synergistic antimicrobial activity from the different combinations of formulated extract.

The study hypothesized that the combination of these common local fruits would have synergistic antimicrobial effects. Once proven, this could be an alternative to commercially available and expensive drugs. The findings can be utilized to develop safer, cost-effective, and locally available antimicrobial agents.

2. MATERIAL and METHODS

2.1 Sample Collection of Peelings from Local Fruits

Common local fruits such as orange, mango, and papaya were purchased from vendors at Batangas City Public Market. They were submitted in the National Museum for authentication purposes.

2.2 Preparation of Fruit Peeling Crude Extracts

All fruit peelings were washed thoroughly with running water to remove dirt and other contaminating foreign particles [13]. They were cut into small pieces and air-dried for 7 days [5]. After a week, all samples were crushed and powdered finely using an electronic blender [14]. Ten grams of each sample were apportioned for pre-evaluation of their microbial density. This was to ensure that the fruit peelings were not heavily contaminated prior to the antimicrobial assay. Sixty grams of each measured powdered peelings were individually treated with 600 mL of 80% methanol to extract the active constituents deemed to have the antimicrobial activity. Solvent extraction was done for a week and filtered using Whatman filter paper. Filtrates were concentrated in rotary evaporator and reconstituted using 20% dimethylsulphoxide (DMSO) [5, 13, 15, 16].

2.3 Preliminary Determination of Microbial Contamination in Fruit Peelings

The ground dried forms of the fruit peelings (orange, mango, and papaya) were tested for microbial contamination on their microbial load through Plate Count Method. Five grams of the powdered dried peelings were transferred to a 45 mL peptone water diluent and serially diluted until 10^6 dilution. One milliliter of the 10^4, 10^5, and 10^6 dilution solution was pour-plated with Plate Count Agar on petri plates in duplicates. The duplicated plates were incubated at 37°C for 48 hours. The presence of colony growth on plates was checked after 24hours of incubation [17].

2.4 Preparation of Stock Crude Extract of Known Concentration

A stock crude extract of known concentration was prepared after weighing each fruit peelings' extracts and reconstituted using 20% dimethylsuphoxide(DMSO). Concentration (%w/v) was computed based on weight and the volume of the resulting solution. The formulated stock solution was further used in the two-fold dilution [18].

2.5 Two-Fold Dilution of Formulated Extract

Six test tubes were labeled as follows: concentrated or 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32. One mL of the formulated stock extract was placed on tube labeled concentrated and 0.5mL of distilled water to the next dilution tubes. A volume of 0.5mL extract in tube 1 was transferred to the next tube and was carefully mixed using Vortex. The 0.5mL of this mixture was transferred to the next higher dilution and repeated up to the last tube. The excess 0.5mL in the 1:32 tube was finally discarded. Each of the resulting solution was membrane filtered prior to microbiological assay. Each volume was proportionately increased in order to dispense as much as 0.5mL of the diluted extract to each combination in the test for synergism [14].

2.6 Synergy Set-Up Of Formulated Extract

Combination of each diluted extract followed the protocol as in Table 1. A volume of 0.5 ml of each original tube of diluted orange formulated extract was combined with 0.5 ml of each original diluted mango formulated extract. To the resulting mixture, 100 µL of the adjusted inoculum was added. A separate tube containing only the solvent (DMSO) and a tube containing nutrient broth were also inoculated with 100 µL of the adjusted inoculum. These served as controls. The tube containing the solvent was used to confirm if the DMSO has no activity against the bacteria. After preparation of all tubes containing extracts and inoculums, they were incubated at 37°C for 24 hours.

Another same combination of each diluted extract was done for mango and papaya as well as orange and papaya.

Table 1. Extracts formulation for synergistic activity [24]

Extract		Orange					
		A1	**A2**	**A3**	**A4**	**A5**	**A6**
Mango	**B1**	A1: B1	A2: B1	A3: B1	A4: B1	A5: B1	A6: B1
	B2	A1: B2	A2: B2	A3: B2	A4: B2	A5: B2	A6: B2
	B3	A1: B3	A2: B3	A3: B3	A4: B3	A5: B3	A6: B3
	B4	A1: B4	A2: B4	A3: B4	A4: B4	A5: B4	A6: B4
	B5	A1: B5	A2: B5	A3: B5	A4: B5	A5: B5	A6: B5
	B6	A1: B6	A2: B6	A3: B6	A4: B6	A5: B6	A6: B6

Note:
A1–A6 original dilution tubes of orange formulated extracts
B1–B6 original dilution tubes of mango formulated extracts

2.7 Test Organisms

Two bacterial strains were used in the study, namely Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 35218 strain which were obtained from the Bacteriology Section of the Batangas Medical Center. They were subcultured on nutrient agar and incubated aerobically at 37°C [19-20].

2.8 Antibacterial Activity

2.8.1 Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined using nutrient broth dilution method. ATCC strains of *Escherichia coli* and *Staphylococcus aureus* were used as test organisms. The turbidity of the inoculawas was adjusted first using 0.5 McFarland standards to achieve cell count of 10^6 CFU/Ml [8]. One hundred microliter (100uL) of this bacterial suspension was added per tube that was previously prepared in the serial dilution of the formulated extract. Tubes were then incubated at 37^oC for 24 hours. At the end of incubation period, MIC values were recorded as the lowest concentration of the substance that gave no visible turbidity. All antibacterial assays were run in duplicate [2, 12].

2.8.2 Determination of minimum bactericidal concentration (MBC)

The concentration of the test compound that completely killed the organism was taken as MBC. Samples were taken in dilution tubes, inoculated on freshly prepared nutrient agar plates and incubated at 37^oC for 24 hours. The lowest concentration that showed no growth on plates was recorded as MBC [2].

2.9 Statistical Analysis

The experimental results obtained were expressed as mean + standard error of mean (SEM). All measurements were carried out in duplicates. The percent yield of fruit peelings' extracts was determined by percentage (%).

3. RESULTS AND DISCUSSION

3.1 Plant Extract

Extraction conditions using 80% methanol for the orange, mango and papaya fruit peelings produced extracts with total weight of 6.3 grams, 8.7grams, and 6.9 grams, respectively. The highest percentage yield was obtained with mango extracts (14.5%) followed by papaya extracts (11.5%) and orange extracts (10.5%). The yields were lower than those produced by Chatterjee et al [20] in 2011, wherein they added chloroform to the methanol during the extraction process. All of the extracts were brown in color with a smooth and syrupy consistency. Orange had a distinct sweet fruity odor due to the presence of essential oils in citrus fruits [21].

3.2 Presence of Microbial Contamination

Table 2 summarized the result of preliminary determination of microbial contamination. The peeling extracts of orange showed minimal growth in 10^4 and 10^5 dilutions while mango had a minimal growth in 10^5 dilutions only. Therewas nogrowth observed in 10^6 dilutions of the orange, mango and papaya fruit peeling extracts. This showed that all of the fruit peeling extracts in 10^6 dilutions were devoid of microbial contamination as observed using plate count method.Hence, they were used in the next phase of the experiment.

Table 2. Summary Result of Preliminary Determination of Microbial Contamination

	Dilution		
Extract	**10^4**	**10^5**	**10^6**
Orange	+/-	+/-	0
Mango	+/-	0	0
Papaya	+/-	0	0

Note:0: no visible growth; +/- : with minimal growth;

3.3 Antimicrobial Activity of Extracts against Test Organisms

Table 3 presented the summary of antimicrobial test against *S. aureus* and *E. coli* for the formulated extracts and its dilution. Orange and papaya extracts when tested individually were able to inhibit the growth of *S. aureus* at 1:1, 1:2 and 1:4 dilutions whereas no inhibition was observed when these extracts were tested against *E. coli.*Orange and papaya were both effective against *S. aureus* up to 1:4 dilution as shown by the presence of growth in higher dilutions of extracts. However, they were not effective against *E. coli* as evidenced by the presence of growth in all dilutions of extracts. Mango extracts had inhibited the growth of microorganisms in six dilutions of extracts for both *S. aureus* and *E. coli.* This revealed that mango was considered most effective against *S. aureus* and *E. coli* since all of its dilutions gave no visible growth after incubation. Orange and papaya extracts were effective against *S. aureus* at higher concentrations only. Generally, all of the fruit peeling extracts had potential antibacterial properties against test organisms. Similar findings were demonstrated in various studies wherein leaf extracts and seed kernel of mango exhibited antimicrobial potential against *S. aureus* and *E. coli* [5, 13]. A study of Khan et al. [22] evaluated the antimicrobial properties of papaya and ripe peelings' extracts showed best antibacterial activity against *S. aureus* and *E. coli.* Furthermore, the results of this study in which orange extracts had a lesser extent of antimicrobial activity against *E. coli* had the same outcome as in a study conducted by Tahera et al. [23].

The inhibitory effects of fruit peelings' extracts against *S. aureus* rather than to *E. coli* could be due to the difference in the structure of the cell wall [19]. Unlike gram-positive bacteria, the lipopolysaccharides layer along with proteins and phospholipids were the major components in the outer surface of gram-negative bacteria. Therefore, the outer

lipopolysaccharide layer hindered access of most compounds to the peptidoglycan layer of the cell wall [5, 19].

Table 3. Summary of antimicrobial test against isolates for the formulated extracts and its dilution

Extract	Control		Dilution					
	Nutrient broth	DMSO	1:1	1:2	1:4	1:8	1:16	1:32
Orange	+	+	0	0	0	+	+	+
	+	+	+	+	+	+	+	+
Mango	+	+	0	0	0	0	0	0
	+	+	0	0	0	0	0	0
Papaya	+	+	0	0	0	+	+	+
	+	+	+	+	+	+	+	+

Note: 0: no visible growth; +: presence of visible growth

Against *S.aureus* Against *E. coli*

3.4 Synergistic Activity of Extracts against Test Organisms

Table 4tabulated the synergistic activity of formulated extracts against *S. aureus.* The combination of orange and mango showed absence of microbial growth in up to 1:4 dilutions of mango and up to 1:2 dilutions for orange. All theother dilutions demonstrated agrowth despite the presence of the combined extracts of orange and mango. Mango and papaya combined extracts showed absence of microbial growth in up to 1:8 dilutions of papaya and up to 1:4 dilutions of mango. Their combination is found to be effective against S. *aureus at* a concentration of 25-100mg/mL. All the other dilutions yielded a positive growth. On theother hand, the synergistic antibacterial activity of orange and papaya extracts was demonstrated in 1:2 dilutions of papaya and up to 1:8 dilutions for orange.Other combination of extracts such as orange and mango, orange and papaya exhibited synergistic antibacterial activity at higher concentrations. It showed that orange extracts when combined with mango and papaya fruit peelings' extracts, affect the antibacterial properties of such extracts against*S. aureus*. In a study conducted by Tahera et al. [23], orange peel extracts did not exhibit antimicrobial properties against *S. aureus* when methanol was used as an extracting agent.Only the ethanolic orange extracts showed inhibition of bacterial growth against *S. aureus.* This indicated that the type of extraction used in demonstrating the antibacterial properties of orange has a crucial role in its antibacterial effects.

Table 4. Synergistic activity of formulated extracts against *S. aureus*

Extract		Orange					
		A1	A2	A3	A4	A5	A6
Mango	B1	0	0	+	+	+	+
	B2	0	+	+	+	+	+
	B3	0	+	+	+	+	+
	B4	+	+	+	+	+	+
	B5	+	+	+	+	+	+
	B6	+	+	+	+	+	+
Extract		Mango					
		B1	B2	B3	B4	B5	B6
Papa	C1	0	0	0	+	+	+
	C2	0	0	0	+	+	+

	C3	0	0	0	+	+	+
	C4	0	+	+	+	+	+
	C5	+	+	+	+	+	+
	C6	+	+	+	+	+	+
Extract		**Orange**					
		A1	**A2**	**A3**	**A4**	**A5**	**A6**
Papaya	**C1**	0	0	0	0	+	+
	C2	0	0	0	+	+	+
	C3	+	+	+	+	+	+
	C4	+	+	+	+	+	+
	C5	+	+	+	+	+	+
	C6	+	+	+	+	+	+

Note : 0 : no visible growth; + : presence of visible growth

Table 5 presented the synergistic activity of formulated extracts against *E. coli*.The combination of orange and mango showed an absence of microbial growth in up to 1:32 dilutions of mango and up to 1:8 dilutions of orange. Mango and papaya combined extracts showed absence of microbial growth in up to 1:32 in both dilutions. All the other dilutions demonstrated a positive growth despite the presence of the combined extracts of orange and mango as well as mango and papaya. Moreover, the antibacterial activity of orange and papaya extracts was demonstrated in 1:2 dilutions of papaya and up to 1:8 dilutions of orange.

The results showed that mango and papaya combination inhibited*E. coli* even at the lowest concentration of 6.25mg/mL. Mango extracts also potentiated the antibacterial properties of orange as shown by the absence of growth in some dilutions when they were combined. Orange and papaya surprisingly suppressed bacterial growth in few dilutions when combined as compared to the resulting growth when tested individually. It was revealed that mango and papaya were the most effective among the three sets of extracts used in the synergistic antibacterial activity. Other combination of extracts such as orange and mango, orange and papaya also exhibited synergistic antibacterial activity but only at higher concentrations. The improvement in the antibacterial activity of orange and papaya was probably due to accumulation of inhibitory concentrations or due to the additional inhibitory effect given by the other extract [24]. The results agreed with other studies that potential synergistic antibacterial properties were present among fruit extracts [11, 19].

Table 5. Synergistic activity of formulated extracts against *E.coli*

Extract		**Orange**					
		A1	**A2**	**A3**	**A4**	**A5**	**A6**
Mango	**B1**	0	0	0	0	+	+
	B2	0	0	0	+	+	+
	B3	0	0	0	+	+	+
	B4	0	0	0	+	+	+
	B5	0	0	+	+	+	+
	B6	0	0	+	+	+	+
Extract		**Mango**					
		B1	**B2**	**B3**	**B4**	**B5**	**B6**
Papaya	**C1**	0	0	0	0	0	0
	C2	0	0	0	0	0	+
	C3	0	0	0	+	+	+

	C4	0	+	+	+	+	+
	C5	0	0	0	+	+	+
	C6	0	+	+	+	+	+
Extract		**Orange**					
		A1	**A2**	**A3**	**A4**	**A5**	**A6**
Papaya	**C1**	0	0	+	+	+	+
	C2	0	+	0	+	+	+
	C3	0	+	+	+	+	+
	C4	+	+	+	+	+	+
	C5	+	+	+	+	+	+
	C6	+	+	+	+	+	+

Note: 0: no visible growth; +: presence of visible growth

Table 6 summarized the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of crude extracts against both isolates; *S. aureus* and *E. coli.* Mango extracts exhibited the lowest MIC of 6.25mg/mL against *S. aureus* whereas both orange and papaya had a higher MIC of 50mg/mL. Same results were found in the MIC of mango extracts against *E. coli* at 6.25mg/mL without antibacterial activity at a maximum concentration in both orange and papaya. Mango extracts also exhibited the lowest MBC of 50mg/mL against *E. coli* whereas both orange and papaya resulted to MBC of 100mg/mL and 200mg/mL, respectively. Same results were found in the MBC of mango extracts against *E. coli* at 100mg/mL without antibacterial activity at a maximum concentration in orange and papaya.

The mango extracts had the highest antimicrobial activity against the test organisms as evidenced as low as 6.25mg/mL concentration that can inhibit and prevent the growth of organisms. From the various researches done on mango leaves, pulp, peel and seeds, polyphenolic compounds were present which were often associated with their antimicrobial and antioxidative activity [5]. Orange and papaya extracts did not exhibit antibacterial activity at a maximum concentration of 200 mg/mL.Higher dilutions of these extracts, however, hadshown antibacterial activities [22, 26].

Table 6. Summary of the MIC and MBC of Crude Extracts against test organisms

Extract	**Against *S. aureus***		**Against *E. coli***	
	MIC	**MBC**	**MIC**	**MBC**
Orange	50 mg/Ml	100 mg/mL	None	None
Mango	6.25 mg/mL	50 mg/mL	6.25 mg/mL	100 mg/mL
Papaya	50 mg/mL	200 mg/mL	None	None

Table 7 summarized the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of combined crude extracts against both isolates; *S. aureus* and *E. coli.* Orange and mango combined extracts had MIC/MBC of 100mg/mL:50mg/mL against *S. aureus* and MIC/MBC of 25mg/mL:6.25mg/mL against *E. coli.* The combination of mango and papaya had MIC/MBC of 25mg/ml:100mg/mL against *S. aureus* and 6.25mg/mL:6.25mg/mL against *E. coli.* Furthermore, orange and papaya extracts when combined, exhibited MIC/MBC of 50mg/mL:25mg/mL against *S. aureus* and 100mg/ml:50mg/mL against *E. coli.*

The combination of mango and papaya had the most antimicrobial activity against the isolates with a very low concentration against *E. coli* than *S aureus*. This study proved that the papaya fruit peelings had antibacterial properties but they can be more effective against

test organisms when combined with mango peelings. This type of synergistic activity had also been demonstrated in other studies of synergism among plant extracts and in combination with known antibiotics [11, 24, 27]. Orange and mango combination had a better synergistic activity against *E. coli* than *S. aureus.* It showed that orange fruit peelings affect the antibacterial properties of mango fruit peelings when they were combined. On the other hand, orange and papaya when combined had a better synergistic activity against *S. aureus* than *E. coli.* This further suggested that certain extracts can possibly potentiate the antimicrobial effects of some plant extracts [28].

Table 7. Summary of MIC and MBC of combined crude extracts against test organisms

Extract	Against *S. aureus*		Against *E. coli*	
	MIC	**MBC**	**MIC**	**MBC**
Orange & Mango	100 mg/mL 50 mg/mL	100 mg/mL 50 mg/mL	25 mg/mL 6.25mg/mL	25 mg/mL 6.25mg/mL
Mango & Papaya	25mg/mL 100 mg/mL	25mg/mL 100mg/mL	6.25mg/mL 6.25mg/mL	6.25mg/mL 6.25mg/mL
Orange & Papaya	50mg/mL 25 mg/mL	50mg/mL 25mg/mL	100 mg/mL 50 mg/mL	100 mg/mL 50 mg/mL

4. CONCLUSION

The combination of the extracts of mango and papaya had a more potential synergistic antimicrobial activity against *S. aureus* and *E. coli*. The combined fruit peeling extracts may have elucidated better antibacterial activity rather than using them alone. However, the exact mechanisms of antibacterial effects need to be further examined for health uses and in-vivo studies need to be carried out to evaluate its safety.

5. RECOMMENDATIONS

Based on the results of the study, the researchers recommended that the fruit peelings' extracts be combined with one or more antibiotics to further evaluate their synergistic effects. Other solvents like chloroform can also be tested and compared for the antimicrobial activity of extracts from peelings of these common local fruits. Finally, extensive research should be carried out on the phytochemicals of these fruits for the development of cost-effective drugs in the future.

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