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Phytochemical screening and Antimicrobial activity of fruit peels of Citrus reticulata, Citrus aurantifolia and fruit of Phyllanthus emblica

Vyshali V M, HOD & Lecturer, Department of Biotechnology, BMS College for Women, Bugle rock road, Basavangudi, Bangalore, Karnataka, India

-Neha Jadhav G, Assistant Professor, Department of Biotechnology, BMS College for Women, Bugle rock road, Basavangudi, Bangalore, Karnataka, India

-Vishnupriya P S, M.Sc Student, Bangalore University, Main Rd, Gnana Bharathi, Bengaluru, Karnataka, India

-Sowmya B S, M.Sc Student, MS Ramaiah College of arts science and commerce

Abstract:

The current study was conducted to determine phytochemical constituents and antimicrobial activity of peels of two Citrus fruits viz., C. reticulata and C. aurantifolia and fruit of Phyllanthus emblica. The peels were separated and dried at 40ºC and extracted using methanol, chloroform and dichloromethane. Phytochemical studies on three organic solvent extracts showed presence of alkaloids, glycosides, flavonoids, mucilage, saponin, steroids, terpenoids, protein, tannins and volatile oils. Methanolic extract was further considered for testing antimicrobial activity because of higher phytochemical compounds observed. Antibacterial and antifungal activity of peel extracts was determined by agar well diffusion assay against two gram positive (Bacillus cereus and Staphylococcus aureus), two gram negative (Pseudomonas aeruginosa and Salmonella typhi) and two fungal (Aspergillus niger and Candida albicans) strains. Phyllanthus emblica extract showed the most antibacterial activity of three samples. Candida albicans was inhibited by all three extracts to a higher extent.

Keywords: Antimicrobial activity, Citrus peels, Minimum Inhibitory Concentration (MIC), Phytochemical screening, *Phyllanthus emblica*

Introduction

Citrus, the genus Citrus L. of the family Rutaceae, is commercially important fruit crops around the globe. It is generally grown in the tropical and subtropical areas of the world, with yearly production of approximately 102 million tons (Zhuo Zo et al., 2016). Citrus contains active phytochemicals that can protect health. In addition to this, it provides an ample supply of vitamin C, folic acid , potassium and pectin (Shafiya Rafiq et al., 2018).

In India, the most commonly used Citrus fruit is *C. aurantifolia* (Lime), *C. reticulata* (Orange), *C. sinensis var. mosambi* (Mosumbi), *C. medica* (Lemon), *C. sinensis var. sinensis* (Malta), *C. limon*

(Galgal), *C. limenta* (Sweet Lime) and *C. paradisi* (Grapefruit) (Shah N. C, 2014). Citrus waste, such as peel flavedo, albedo and seeds are non-edible parts of the citrus fruits and contain chemical compounds like soluble sugar, starch, fiber including cellulose, hemicellulose, lignin and pectin, ash, fat and protein and many bioactive compounds. Polyphenols, carotenoids and essential oils are considered to be the most biologically active compounds (BAC) in the citrus by-products (Neelima Mahato et.al., 2018). Fruit and vegetable peels are considered agro waste and are thrown into the environment instead of being used as a source of antimicrobial agents (Muhammed Saleem et al., 2020).

Another non-citrus fruit popular in India is Phyllanthus emblica also known as amla, is widely distributed in most tropical and subtropical countries. P. emblica has a lot of therapeutic potential against deleterious diseases. The phytochemical analysis revealed it is also rich in biologically active compounds like vitamin C, polyphenols such as tannins, gallic acid, ellagic acid, flavonoids like quercetin and rutin (Mirunalini S et al., 2013). Of these tannins have been found to form irreversible complexes with proline-rich proteins resulting in the inhibition of the cell protein synthesis. Apart from antimicrobial activity exhibited by tannins, they also react with proteins to provide the typical tanning effect (Zakaria Ahmed et al., 2012).

Antioxidant activity denotes the ability of a bioactive compound to maintain cell structure and function by effectively clearing free radicals, inhibiting lipid peroxidation reactions, and preventing other oxidative damage (Bravo, 1998). There are more than 170 antioxidants from Citrus fruits that have been reported in the current literature, including vitamins, mineral elements, phenolic compounds, terpenoids and pectin (Zhou, 2012).

The use of synthetic compounds to inhibit microbial activity is one of the conventional methods for controlling microbial growth. One of the applications of synthetic agents is in food preservatives, where safety is to be maintained, here synthetic fungicides are found to be troublesome due to their residual nature and high toxicity to mammals . So a shift from synthetic chemicals to botanical antimicrobial compounds is gaining popularity because of their environmental safety. Phytochemical in citrus peel from different plant sources can inhibit various pathogenic bacteria, and total phenolic compounds and limonoids are highly correlated with antibacterial activity (Ghada M. Kholaf et al., 2017).

The aim of the present study was to evaluate the phytochemicals constituents of *Phyllanthus emblica* (amla), *Citrus reticulata* (orange) *and Citrus aurantifolia* (lime) solvent extracts. In addition, their influence on antibacterial activity against Gram-negative bacteria, Gram-positive bacteria and fungi was investigated.

Materials and Methods

Collection of the plant materials:

Phyllanthus emblica (amla), *C. reticulata* (orange) *and C. aurantifolia* (lime), were collected from local market in Bangalore, Karnataka, India.The peels of oranges

and limes, as well as amla excluding seed were chopped into small pieces and dried at 40ºC and then homogenized to fine powder and stored in airtight containers at 4ºC.

Preparation of plant extracts:
The dried and powdered plant materials (10 g) were extracted by maceration method using three different organic solvents: Methanol, Chloroform and Dichloromethane. 25 gm of dried fruit peel powder of plant material was soaked in 125 ml of solvent for 48 hrs at room temperature. This solution was filtered with the help of whatman No. 1 filter paper. The filtrate was collected in 15 cm petri dishes and evaporated the solvent at room temperature. The solid dried extract was stored in 2 ml eppendorf tube and powder was used for antimicrobial assays after dilution. The filter cake was dried at room temperature and stored separately (Dharajiya et al., 2015).
Yield of the extract obtained was calculated as:
$$Yield\ \% = weight\ of\ extract\ recovered / weight\ of\ dried\ powder \times 100$$
Phytochemical Screening:
The phytochemical screening of the sample was carried out as described by Nweze et al., (2004) and Senthilkumar and Reetha (2009). The samples were screened for the presence of carbohydrates, alkaloids, flavonoids, steroids, phenols, tannins, saponin, glycosides, and proteins.
a) Test for Alkaloids:
To 2 ml of plant extract, 2 ml of concentrated hydrochloric acid was added. Then 3 drops of Mayer's reagent were added. Presence of green colour or white precipitate indicates the presence of alkaloids.
b) Test for Carbohydrate:
To 2 ml of plant extract, 1 ml of Molisch reagent and 4 drops of concentrated Sulphuric acid were added. Formation of purple or reddish ring indicates the presence of carbohydrates.
c) Test for tannins:
To 1 ml of plant extract, 2 ml of 5 % ferric chloride was added. Formation of dark blue or greenish black indicates the presence of tannins.
d) Test for terpenoids:
To 1 ml of plant extract, 1 ml of chloroform was added with a few drops of Sulphuric acid added along the sides of test tube walls and left for a few mins. Yellow or brown colour formed at the lower layer indicates the test is positive.
e) Test for glycosides:
To 2 ml of plant extract, 1 ml of glacial acetic acid and 5% ferric chloride was added. To this, 3 drops of concentrated Sulphuric acid was added. Presence of greenish blue colour indicates the presence of glycosides.
f) Test for steroids:
To 1 ml of plant extract, equal volume of chloroform and 3 drops of concentrated Sulphuric acid were added. Formation of brown ring indicates the presence of steroids.
g) Test for saponins:
To 1 ml of plant extract, 5-10 ml of distilled water was added and shaken in a graduated cylinder for 15 min lengthwise. Formation of 1 cm layer of foam indicates the presence of saponin.
h) Test for flavonoids:
To 2 ml of plant extract 1 ml of 1N aqueous Sodium Hydroxide solution was added and observed for the formation of yellow-orange colouration.
i) Test for proteins:

To 2 ml of plant extract, 4 drops of 0.2% Ninhydrin was added and heated to 100℃. Formation of blue colour indicates the presence of proteins.

j) Test for mucilage:

To 1 ml of plant extract, 250 µl of absolute ethanol is added with constant stirring. Presence of white cloudy precipitate precipitate gums and mucilage.

k) Test for volatile substance:

To 1 ml of plant extract, dilute Hydrochloric acid is added dropwise. Formation of white precipitate indicates presence of volatile compounds.

Preparation of media

For Bacterial culture, Luria Bertani (LB) agar media (tryptone 10 g, sodium chloride 10 g, yeast extract 6 g, agar 15 g, distilled water 1000 ml) was prepared and autoclaved at 121°C for 15 mins. For fungal culture, Potato Dextrose Agar (PDA: Potato-200 g, dextrose-20 g, agar-20 g, distilled water-1000 ml) 150 ml of the media was prepared by boiling 30 g of potato in 100 ml distilled water and filtered. The remaining components: Agar- 3 g, Dextrose- 3 g was added into the filtrate and the volume was made upto 150 ml with distilled water and autoclaved at 121°C for 15 mins.

Antimicrobial activity by Well diffusion method

The three samples (Amla, Orange and Lime) were assessed in triplicates for their MIC property against microorganisms (*Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Aspergillus niger* and *Candida albicans*). 100 µg, 200 µg, 300 µg, and 400 µg samples were made using DMSO. Ampicillin and fluconazole were used as bacterial and fungal control respectively. Approximately 25 ml of the media was poured into the sterilized petri plates and allowed it to solidify, later 24 hrs cultured 100 µl inoculum of *Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Aspergillus niger* and *Candida albicans* poured into the respective plates and spread throughout the plate using spreader. Five wells were made using well borer and the samples containing 100 µg, 200 µg, 300 µg, and 400 µg were loaded into the respective wells and 50 µl of DMSO was loaded in the center well as control blank and incubated at 37°C for 24 hrs. Zone of inhibition was recorded in mm (millimeter). All the experiments are done in triplicates.

Results:

The present systematic examination shows the phytochemical analysis, antimicrobial activity of the methanol, chloroform and dichloromethane extract of the fruit of *Phyllanthus emblica* (amla) and fruit peel of *Citrus reticulata* (orange) and *Citrus aurantifolia* (lime).

Phytochemical analysis

The phytochemical analysis with the methanol(M), chloroform(C) and dichloromethane(DCM) extract of the dried fruit of *Phyllanthus emblica* (amla) and dried fruit peel of *Citrus reticulata* (orange) and *Citrus aurantifolia* (lime) showed the presence of alkaloids, glycosides, flavonoids, mucilage, saponin, steroids, terpenoids, protein, tannins and volatile oils are given in (table 1).

Antimicrobial activity

The in-vitro antimicrobial activity of methanolic extracts of *C. reticulata* (orange) and *C. aurantifolia* (lime) and *P.*

emblica (amla) under different concentrations (100 ug, 200 ug, 300 ug and 400 ug) against two standards are mentioned in table 2. Table 2 presents the diameter of inhibition of the various extracts on selected gram positive bacterial (*Bacillus cereus and Staphylococcus aureus)* and gram negative bacterial *(Pseudomonas aeruginosa and Salmonella typhi) and* fungal strains *(Aspergillus niger* and *Candida albicans)* species against the standards and fruit extracts.

Results of the Most Inhibitory Concentration (MIC) by a well diffusion method of the methanolic extract of Amla, Orange peel and lime peel worked best against *Candida albicans*, *Aspergillus niger* and *Staphylococcus aureus* respectively. The Amla extract was effective in inhibiting all test bacterial and fungal strains but to a varied extent, while it showed highest inhibition against *Candida albicans* (Mean(SD)-26.6(0.57) at 400 µg/ml). On the other hand, citrus extracts: *C. reticulata* showed inhibition only against fungal strains with highest inhibition against *Aspergillus niger(*Mean(SD)-23.6(0.57) at 300 µg/ml). *C. aurantifolia* showed inhibition against all test organisms except *Aspergillus niger,* it was found to be most effective against *Staphylococcus aureus, Salmonella typhi* and *Candida albicans.* Table.2 represents most significant values of inhibition against test organisms and details of inhibition at all concentrations can be seen in Fig.1.

Statistical analysis: Mean value and standard deviation were calculated for each test bacterial and fungal strains.

Plant material	Extract	Name of Phytochemical									
		Alkaloids	Tannins	Terpenoids	Glycosides	Steroid	Saponin	Flavonoids	Protein	Mucilage	Volatile oils
Phyllanthus emblica	M	+	+	+	-	+	-	+	+	+	-
	C	+	+	+	-	+	+	-	+	-	-
	DCM	-	-	-	-	-	+	-	+	-	-
Citrus sinensis	M	+	+	+	-		-	+	+	-	-
	C	+	-	+	+	+	-	-	+	-	+
	DCM	-	-	+	-	+	+	-	+	-	-
Citrus aurantifolia	M	+	-	+	+	+	+	+	+	-	+
	C	+	-	+	-	+	+	+	+	-	+
	DCM	+	+	+	-	+	-	-	+	+	-
(M) = Methanol, (C) = Chloroform, (DCM) = Dichloromethane, (+) = Present and (-) = Absent											

Table.1: Phytochemical screening of dried fruits peel of Citrus ***reticulata and Citrus aurantifolia and Phyllanthus emblica.***

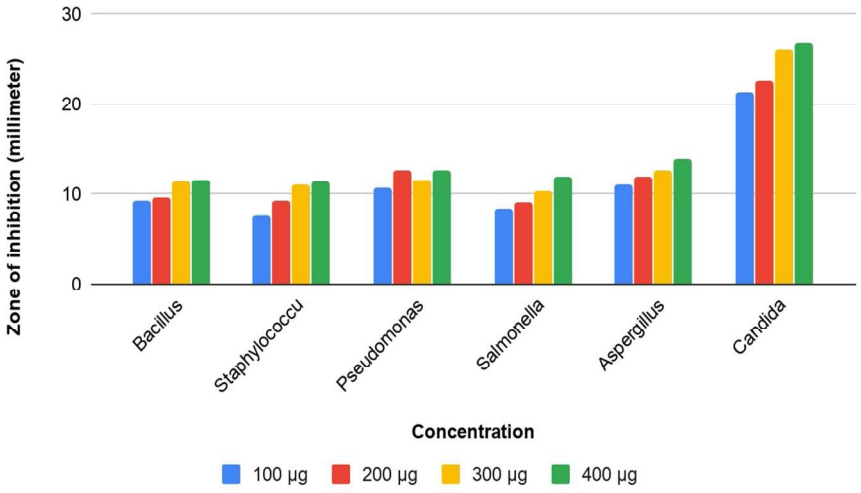
1) Table 2: Antimicrobial activity of dried fruits peel of Citrus *reticulata and Citrus aurantifolia and Phyllanthus emblica* by well diffusion assay

Name of the compounds and their concentration	Antibacterial activity diameter of inhibition zone(mm), Mean(SD)				Antifungal activity diameter of inhibition zone(mm), Mean(SD)	
	Bacillus cereus	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Salmonella typhi*	*Aspergillus niger*	*Candida albicans*
Standard - ampicillin(40 µg)	26(1.0)	20.6(1.15)	27(2.64)	28(0.0)	-	-
Standard fluconazole(400 µg)	-	-	-	-	24(1.0)	35(1.73)
Methanolic extract Amla(400µg)	11.6(0.57)	11.3(0.57)	12.6(0.57)	12(0.0)	14(0.0)	26.6(0.57)
Methanolic extract Orange(300µg)	-	-	-	-	23.6(0.57)	15.3(0.57)
Methanolic extract lime(300µg)	14.3(0.57)	17.3(1.15)	12(0.0)	16(0.0)	-	16.3(1.15)

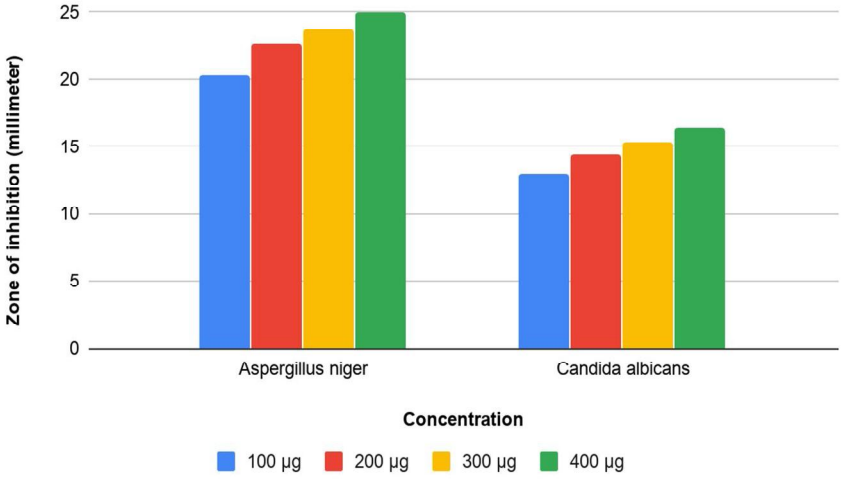
*Most significant activity, (-) = No Zone of inhibition

Fig.1: Zone of inhibition vs concentration of methanolic extract of sample and standards(A: Phyllanthus emblica, B: Citrus reticulata, C: Citrus aurantifolia, D: Ampicillin and E: Fuconazole)

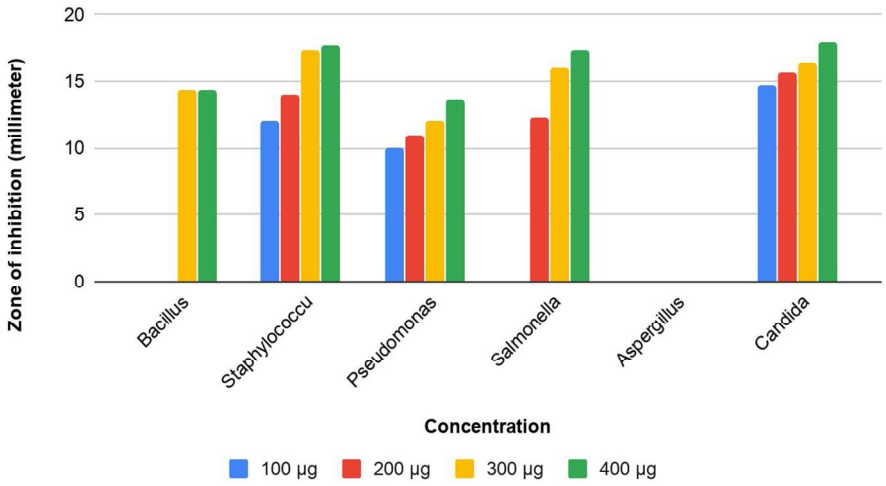
A] MIC of Phyllanthus emblica (Amla) extract in µg



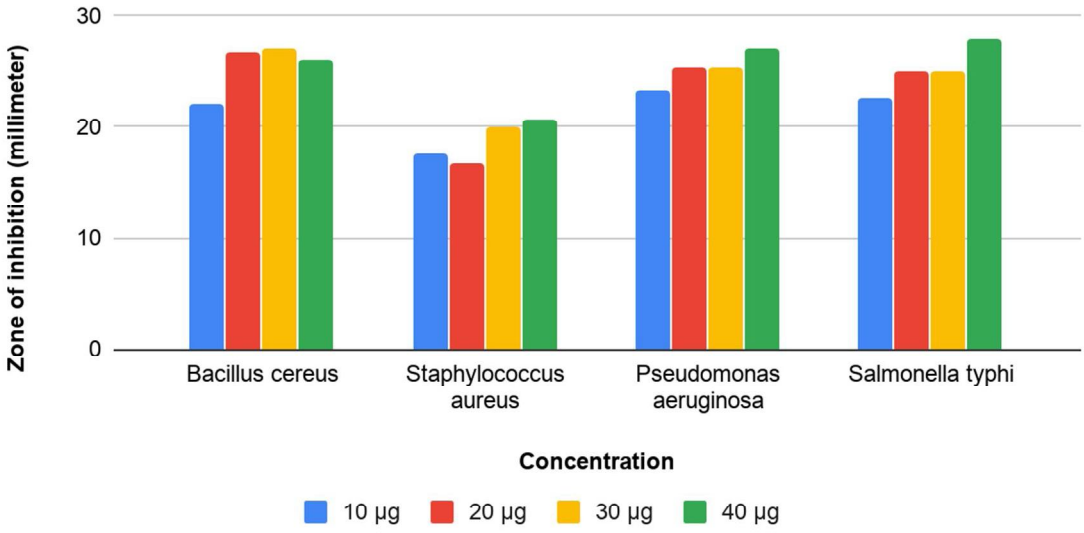
B] MIC of Citrus reticulata (Orange) extract in µg

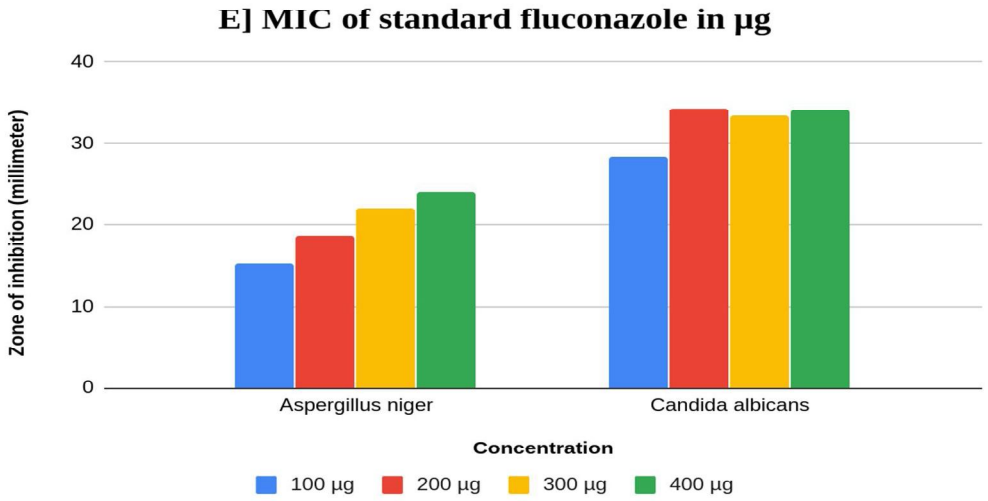


C] MIC of Citrus aurantifolia (lime) extract in µg



D] MIC of standard Ampicillin in µg





Discussion:

Citrus fruits are known to have therapeutic properties and this is linked to the high amounts of photochemical and bioactive compounds such as flavonoids, carotenoids, vitamins and minerals available in citrus fruits(D. E. Okwu, 2008; Dhanavade, 2011) as also shown in this experiment. All the three samples, dried fruits peel of C. *reticulata, C. aurantifolia and Phyllanthus emblica* extracts were rich in phytochemicals such as alkaloids, terpenoids, steroids, saponins, flavonoids and proteins. Three organic solvents like chloroform, methanol and dichloromethane were used for extraction, off these methanolic extracts showed positive for most of the phytochemicals hence it was further used for testing antimicrobial activity.

Antimicrobial activity was done against two Gram-positive bacteria and two Gram-negative bacteria and two fungal strains as mentioned above. Results of this study shows that the methanolic extracts of the fruit Phyllanthus emblica showed antimicrobial activity against all the six test samples and showed maximum activity against Candida albicans(26 and 26.6 mm(mean) at 300 and 400 µg respectively) as shown in Fig.1. On the other hand, Citrus reticulata showed activity only against two fungal stains, and maximum activity against Aspergillus niger(25mm(mean) at 400 µg). While Citrus aurantifolia extract, showed good inhibition against all test organisms except Aspergillus niger. These results are further supported by (Kumar A et al., 2011; Sini. G Nath et al., 2019; Rehab MA El-Desoukey., et al, 2018)

Conclusion:

In general, using peels of fruit wastes that makes significant solid waste in the environment not only is supportive to the ecosystem but also has potential antimicrobial activity against mutli-drug

resistant microorganisms. The results obtained in this study is convincing to explore the possibility for their future use, as also supported by many researches(Muhammed Saleem et al., 2020). In the present study, the peel of extracts of Phyllanthus emblica, Citrus reticulata and Citrus aurantifolia exhibited inhibitory effect against bacterial and fungal strains even at 100 µg/ml . The antimicrobial efficacy can be related to the presence of potential phytochemicals as shown in Table.1.

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