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Bioprospecting of pods of *Moringa oleifera* Lam. as novel antibacterial agent

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In the treatment of bacterial diseases, increasing resistance to traditional chemotherapeutics has drawn the necessity for substitute remedies. In this context, here, we evaluated the bactericidal activity of pods of *Moringa oleifera* Lam., an ethno medicinal plant, against eight pathogenic bacterial strains, both Gram positive (*Bacillus licheniformis*, *B. mycoides*, *B. subtilis* and *Staphylococcus aureus*) and Gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida*). Different organic solvent extracts, like ethyl acetate, acetone and alcohol, of pods of *M. oleifera* were examined for bactericidal activity against test microorganisms. Minimum inhibitory concentration, chromatographic analyses along with infrared spectroscopy, gas chromatography-mass spectroscopy and nuclear magnetic resonance spectroscopy was carried out for chemical characterization of active ingredient responsible for antibacterial activity. Both the Gram positive and Gram negative organisms showed variable sensitivity to different solvent extracts of *M. oleifera* pods. Ethyl acetate extracts showed maximum antibacterial activity with MIC value ranging from 1.30 to 4.10 mg/mL. IR analysis provided preliminary information about the amines, amides, aromatics and sulphur containing compounds of the active ingredient. GC-MS and NMR analyses indicated the presence of principal bioactive antibacterial compound 2-(benzoylsulfanyl)-1,3-thiazol,4-yl, benzoate with molecular formula $C_{17}H_{11}NO_3S_2$ from ethyl acetate extract of *M. oleifera* pods. The study concludes that the compound 2-(benzoylsulfanyl)-1,3-thiazol,4-yl, benzoate from ethyl acetate extract of pods of *M. oleifera* possess the antibacterial activity against the tested strains.

Keywords: Antibacterial activity, Ben oil tree, Drumstick tree, Horseradish tree

Antibiotics are one of our most imperative weapons in combating bacterial infections and have significantly benefited the health-related eminence of human life since their introduction. The widespread exploitation of synthetic drugs, disproportionate use of unwanted medication, increasing side effects, overpriced chemical drugs have become much more serious problems than the disease itself. Additionally development of antibiotic resistance created a global public health problem. WHO recommended traditional medicines as safe remedies for ailments of both microbial and non-microbial origins[1]. More than 6000 medicinal plant species have been known to produce chemical compounds and metabolites with preventive and curative properties against antifungal, antibacterial diseases[2]. Plant-based therapeutics have been the conventional source of raw materials for medicines since ancient period as they are natural products, biodegradable, non-narcotic, and easily available at affordable prices and, in most cases, they have no side effects. Medicinal plants represent a rich source of antimicrobial activities[3] to diminish infectious diseases. Considering the vast potentiality of plants as sources for antimicrobial drugs with reference to antibacterial[4] and antifungal agents, a systematic investigation was undertaken to screen the antibacterial activity from edible pods of *Moringa oleifera* Lam.

Ethno pharmacologically important plant, *M. oleifera* is commonly known as Miracle tree or Horseradish tree or Ben oil tree. It is a medium sized (10 m) widely grown soft wooded easily cultivable tree, found mainly in the tropics and subtropical area[5]. Almost all parts are vividly used for different nutritional prospects. The leaves and fruits acquire cytotoxicity, antistress, antioxidant, antimicrobial, anticonvulsant, antidepressant, antipyretic, antiasthmatic, anti-inflammatory, antiarthritic and analgesic properties[6-8] along with neuroprotection in Alzheimer's disease[9]. The pods of *M. oleifera* can be cooked, or stored as a dried powder for several months without any major loss of its dietary value[10]. The powdered form is utilized by pregnant women and lactating mothers for improvement of their

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children's sustenance[11]. The high nutritive value of pods of *M. oleifera* remains undisturbed even after thermal processing[12]. A total of 44 compounds were isolated from the leaves and four of them showed blood pressure reducing effect[13]. In the present study, we have made an attempt to explore the bioactive compounds from the ethyl acetate extract of immature edible pods of *Moringa oleifera* responsible for bactericidal activity against four pathogenic Gram positive (*Bacillus licheniformis*, *B. mycoides*, *B. subtilis* and *Staphylococcus aureus*) and four Gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida*) bacteria under laboratory condition.

Materials and Methods

Plant material

The immature pods of *M. oleifera*, called "drumsticks", were picked up from outskirts of Burdwan district (23°16'N, 87°54'E), WB, India during mid-March to mid-April. The specimen was taxonomically categorized and herbarium has been kept in the Department of Zoology, The University of Burdwan, having the Voucher specimen no. GCZD-09. In the beginning, the pods were rinsed off with tap water followed by drying on paper towel in the laboratory at (37°C) for 24 h.

Test microorganism

Eight bacterial strains namely, *Bacillus licheniformis* (MTCC 530), *B. mycoides* (MTCC 7343), *B. subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 2940), *Escherichia coli* (MTCC 739) *Pseudomonas aeruginosa* (MTCC 2453) *P. fluorescens* (MTCC 103) and *P. putida* (MTCC 1654), were collected from Mosquito, Microbiology and Nanotechnology Research Units, Parasitology Laboratory. The microorganisms were cultured in nutrient broth Hi-Media, M002 for their optimum growth at 37°C for *B. licheniformis*, *B. mycoides*, *E. coli*, *P. aeruginosa*, *P. fluorescens* and *S. aureus*, and at 30°C for *B. subtilis* and *P. putida*. All the strains were maintained with periodic subculture on nutrient agar slants at 4°C for further examination.

Antibiotics

Different concentration antibiotic discs (Span Diagnostics Limited, Surat, India) were used during the antibiogram bioassay. Amoxycillin (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), tetracycline (30 µg), gentamycin (10 µg), norfloxacin (10 µg), ampicillin (10 µg), penicillin g (10 µg), ofloxacin (5 µg) and ciprofloxacin (5µg) were applied for the test.

Plant extracts procurements

Preparation of the plant extracts

Solvent extraction preparation was carried out with clean, air dried 200 g pods of *M. oleifera* were put into the thimble of the Soxhlet apparatus and in a ratio of 1:10, 2 L ethyl acetate solvent loaded in the still pot[14]. The extraction period was fixed at a temperature around 77°C with maximum 8 h a day for 72 h. Elutes were collected from chamber of the still pot and made concentrated through evaporation. At 4°C the extractives were preserved for further bioassay.

Antibacterial Bioassay and Sensitivity test

Antibiogram and antibacterial bioassay were carried out through disc diffusion and agar well method[15]. Antibiotic sensitivity test discs were placed on the surface of solid agar. Wells of 5 mm diameter were punched by cork borer after agar solidification and filled with the organic ethyl acetate extracts (10-50 mg/mL) aided with water against all the 8 seleected bacterial strains. The surface of the different plates were inoculated with respective strains from broth culture and incubated for 24 h at 37°C for all the said bacteria except *P. putida* and *B. subtilis* which was kept at 30°C. Control experiments was also set in accordance. Clear zones of inhibition formed in the plates around the disc and ethyl acetate extract were measured in millimetres. The experiments were repeated thrice.

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of natural compounds was established by broth micro-dilution method, according to Clinical and Laboratory Standards Institute[15], with some modifications. The cultures were diluted in Müeller-Hinton broth at a density adjusted to turbidity of 0.5 MacFarland standards. Equal volume of 0.5 mL of each extract (by serial dilutions from the suspension of ethyl acetate plant extract stock solution) and nutrient broth were mixed in test tubes. The tubes were incubated at 37°C and 30°C, respectively for respective bacteria for 24 h. Two control tubes were maintained which included antibiotic control (tube containing extract and the growth medium without inoculums) and bacterium control (tube containing physiological saline, growth medium with inoculums). The lowest concentration of the extract which produced no visible bacterial growth when compared with the control tube was regarded as MIC value.

Phytochemical screening

Preliminary phytochemical analysis of ethyl acetate extracts of pods of *M. oleifera* was examined for the existence of for flavonoids, alkaloids, reducing sugar, saponins, tannins, terpenoids, steroids etc using several qualitative tests[16,17].

Column Chromatography (CC) analysis

About 9.1 g of the ethyl acetate fraction of *M. oleifera* pods was adsorbed on silica gel and subjected to column chromatography (60-120; Merck, India) column (50 × 4.5 cm). Single and mixture of solvent in different ratios with increased polarity like petroleum ether, petroleum ether: n-hexane, n-hexane, n-hexane: ethyl acetate, ethyl acetate, ethyl acetate: chloroform, chloroform, chloroform: methanol, methanol, methanol: acetone, acetone were used as eluting solvent. The flow rate was 2 mL/min and fractions were collected separately[18]. Six fractions of *M. oleifera* residues obtained EA1 (1.8 g), EA2 (1.1 g), EA3 (0.9 g), EA4 (3.8 g), EA5 (0.5 g) and EA6 (1.0 g) were tested for antibacterial activity against eight bacterial isolates. EA4 fraction exhibited significant activity and was selected for further isolation through TLC.

Thin Layered Chromatography (TLC) analysis

The bioactive fraction analysis was carried out using one to two drops of organic plant extract of the green pods of *M. oleifera* using a capillary tube to the bottom of each of the pre-coated (prepared with silica gel "G", Merck, India, 0.5 mm thickness) and pre-heated (100°C for 30 min) eight glass plates at equal distance. After few minutes of drying, each plate was placed in separate glass chamber filled with solvent mixtures in different ratio. Finally, mobile phase n-hexane: ethyl acetate: ethanol in the ratio 1:7:2 acts as gradient eluent solvent and separated the fractions, which were scrapped off the plates. After 55 plates were done the material was collected having same R_f values. The separated chromatographic fraction was made silica free by dissolving in absolute alcohol and tested for antibacterial activity. The underneath solid fraction was further analysed to isolate the active ingredient.

IR analysis of bio active principle

FTIR analysis was carried out by the fraction of the dried sample containing active ingredient. The sample was encapsulated with potassium bromide using hydraulic press to form KBr pellets. For control, a pellet containing only KBr was also prepared[18]. IR spectral analyses were carried out in Jasco, FT/IR 4700 spectrophotometer with a scanning range of 400-4500 cm^{-1} at room temperature (37°C). The spectral wavelength were recorded and identified by comparing the vibrational stretch with computer library search[19].

Gas Chromatography – Mass Spectroscopy (GC-MS) and Nuclear Magnetic Resonance (NMR) analyses

The purified fraction of pod extracts was further analysed by Gas Chromatography Mass Spectroscopy, ION TRAPE technology[19,20]. The temperature of column oven was set at 60°C initially for 2 min and then enhanced to 270°C and finally raised to 350°C with 10 min preparation time run out. Split injection mode was applied. Column used was TR-WAXMS, from Thermo Fisher Scientific India Pvt. Ltd. Helium acted as carrier gas. The mass spectra and retention indices of the compounds identified in the samples were determined by comparing with those of standard NIST library. X CALIBUR was the data store software. Nuclear magnetic resonance analyses were carried out by ^1H NMR spectra operating at 400 MHz spectrometer. Chemical shifts were reported in ppm using $CDCl_3$ (δ = 7.26 for ^1H) as solvent and internal standard solvent signal.

Determination of MIC of bioactive fraction

The minimum inhibitory concentrations of the isolated compounds against bacterial strains were determined by following standard method[18]. Standard antibiotic disc (Tetracycline) was used as reference bacteria for control experiment. Analysis was performed in three replicates.

Statistical analysis

The results are presented as mean ±SD using statistical tool like student T-test and Welch Analysis of Variances (one way analysis) followed by Games-Howell post-hoc test. Significant differences between the means of different concentrations were considered noteworthy when P value is <0.05[21].

Results

The laboratory bioassay showed that the ethyl acetate extracts of the pods of *Moringa oleifera* exhibited maximum antibacterial activity against tested strains among different solvents. The results of phytochemical analysis of the ethyl acetate extract showed that with increasing concentration the inhibition zones diameter also increased (Table 1). Profiles of antibiotic sensitivity of the tested bacteria against commercially accessible antibiotic discs were presented in Table 2. Comparing the data of inhibition

Table 1 — *In vitro* antimicrobial activity of ethyl acetate extracts of pods of *Moringa oleifera* by agar well diffusion assay

Conc. (mg/mL)	Diameter of the inhibitory zones (mm)							
	Bacillus licheniformis	*B. mycoides*	*B. subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*P. fluorescens*	*P. putida*
10	22.33±0.58	27.17±0.29	25.00±0.00	24.00±0.00	20.00±0.00	24.33±0.58	28.50±0.50	27.00±0.00
20	23.83±0.29	28.27±0.46	26.33±0.29	25.17±0.29	20.83±0.29	25.17±0.29	29.33±0.58	28.17±0.29
30	25.33±0.58	29.67±0.58	28.00±0.00	26.67±0.58	21.67±0.58	26.00±0.00	30.00±0.00	29.67±0.58
40	26.00±0.00	30.67±0.29	28.83±0.29	27.33±0.29	22.33±0.58	27.50±0.50	30.33±0.58	30.17±0.29
50	27.67±0.58	31.00±0.00	29.00±0.00	28.17±0.29	23.33±0.58	28.33±0.58	31.00±0.00	31.00±0.00

Table 2 — Susceptibility of eight reference bacterial strains to some antibiotics by agar well diffusion assay

Antibiotics (µg/mL)	Diameter of the inhibitory zones (mm)							
	Bacillus licheniformis	*B. mycoides*	*B. subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*P. fluorescens*	*P. putida*
Amoxicillin (30)	9	0	0	0	5	13	15	0
Chloramphenicol (30)	22	20	22	25	0	20	30	25
Nalidixic acid (30)	20	0	20	0	11	0	0	11
Tetracyclin (30)	25	0	25	0	12	0	12	0
Ampicillin (10)	0	0	0	23	15	0	0	7
Gentamycin (10)	20	17	20	19	16	17	19	16
Norfloxacin (10)	0	8	0	24	16	8	24	16
Penicillin G (10)	0	0	0	0	0	0	0	0
Ciprofloxacin (5)	11	17	22	19	21	20	19	22
Ofloxacin (5)	20	19	20	18	20	17	17	18

Table 3 — Minimum inhibitory concentration of bioactive fraction of ethyl acetate from pods of *Moringa oleifera*

Bacteria	MIC value (mg/mL)
Bacillus licheniformis	1.30
B. mycoides	1.70
B. subtilis	2.10
Staphylococcus aureus	2.90
Escherichia coli	4.10
Pseudomonas aeruginosa	1.90
P. fluorescens	1.90
P. putida	2.10

Table 4 — Phytochemical constituents of pods of *Moringa oleifera*

Constituents	Phytochemical Test	Result
Flavonoids	Ferric chloride	+
Alkaloids	Wagner's test/ Mayer's test	+
Free reducing sugar	Fehling's test	+
Saponins	Frothing test	–
Tannins	Ferric chloride reagent test	-
Terpenoids	5 mL Chloroform + 3 mL conc. H_2SO_4	+
Cardiac glycosides	GAA + Ferric chloride + conc. H_2SO_4	+
Anthraquinones	Chloroform+10% ammonia solution	-
Steroids	Acetic anhydride +Sulphuric acid	-

[GAA, Glacial acetic acid; +, Present; and -,Absent]

zone, penicillin was found to be resistant among all the reference bacteria whereas gentamycin, ofloxacin and ciprofloxacin were effective against all (Table 2). Efficiency of the ethyl acetate pod extracts was determined by calculating the MIC value (Table 3). Preliminary secondary phytochemical analysis revealed the presence of alkaloids, free reducing sugar groups, terpenoids, and cardiac glycosides from ethyl acetate extracts of immature pods of *M. oleifera* (Table 4). Isolation of active constituent from column chromatography showed EA-4 fractions with bioactivity (Table 5) and from thin layer chromatography, R_f value of 0.83 (R_f = 12.5/15 = 0.83) confirmed antibacterial activity.

From the IR analysis (Suppl. Fig S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*) following functional group were detected: 3382 cm^{-1} (N-H stretch, primary amine), 3008 cm^{-1} (-CH stretch, aromatics), 2914 cm^{-1} and 2847 cm^{-1} (-CH stretch, aldehyde), 2364 cm^{-1} (O-H stretching, sulphur compounds), 1707 cm^{-1} (C=O stretch, ketones), 1461 cm^{-1} (ring, aromatics), 1038 cm^{-1} (-CH bending, aromatics). Further mass spectral analysis of the chromatographed purified fraction displayed a major peak (relative abundance) at retention time of 10.35 min (Suppl. Fig. S2). Further mass spectral analysis of the chromatographed purified fraction displayed a major peak (relative abundance) at retention time of 10.35 min (Suppl. Fig. S2). The H^1 NMR experiment was conducted to confirm spectral assignments (Fig 4). From the GC-MS (Suppl. Fig. S3) and NMR analyses (Suppl. Fig. S4) active ingredient figured out encompass chemical formula as $C_{17}H_{11}NO_3S_2$ with a molecular weight of 341. The novel isolated compound 2-(benzoylsulfanyl)-1,3-thiazol,4-yl, benzoate exhibited higher antimicrobial activity when MIC value was determined (Table 6).

Table 5 — Antibacterial activity of column fractions (EA1-EA6)

Bacteria	Diameter of zone of inhibition (mm)						
	EA1	EA2	EA3	EA4	EA5	EA6	Tetracycline
Bacillus subtilis	0	0	0	30.33±0.58	0	0	25.01±0.58
B. licheniformis	6.04±0.58	0	0	33.50±0.58	0	0	24.33±0.00
B. mycoides	3.00±0.58	0	0	32.00±0.01	0	0	11.00±0.00
Staphylococcus aureus	0	0	0	29.00±0.58	0	0	12.50±0.58
Escherichia coli	0	0	0	29.50±0.00	0	0	13.83±0.00
Pseudomonas aeruginosa	0	0	0	31.33±0.00	0	0	16.00±0.00
P. fluorescens	0	0	0	28.33±0.01	0	0	12.33±0.29
P. putida	0	0	0	29.50±0.58	0	0	13.53±0.06

Table 6 — Minimum inhibitory concentration of 2-(benzoylsulfanyl)-1,3-thiazol,4-yl,benzoate of pods of *Moringa oleifera*

Bact. strains		Fraction concentrations (mg/mL)								
		50	25	12.5	6.25	3.125	1.5625	0.78125	0.390625	Water*
BL	MO	-	-	-	-	-	-	+	+	-
	TC	-	-	-	-	-	+	+	+	-
BM	MO	-	-	-	-	-	+	+	+	-
	TC	-	-	-	-	+	+	+	+	-
BS	MO	-	-	-	-	-	+	+	+	-
	TC	-	-	-	-	+	+	+	+	-
SA	MO	-	-	-	-	-	+	+	+	-
	TC	-	-	-	+	+	+	+	+	-
EC	MO	-	-	-	-	+	+	+	+	-
	TC	-	-	+	+	+	+	+	+	-
PA	MO	-	-	-	-	-	-	+	+	-
	TC	-	-	-	+	+	+	+	+	-
PF	MO	-	-	-	-	-	-	-	+	-
	TC	-	-	-	-	+	+	+	+	-
PP	MO	-	-	-	-	-	-	+	+	-
	TC	-	-	-	+	+	+	+	+	-

[Bacterial strains: BL, *Bacillus licheniformis*; BM, *B. mycoides*; BS, *B. subtilis*; SA, *Staphylococcus aureus*; EC, *Escherichia coli*; PA, *Pseudomonas aeruginosa*; PF, *P. fluorescens*; and PP, *P. putida*. MO, Active ingredient fraction of *Moringa oleifera* pods extract; TC, Tetracycline as control; -, No growth of bacteria; and +, Growth of bacteria. *, Negative control]

Discussion

The present investigation elucidates the antibacterial activity of immature pods of *M. oleifera* against eight microorganisms at different concentrations of the extract. Different parts of the plant contain various phytochemicals which acts as valuable therapeutic key. Therefore, several solvents are used to extort phytochemicals to extract all the phytoconstituents. Methanol extract of root showed sedative action on central nervous system whereas aqueous extract possess antifertility property. Besides, seeds of *Moringa oleifera* show cyanobacteriacidal activity. Few bacteria show sensitivity to ethyl acetate and acetone extracts of *M. oleifera* root barks, while other pathogens showed response to methanolic extracts of stem bark[22]. The present study was performed by ethyl acetate extracts of immature pods of *M. oleifera* that showed antibacterial activity against eight bacterial strains, namely *Bacillus licheniformis*, *B. mycoides*, *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida* at 10 mg/mL concentration. *P. fluorescens* showed highest zone of inhibition (28.50±0.50 mm) with a MIC value of 1.60 mg/mL while *E. coli* showed lowest inhibition zone (20.00±0.00 mm) having MIC 4.10 mg/mL among them. It may be foresighted that difference in the sensitivity of extracts prepared from different parts of *Moringa* is directly related to the concentration of antibacterial compounds in different parts of the same plant. Furthermore, the growth stage of plant, environmental factors, storage conditions, and difference in extract preparation can also influence the antimicrobial compounds in extracts of same part of same plant.

Preliminary essential information regarding chemical constituents of plant extracts can be achieved through qualitative phytochemical screening[23]. In this experiment, qualitative tests indicate appreciable amount of alkaloids, cardiac glycosides, free reducing sugars and terpenoids as chief metabolites. Different solvents in different ratio with variable polarity elucidate separation of pure compound from plant extracts[24]. In this experiment, 1:7:2 ratio of three solvents n-hexane: ethyl acetate: ethanol separates and a single band derived with R_f value 0.83. The fraction was found most effective in antibacterial activity. IR spectrum analyses revealed the presence of –OH stretching sulphur compound, primary amines. All these can be correlated with the presence of alkaloids as active principal in immature pods of *M. oleifera*.

Alkaloids are naturally occurring groups containing nitrogen atoms. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain sulphur, phosphorus and halogen elements[25]. Based on all the spectral data of GC-MS as shown in Suppl. Fig.

S3, 45 peaks were detected from which 2-(benzoyl-sulfanyl)-1,3-thiazol,4-yl,benzoate ($C_{17}H_{11}NO_3S_2$) represented a major compound with molecular weight of 341 from the TLC fraction (RF= 0.47) of ethyl acetate extracts of pods of *M. oleifera*. Meyer and his co-workers[26] reported potent cytotoxicity activity of the compound against brine shrimp larvae, with ED_{50} values of 4.00±0.25. The compound finds its novelty in this experiment as no data was reported in terms of antibacterial potentiality. Efforts are required for wholesome research to test the mode and site of action of the isolated compound. The prospective for developing antimicrobials from botanicals appears rewarding, as it shows the way for the development of a phytomedicine to act against microbes. Hence, the active ingredient isolated from the pods of *M. oleifera* may be used to the advancement of new pharmaceuticals. Although the effect was observed *in vitro*, the benefits may be extended in dietary supplements form to the community in prospect.

Conclusion

The study depicts *in vitro* antibacterial activity of ethyl acetate extracts of *Moringa oleifera* pods against four Gram positive (*Bacillus licheniformis*, *B. mycoides*, *B. subtilis* and *Staphylococcus aureus*) and four Gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida*) bacterial strains. GC-MS and NMR spectroscopic analysis confirm the presence of 2-(benzoylsulfanyl)-1,3-thiazol,4-yl,benzoate as principle bioactive compound with its signature peak at specific wavelength. The minimum inhibitory concentration portrays the potentiality of the compound as antibacterial agent. The experiment, thus enables development of more effective and therapeutic formulations in the field of wide-spectrum antibacterial activity.

Conflict of Interest

Authors declare no competing interests.

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