Interaction of some plant extracts with some antibiotics against *Listeria monocytogenes from rabbits*

Ashraf A. Abd El- Tawab[1], Ahmed A. Ammar [2], Ahmed M. Hamouda[3] and Safinaz A. M.Elhawary [3]

[1]*Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Benha University, Egypt.*

[2]*Microbiology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt.*

[3]*Animal Health Research Institute, Zagazig branch.*

A B S T R A C T

Multidrug-resistant bacteria strains is becoming a serious problem, so new technology applied plant extract as a natural antimicrobial source combined with antibiotics to overcome this problem by using new method as the Decimal Assay for Additivity (DAA) to define the end point for additivity as the interactions could be respectively defined as synergism or antagonism. The present study was carried out to evaluate the antimicrobial activity of five plant extracts prepared by the ultrasonic-assisted methanol extract (UAE) as z*ingeber officinalis* (Ginger), *camellia sinensis* (Green tea), *curcuma longa* (Turmeric), *pelargonium graveolens* (Geranium) and *thymus vulgaris* (Thyme) combined with antibiotics like amoxicillin, doxycycline , gentamicin and difloxacin against 90 samples from dead and diseased rabbits with (10) isolates belonging to one Gram positive field strain of *Listeria monocytogen* by using agar diffusion method. The mean zone of inhibition (IZ) of methanol plant extracts and antimicrobial agents determined at different concentrations, Also, the minimum inhibitory concentration (MIC) (the lowest concentration which prevent visible growth of the bacteria) was determined for the tested plant extracts and the used antibiotics and for combination between them by the twofold dilution method, as the antimicrobial activities were assessed by using disc diffusion method. Total phenolic content (TPC) of plant extracts was determined by the Folin–Ciocalteu method, also the antioxidant activity of the extract was determined by the (DPPH) assay. Results revealed synergistic effects appear in thyme with amoxicillin by ratio (6:4),(5:5) while green tea with amoxicillin by ratio (7:3),(6:4) and gentamicin by ratio (6:4) finally turmeric with doxycyclin by ratio (5:5) , with gentamicin with (6:4) ,while with difloxacin by ratio(6:4), (5:5).

Keywords: *Listeria monocytogens, plant extracts, Total phenolic compound, antioxidant activity.*

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1. INTRODUCTION

Listeriosis is an infection of human and animal importance. It is caused by *Listeria monocytogens*. Which widely distributed in the environment (Abd El-Motelib et al., 1990). The incorrect and overuse of existing antimicrobials is becoming a formidable threat in the fight against disease due to the emergence of multi-drug resistant strains. (Lambert, 2000 and Van Vuuren *et al*., 2009). Drug synergism between known antimicrobial

agents and bioactive plant extracts is a novel concept and has been recently reported (Nascimento *et al*., 2000, Aqil *et al*., 2005, Betoni *et al*., 2006 and Mhanna and Adwan, 2008).

In the recent time, spread of multidrug resistance as a phenomenon among bacterial pathogens has been a major problem confronting the field of antibacterial chemotherapy (Stefanovic and Comic, 2012).

Listeria monocytogenes had resistance to one or two antimicrobials. Oxacillin resistance was the most common resistance phenotype against *Listeria* isolates. A medium prevalence of resistance to clindamycin and low incidence of resistance to tetracycline were also detected (Diego, et al., 2014).

The highest resistance of *Listeria monocytogen* against penicillin, erythromycin and nalidixic acid, with all *Listeria* spp displaying resistance, followed by ampicillin , trimethoprim, nitrofurantoin and cephalosporin Olanirian et al., (2015) .

To overcome this problem some medicinal plants also produce multidrug resistance inhibitors (Eze et al., 2013).

Methanol and aqueous extract of *Camellia sinensis* had antibacterial activity against *Listeria monocytogenes*. The methanol extracts of the test plant produce larger zones of inhibition against the bacteria than the water extract. **(**Mbata et al., 2008)

Myristica fragrans (Nutmeg) showed good anti-listerial activity, although, extracts of onion and pepper did not show any antibacterial activity against *Listeria monocytogens* (Indu et al., 2006)

Twenty eight essential oils had antibacterial properties, against four pathogenic bacteria (*Escherichia coli*, *Listeria monocytogenes*, *Salmonella Typhimurium* and *Staphylococcus aureus*) to determine the minimum inhibitory concentration (MIC) for each pathogen evaluated (Oussalah et al., 2007).

Thyme essential oil had antimicrobial effect against *Listeria monocytogenes* .It had synergestic effect with nisin against *Listeria monocytogenes* **(**Solomakos et al., 2008).

There were synergstic effects of the combination of subtilosin with natural antimicrobials of curcuminum against *Listeria monocytogenes*. **(**Tahar et al., 2010).

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism by overnight incubation, usually reported as mg/L (Delaquis *et al*., 2002) as represent a monitor resistance to antimicrobial agents and done by broth dilution methods (Handa *et al.*, 2008).

Nowadays to overcome environmental pollution by aromatic plant extract residues, numerous studies focuses in recovering, recycling of residues as it has potential biological (Cioffi *et al*., 2010;Gavaric *et al.,* 2015).As 99% of residues after extraction rich with secondary metabolites and bioactive compounds e.g. natural antioxidants and phenolic compounds (Zhao and Gao, 2014) which play an important role in protection against infection, preventing oxidation and degenerative diseases (Singleton *et al.,* 1965 and Valko *et al*., 2006). Finally, this study was carried out to evaluate the Interaction of some plant extracts with some antibiotics against *Listeria* from rabbits and determination of MIC for each antibiotics and plant extracts by using DAA method to detect effect of interaction between antibiotics and plant extracts and detection of antioxidant and total phenolic compound.

2. Materials and methods

2.1. Bacterial strains:

a) Standard strain: The tested microorganisms were provided from the culture collections of the Microbiological Dept. National Research

Center (NRC) Dokki, Giza, Egypt. These include *Listeria monocytogens* ATCC19112.

b) Field strain: 10 isolates out of 90 samples which were isolated from visceral organs of clinically diseased and dead rabbits of different ages reared in farms located in Sharkia and Dakahlia governorates.

Under aseptic condition, samples were transferred to bacteriological laboratory for bacteriological examination.

2.2. Plants:

a) Plant materials: Five plant samples: [Green tea leaves (*Camellia sinensis*), Thyme leaves (*Thymus vulgaris*), Ginger rhizomes (*Zingiber officinale*), Turmeric rhizomes (*Curcuma longa*) and Geranium leaves (*Pelargonium graveolens*).

b) Preparation of the ultrasonic-assist methanol 80% extract: Modern techniques as extraction by ultrasound to overcome (time-solvent) consuming and increase extraction efficiency (Betancount, 2008).

2.3. Isolation and Identification of the suspected bacteria:

1-Media:

A-Liquid media: Fraser broth, semisolid agar, 1% peptone broth, sugars (dextrose, maltose, sucrose, sorbitol, xylose, Lactose and mannitol). (Himedia).

B-Solid media: PALCAM, blood agar, triple sugar iron agar and urea agar base. (Himedia)

2-Reagents, chemicals and stains:

Kovac's, urea, Andrade's indicator, H2O2, Gram's stain (Quinine et al. (2002) and Stanley, (1986).

3-Isolation and Identification of the suspected bacteria:

Samples were identified by gram staining, colony morphology, motility test (listeria show umbrella shape), catalase reaction, Vogues-proskaur test, sugar Fermentation and the Christie- Atkins-Munch-Peterson (CAMP test). Quinine et al. (2002) and Warburton et al. (2003).

2.4. Antimicrobial susceptibility testing:

2.4.1. Disk Diffusion Method:

According to guidelines set by the Clinical Laboratory Standards Institute (CLSI, 2011) the diameters of the zones of inhibition were measured in millimeter and classified as resistant, intermediate or sensitive and done to every plant extract and, antibiotics alone and also to combination between them by disk diffusion method to detect the effect of ten standard antibiotic discs (Oxoid®) and five selected extracts against *Listeria monocytogens*.

2.4.2. Minimal inhibitory concentration (MIC):

The isolated strain matches the 0.5 McFarland standards ($1.5*10^8$CFU mL $^{-}$1) and results of antibiotics and plant extracts showed no visible bacterial growth considered as MIC and interpreted with recommendations of the National Committee for Clinical Laboratory standards (CLSI,2011).

2.5. Evaluation of combined activity of antibiotics and plant extracts using Decimal Assay for Additivity (DAA):

As described by Sanders *et al.* (1993) to detect end point for additivity so that interactions greater or less than additivity defined as synergism and antagonism respectively.

2.6. Determination of total phenolic compounds (TPC):

TPC measured by UV spectrophotometer Škerget *et al.,(* 2005) used Folin-Ciocalteu reagent (AOAS, 1990) results expressed as mg gallic acid equivalents (GAE) per gram of dry weight (mg GAE g-1 DW) using a calibration curve.

2.7. Antioxidant DPPH radical-scavenging activity:

The ability of the extracts for electron donation was measured by bleaching of the purple colored solution of DPPH˙ (2, 2- diphenyl-1-picrylhydrazyl) to the yellow colored. (Gulcin et al., 2004) color intensity varies according to the amount of oxidant in the sample. The absorbance of this color is measured spectrophotometrically at 530 nm Dikilitas et al. (2011).

3. RESULTS

This study focused on the prevalence of *Listeria monocytogenes* and resistance patterns in *Listeria monocytogenes*. A total of 90 samples were aseptically collected from visceral organs of clinically diseased and dead rabbits of different ages reared in farms located in Sharkia and Dakahlia governorates with bacteriological examination of these Gram positive samples revealed the presence of 10 Listeriosis out of 90 specimens and isolates with percentages of (11.11 %) (Table1).

Fermentation and the Christie- Atkins-Munch-Peterson (CAMP test)were used, under standard conditions (Table 3), as *Listeria monocytogenes* show positive results with Catalase and Voges-Proskauer, also positive result with methyl red and haemolysis test which recorded by Quinine et al. (2002) and Warburton et al. (2003)

Antimicrobial susceptibility testing showed the sensitivity of the *Listeria monocytogenes* strains tested, which exhibited respectively , as the highest sensitivity rate was recorded to flurophenicol , cefotaxime and erythromycin with (7, 6 and 5) of sensitive strains .The highest intermediate rate was recorded to amoxicillin and doxycycline with (6, 4 and) of intermediate strains. The highest resistant rate was recorded to colistin, and streptomycin with 9, and 8 of resistant strains which shown in (Table 4).

As for *Listeria monocytogenes,* the extract of ginger with the lowest concentration had an Inhibition Zone of 11 mm, the green tea with the lowest concentration had an Inhibition Zone of 13 mm, the thyme with the lowest concentration had an Inhibition Zone of 14 mm, the gernium with the lowest Concentration had an Inhibition Zone of 10 mm and the turmeric with the lowest concentration had an Inhibition Zone of 13 mm showed in (Table 5).

On the other hand, the clear zones around each antibiotic discs were the zones of inhibition that indicated the extent of the test organism's inability to survive in the presence of the test antibiotic. So in this study the four antibiotics showed different Inhibition Zone on *Listeria Monocytogenes* like (13- 17 mm) around amoxicillin, (11-16 mm) around doxycycline, (12 mm) around gentamycin and (14- 22 mm) around difloxacin (Table 5) .

Five plant extracts were subjected to a broth macro dilution assay and after twenty four hours observation of *Listeria monocytogenes* growth to determine the MIC values, as the MIC of Geranium was 2µg/ ml for *Listeria monocytogenes,* ginger was >16 µg/ ml for *Listeria monocytogens,* turmeric was >16 µg/ ml for *Listeria monocytogenes,* thyme was 4 µg/ ml for *Listeria monocytogens*, and green tea was >16 µg/ ml for *Listeria monocytogenes.*

In this study, each 4 antibiotics was subjected to a broth macro dilution assay and after twenty four hours observation of bacterial growth to determine the MIC values on *Listeria monocytogenes*. MIC values of amoxicillin was 0.5 µg/ ml for *Listeria monocytogenes,* doxycycline was 2 µg/ml for *Listeria monocytogens,* gentamicin was 8 µg/ ml for *Listeria monocytogens* and of difloxacin was 0. 5 µg/ ml for *Listeria monocytogens* (Table7).

Antimicrobial activities of methanol different plant extracts in combination with antimicrobial agents on selected *Listeria*

monocytogenes isolates as interactions between these components lead to antagonistic, additive and synergistic effects. Additive effect was observed when the combined effect was equal to the sum of the individual effects, antagonism was observed when the effect of one or both compounds was less when they were applied together than when individually applied and synergism was observed when the effect of the combined substances was greater than the sum of the individual effects.

Results of synergy between antibiotics/plant extract on *Listeria monocytogenes* were presented in table (8).

Then, Antibacterial activity of all Antibiotics alone and in combination with plant extracts on *Listeria monocytogenes* shown in (Table 8).

Amoxicillin shows synergistic action in combination of with green tea by level (7:3) and by (6:4), also shows synergistic action in combination with thyme by level (6:4) and (5:5). While doxycycline shows synergistic action in combination with turmeric by level by (5:5), while gentamicin shows synergistic action in combination with green tea by level (6:4), also shows synergistic action in combination with turmeric by level (6:4). Finally difloxacin shows synergistic action in combination with turmeric by level (6:4),(5:5).

According to total phenolic compound (Table 9) illustrated that thyme and geranium had high phenolic compounds than green tea. Finally turmeric and ginger had nearly the same results with respective values of 253.01, 219.3, 190.33, 43.96 and 41.92 mg GAE g^{-1} extract TPC expressed as Gallic acid equivalent (GAE) was calculated using the following linear equation based on the calibration curve.

According to DPPH result, ginger and turmeric showed higher extension than green tea than geranium, Also, thyme extract had shown lower extent of DPPH neutralization (EC50 = 128.49 mg/mL) than oil obtained (Fig.1).

Table 1: Number of *Listeria i*solates obtained from various specimens collected from different localities in Sharkia and Dakahlia governorates.

Locality		No. of cases	*Listeria monocytogens 01*
Dakhlia governate	Private farms (Gamsa,Sherbin)	40	4
Sharkia governate	Private farms in (Sharkia governate)	50	6
Total		90	10(11.11%)

Table 2: List of the methanolic extract obtained from different plant parts g/ml spices in stock solution.

Scientific name	Family	Local name	Wt. of extract in tube (g)	Wt. of empty tube (g)	Total Wt. of extract (g)
Pelargonium graveolens	Geraniaceae	Geranium	138.0542	134.5216	3.53
Camellia sinensis	Theaceae	Green tea	172.3789	169.9189	8.9
Zingiber officinale	Zingiberiaceae	Ginger	177.5154	176.9858	1.02
Thymus vulgaris	Thymeleaceae	Thyme	191.9960	187.3328	4.66
Curuma longa	Zingiberiaceae	Turmeric	193.6245	192.6999	0.42

Table 3a: Morphological and Biochemical characteristics of isolated Listeria *monocytogenes*.

Growth on enriched fraser broth	Blackening
Growth on Palcam media	Gray green colonies surrounded by dark brown to black halos in medium
Gram staining	Purple gram positive rodes
Catalase	Positive
Oxidase	Negative
Indole	Negative
Methyl Red	Positive
Voges-Proskauer	Positive
Urease production	Negative
Citrate	Negative
Haemoylsis	Positive

Table 3b: Results of carbohydrate fermentation test for *Listeria monocytogenes*.

CARBOHYDRATES TEST	Result
Rhamnose	Positive
Xylose	Negative
Glucose	Positive
Manitol	Negative
Arabinose	Positive
Sorbitol	Positive
Fructose	Positive

Table 4: Antimicrobial susceptibility of *Listeria* spp. by agar disc diffusion method.

Antimicrobial agents	Antibiotic disc/conc.(µg)	S	I	R
Amoxicillin	AML-25	2	6	2
Colistin	CT-10	0	1	9
Difloxacin	INN-5	1	5	4
Doxycycline	DO-30	2	4	4
Gentamycin	CN-10	3	4	3
Erythromycin	E-15	5	3	2
Flurophenicol	F-30	7	0	3
Cefotaxime	CTX-30	6	0	4
Streptomycin	S-10	0	2	8

R (resistance), S (susceptibility), I (intermediate)

Table 5: Zone of inhibition (mm) of antibiotics and extracts alone and in combination on field strain of *Listeria monocytogenes*.

Isolates		Inhibition zone (mm)							
		Antibiotics alone				Combination			
Plants	Plant	AML/ 18 (mm)	DO/ 1 8 (mm)	CN/ 15 (mm)	INN/2 2 (mm)	AML	DO	CN	INN
Geranium	10	13	11	12	22	16	19	13	20
Green tea	13	15	14	12	15	19	12	16	14
Ginbger	11	16	14	12	16	16	13	11	22
Thyme	14	17	16	12	14	18	16	17	22
Turmeric	13	15	13	12	19	14	18	15	22

AML: amoxicillin. CN: gentamicin.

DO : doxycyciline. INN: difloxacin.

Table 6: Minimum inhibitory concentration (MIC) of plant extracts on *Listeria monocytogenes (*Field and Standard Strains).

Plants	*Listeria monocytogenes*	MIC µg / ml
Geranium	Field Strain	2
	Standard strain	1
Green tea	Field Strain	>16
	Standard strain	8
Ginger	Field Strain	>16
	Standard strain	8
Thyme	Field Strain	4
	Standard strain	2
Turmeric	Field Strain	>16
	Standard strain	8

Table 7: Minimum inhibitory concentration (MIC) of antibiotics on *Listeria monocytogens (field and standard strains).*

Antibiotic	*Listeria monocytogenes*	MIC µg / ml
Amoxicillin (20%)	Field Strain	0.5
	Standard strain	0.25
doxcyciline (20%)	Field Strain.	2
	Standard strain	1
Gentamicin (10%)	Field Strain	8
	Standard strain	0.5
difloxacin (10%)	Field Strain	0.5
	Standard strain	0.25

Table 8: Combination activity of antibiotics with extracts using Decimal Assay for Additivity (DAA).

Listeria monocytogenes

Plant extracts	Antibiotics	DAA			MIC	Effect
		AB	E	DAA	AB alone	
Thyme	**a)** Amoxicillin	6	4	0.55	1	Synergy (S)
		5	5		1	

Green tea	a)Amoxicillin	7	3	0. 25	0. 5	Synergy (S)
		6	4			
	b) Gentamicin	6	4	4	8	
Turmeric	a) Doxycycline	5	5	0. 25	0.5	Synergy (S)
	b) Gentamicin	6	4	2	4	
	c) Difloxacin	6	4	0. 25	0. 5	
		5	5			

Table 9: Yield of extracts (g/100g) for different plants.

Plants	extract yield	% Extract	TPC mg GAE/g extract
Green tea	8	40	190.33
Thyme	4.66	23.3	253.01
Ginger	1.02	5.1	43.96
Turmeric	0.42	2.1	41.92
Geranium	3.53	17.65	219.38

TPC: Total phenolic compound, GAE: Gallic acid equivalents

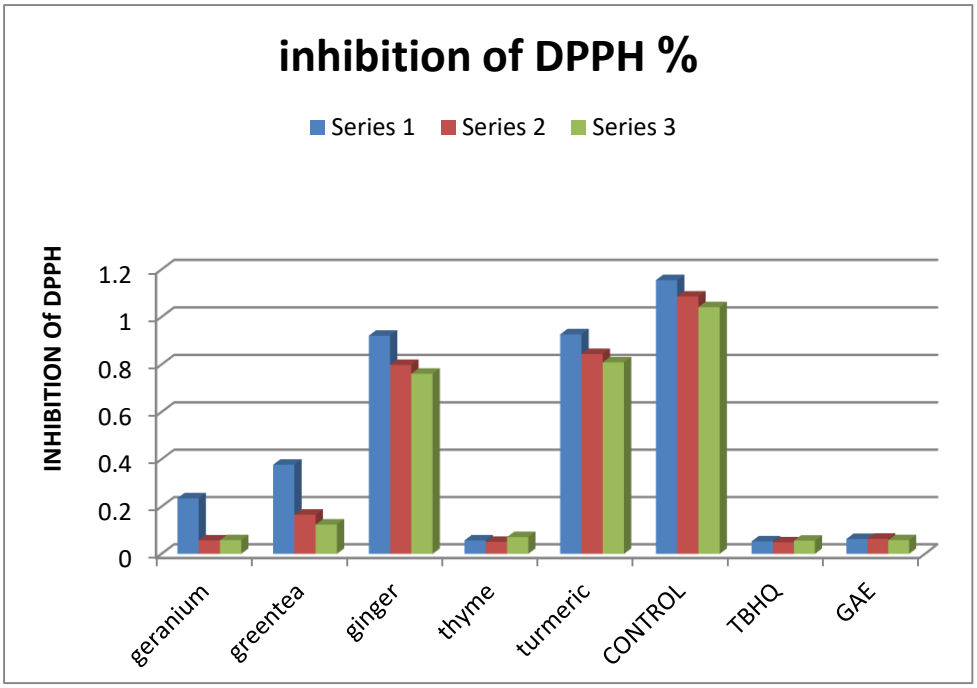

Fig.1. Antioxidant DPPH˙ radical-scavenging activity.

GAE: Gallic acid equivalents.

TBHQ: Tert-butyl hydroquinone.

4. DISCUSSION

L. monocytogenes still occurs at a low prevalence Diego et al., (2014). Biochemical test as catalase, Voges-Proskauer,methyle red and hemolysis test with recorded results agreed with Quinine et al. (2002) and Warburton et al. (2003).

Antimicrobial susceptibility testing showed the highest sensitivity rate of the *Listeria monocytogenes* strains to flurophenicol, cefotaxime and erythromycin this result agreed with Escolar et al. (2017).

The highest intermediate rate of *Listeria monocytogenes* was recorded to amoxicillin and doxycycline of intermediate strains this result disagreed with Vitas etal (2007)

The Zone of Inhibition was obtained for *Listeria monocytogenes* to show antibacterial activity of different plant extracts and antibiotics on it and the results revealed that the diameter Inhibition Zone of ginger(11)mm, disagreed with Hossain et al. (2017), the diameter Inhibition Zone of green tea (13) mm , Inhibition Zone of thyme(14)mm, Inhibition Zone of geranium pelargonium(10)mm disagreed with Ghannadi *et al* (2012) .

Inhibition Zone of the four antibiotics revealed that diameter of Inhibition Zone for *Listeria Monocytogens* ranged from (13-17 mm) around amoxicillin, (11-16 mm) around doxycycline, (12 mm) around gentamicin and (14- 22 mm) around difloxacin, this result agreed with Hossain et al. (2017).

The MIC values for *Listeria monocytogenes* revealed that MIC value of amoxicillin was 0.5 µg/ ml for *Listeria monocytogenes* this result agreed with morvan et al. (2010), (DO) doxycycline was 2 µg/ ml for *Listeria monocytogens*, (CN) gentamicin was 8 µg/ml for *Listeria monocytogenes* and of (INN) Difloxacin was 0. 5 µg/ ml for *Listeria monocytogenes* this result disagreed with (Siporin et al., 1990).

Antibacterial activity of all Antibiotics alone and in combination with plant extracts on *Listeria monocytogenes* revealed that amoxicillin shows synergistic action in combination of with green tea by level (7:3) and by (6:4) this result agreed with Wanda (2018).

Activity of extract compared to unstable oil is probably due to presence of nonvolatile phenol compounds. In addition, some of the compounds with a different polarity, which are present in very small amounts in the extract, are also able to contribute to better an oxidative activity of extract. Some compounds can originate in extract during hydrolysis or other processes of decomposition. Some chemical reactions initiated by heating can also drive up to activities changes of complex extract, composed of a number of compounds with different chemical and physical properties Singh et al. (2005).

The free radical activity of the plant extracts was performed according to the DPPH free radical method, described by Brand-Williams *et al.* (1995).

It can be noticed that the degree of DPPH neutralization depended on incubation time, for all investigated concentrations of oil. The highest degree of DPPH radicals' neutralization is for 60 minutes incubation.

5. Conclusion

The demonstration of synergistic activity by the antibiotic-plant against Gram positive bacteria is an indication that the plant can be a source of bioactive substances that could possess broad spectrum of activity most especially when it is combined with antibiotic. Thus, there is increasing need for researchers

to investigate the synergistic capacity of plants or other natural products, independent of the antimicrobial activity. These findings also suggested that the need for understanding of synergism mechanism is fundamental to development of pharmacological agents to treat diseases by various bacteria using medicinal plants in combination with antibiotics.

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