Original Article

In silico and Molecular Docking Studies of Black Pepper Phyto-constituents against EmrD Efflux Pump of *E. coli*

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ABSTRACT

Multidrug resistance (MDR) bacterial infection is the next pandemic waiting behind the COVID-19 with annual mortality rate 700000 worldwide. Among the MDR bacteria, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococcus faecium* and *Enterococcus faecalis* are showing average resistance of 50 to 80% to ampicillin, amoxicillin, third-generation cephalosporin's and fluoroquinolone and even to combinations antibiotics such as amoxicillin-clavulanic acid. To make the antibiotic resistance issue worst, pharmaceutical industry is reluctant to invest in research and development of new antibiotic typically due to low returns on investment. Accordingly, use of combination of two or more antibiotics or use of the antibiotic adjuvants are only available ways in combatting the ever growing Multidrug resistance (MDR) in bacteria. The present paper is designed to analyze the synergistic potential of black pepper phyto-constituents as the amoxicillin adjuvants in comparison with isolated piperine against the MDR *E. coli*. using *in-silico* molecular docking. The result indicates that binding energy (Kcal/mol) and torsion free energy (Kcal/mol) of piperine (-6.23, +0.89), beta caryophyllene (-6.36, +0.00), beta selinene (-6.93, +0.30), beta-Thujene (-5.42, +0.30) is less for the emrD efflux pump as compared to amoxicillin (-5.85, +2.93) respectively indicating strong inhibition for EmrD of MDR *E-coli* than amoxicillin. The results are also indicating that black pepper extract containing all aforementioned phyto-constituents has synergistic effect in comparison with isolated piperine against the MDR *E. coli*. ADMET of these phyto-constituents also indicates their safety profile in combination with amoxicillin.

Key words: Multidrug resistance (MDR), Piperine, *In silico* molecular docking, Efflux pump, *E. coli*, Black pepper, Amoxicillin.

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INTRODUCTION

The resistance to antibiotics is a natural survival manifestation developed by the bacteria to resist the prejudicial effect of the antibiotics. However, anthropogenic influences, such as antibiotic overuse, inappropriate prescriptions, irresponsible agricultural practices of using farm antibiotics to avoid plant diseases, using antibiotics to treat or prevent diseases in animals, further contributes to the emergence of Multi Drug Resistance (MDR) in bacteria. It is evident that, 65% increase in global antibiotic consumption in humans between 2000 and 2015, 11.5% increase in use of antibiotics in animals between 2017 and 2030 directing global antibiotic consumption to increase by 200 percent between 2015 and 2030.[1,2] Further, MDR bacterial infections are estimated to rise to 10 million by 2050, indicating to surpass cancer morbidity.

According to aforementioned trajectories MDR bacterial infection will be one of the biggest threats to the public health

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with annual mortality rate 700 000 worldwide. Recent reports of WHO indicates that India lead the world in the antibiotic consumption with widespread use of broad-spectrum antibiotics as penicillin's, cephalosporin's and Fluoroquinolones, typically due to its easy accessibility and affordability. To make matters worse, COVID-19 pandemic is paving the way for MDR in bacteria, typically due to increased prescriptions and dispensing of antibiotics, though COVID-19 is caused by SARS-CoV-2 virus and not by bacteria.

Consequently, India support and move the considerable burden of drug-resistant pathogens especially, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococcus faecium* and *Enterococcus faecalis*.[1] As per the Resistance map developed by the center for disease dynamics economics and policy (CDDEP) for India, these bacteria shows average resistance of 50 to 80% to penicillin's like ampicillin, amoxicillin, third-generation cephalosporin's and fluoroquinolone and even to combinations antibiotics such as amoxicillin-clavulanic acid.

The prior studies on bacterial resistance indicates that Gram negative bacteria like *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* develops resistance by limiting uptake of antibiotics in to the cell, inactivation or modification of antibiotics and efflux pump activation. In contrast, Gram positive bacteria like *Enterococcus faecium*, *Enterococcus faecalis* and *Staphylococcus aureus* develops resistance by inactivation or modification of the antibiotics and less likely through activation of efflux pump and limiting uptake of antibiotics.[3-5]

Prior studies on resistance mechanism of bacteria also depicts that majority of the MDR bacteria reduces the efficacy of the antibiotic through efflux pump activation or over-expression. Typically by extruding the antibiotic out of the bacterial cell, thereby reducing the concentration of antibiotics at the site of action required for relevant antibacterial activity.[6,7]

In the year 2010, with the successful launch of Resorine by Cadila Pharmaceutical as anti-tuberculosis drug comprising combination of antibiotics (Rifampicin-200mg, Isoniazid-300mg) and efflux pump inhibitor (Piperine-10mg),[8,9] has created boost in the scientific world to analyze potential of various phyto-constituents of medicinal plants. Scientist from the various parts of the globe started evaluating the efflux pump inhibiting potential of isolated phyto-constituents mainly phenolic, flavonoids and alkaloids such as piperine, curcumin, reserpine, gingerol, shagol, quercetin, rutin and the like. In addition, scientist used computational tools such as *in-silico* molecular docking to identify the efflux pump inhibiting potential of the various phyto-constituents. But even after 10 years from Resorine discovery, no new combination of antibiotic and efflux pump inhibitor has been launched mainly due to complexity of the phyto-constituent structures, its yield after isolation, lack of robustness in the *in-vitro* and *in-vivo* methods. In addition, the prior art is focused only on isolated phyto-constituents and not on the use of combination of phyto-constitutes present in the whole plant.

Accordingly, the present paper is focused on comparative analysis of black pepper phyto-constitutes as *E. coli* efflux pump inhibitors using *in-silico* molecular docking.

MATERIALS AND METHODS

In the present paper, the *in-silico* molecular docking was used as a tool to predict the binding affinity between the phyto-constitutes of the black pepper fruits (Ligand) and the efflux pump of the *E. coli* (Protein) was analyzed.

Ligand Selection

The literature work published in the SOCPUS, Web of Science and PubMed, more than 30% of research papers are exploring either usage of piperine as an efflux pump inhibitor for gram negative bacteria such as *Pseudomonas aeruginosa* and *E. coli* or possible use of black pepper fruit extract and piperine as an antibacterial. In addition, the phyto-constitutes explored in the literature as the efflux pump inhibitor comprises either two co-planner aromatic ring or positively charged nitrogen or carbonyl group separated by hydrophilic and lipophilic moiety.[10,11]

Accordingly, the alkaloids and the phenolic compound present in the fruit of the black pepper such as piperine, beta-thujene, pinene, limonene, beta- caryophyllene, linalool, beta-selinene were selected as Ligands for the *in-silico* molecular docking.[12,13] The chemical structures and the hydrophilic and lipophilic moiety present in the ligand phyto-constituents are shown in Table 1.

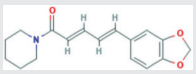
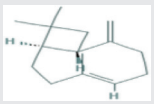
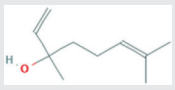
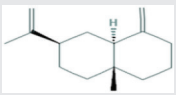
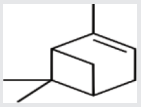
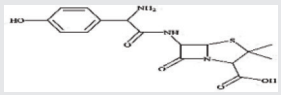
Selection of antibiotic

With reference to the Resistance map developed by CDDEP, 80% to 90% is the resistance rate of *E. coli* to aminopenicillin such as ampicillin and amoxicillin, as well as to the combination of amoxicillin and clavulanic acid. Accordingly, amoxicillin is selected as a ligand for EmrD.

Efflux Pump of the *E-coli* (Protein)

E. coli, comprise 3 efflux pump families namely, the major facilitator superfamily (MFS), the resistance nodulation-cell division (RND) family and the small

Table 1: Structural elucidation of ligands.

Ligand (PubChem CID)	Molecular Structures	Molecular Formula	Molecular weight
Piperine 638024		$C_{17}H_{19}NO_3$	285.34 g/mol
Limonene 22311		$C_{10}H_{16}$	136.23 g/mol
β-Thujene 520384		$C_{10}H_{16}$	136.23 g/mol
β-Caryophyllene 5281515		$C_{15}H_2$	204.35 g/mol
Linalool 6549		$C_{10}H_{18}O$	154.25 g/mol
β-Selinene 348290135		$C_{15}H_{24}$	204.35 g/mol
Pinene 440967		$C_{10}H_{16}$	136.23 g/mol
Amoxicillin 62883		$C_{16}H_{19}N_3O_5S$	365.4 g/mol

multidrug resistance (SMR). Each family comprise of different transporters such as MFS incudes EmrD, mdfA, emrB, RND has acrB, acrF and SMR has emrE (mvrC) and tehA.[14]

Amongst these transporters EmrD is capable to export a broad spectrum of antibiotic molecules from the cell wall of *E. coli*. Therefore, EmrD of *E. coli* with 394 amino acids, 12 transmembrane α-helices and a molecular weight of 42.2 kD is selected for the molecular docking studies.

Preparation Ligands and Protein for Docking

The structure of Ligands with their respective PubChem CID as depicted in Table 1 were saved in SDF format. These structure were converted to PDB format using Pymol software. The 3D structure of EmrD was retrieved using RCSB (http://www.rcsb.org/pdb/) structure file (PDB ID: **2GFP**)

AutoDock 4.2.6 program was used to obtain the docking of EmrD *of E. coli* with the ligands. The implemented empirical free energy function and the Lamarckian Genetic Algorithm (LGA) and the grid maps as depicted in Table 2 was used to obtain binding energy, H. bonds, interacting residues, intermolecular energy and Torsion free energy.

The interactions of complex EmrD and ligand conformations comprising hydrogen bonds and bond lengths were analyzed using Pymol software, UCSF Chimera, Molegro Molecular Viewer and Accelrys DS Visualizer software. Discovery studio 2020 Client and Chimera softwares were also used to depict Hydrogen bonds, 2-D images and protein-ligand interactions images for a visualization of the docking. The best conformation with the lowest docked energy was chosen from the docking search. Number of torsions are chosen from 0-6 and any ligand showed more than 6 was adjusted to 6.

Drug-like properties of the Ligands

Lipinski's filters were used to determine Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) and Drug-like properties of the Ligand Phyto-compounds Piperine, B- thujene, α- and β-pinene, myrcene, α-phellandrene, limonene, B caryophyllene, linalool and B-selinene.

Table 2: Molecular Docking Grid data for EmrD.

Sr. No.	Grid Data	Details
1	PDBID	2GFP
2	Resolution:	3.50 Å
3	Sequence Length	375
4	Grid Point Spacing	1.00 Angstroms
5	Even Number of User-specified Grid Points	50 x-points, 50 y-points, 50 z-points
6	Coordinates of Central Grid Point of Maps	(-4.221, -15.995, 27.731)
7	Minimum coordinates in grid	(-29.221, -40.995, 2.731)
8	Maximum coordinates in grid	(20.779, 9.005, 52.731)

RESULTS

The docking interactions between ligand phyto-compounds, amoxicillin and targeted E-coli EmrD MDR protein are shown in Table 3 and Table 4. It was found that phyto-compound ligands piperine (-6.23kcal/mol), beta caryophyllene (-6.36 kcal/mol), beta selinene (-6.93 kcal/mol), beta-Thujene (-5.42 kcal/mol) requires less binding energy for the EmrD as compared to Amoxicillin (-5.85 kcal/mol). This indicates that the phyto-compounds will bind easily with *E. coli* EmrD as compared to amoxicillin. Consequently, reducing the expulsion of amoxicillin out of the bacterial cell and thereby maintaining the therapeutic concentration of amoxicillin at the site of action required for expected antibacterial activity.[15,16]

The Torsional free energy of the phyto-constitutes typically, piperine (+0.89), beta caryophyllene (+0.00 kcal/mol), beta selinene (+0.30), beta-Thujene (+0.30 kcal/mol) in comparison with amoxicillin (+2.93 kcal/mol) further provides evidence for strong binding affinity with EmrD and phyto-constituents as compared to amoxicillin.

Though phyto constituents such as Limonene (-5.49 kcal/mol), Pinene (-5.69 kcal/mol) showed less binding energy than Amoxicillin (-5.85 kcal/mol), but, the Torsional free energy for these phyto-constituents is +0.00 kcal/mol as compared to amoxicillin +2.93 kcal/mol providing further support for use of combination of phyto-constituents of black pepper with amoxicillin in the management of MDR *E. coli*.

The ADMET properties of the ligands showed that optimum human intestinal solubility (HIA), blood brain barrier (BBB) penetration with no carcinogenicity.

Ligand Protein Interactions

Lipinski's Rule: Drug-like properties of the Phyto-compounds

Lipinski's rule was used to analyze the drug likeness attributes such as human intestinal absorptivity, permeation through Blood Brain Barrier (BBB), Carcinogenicity and Toxicity-Lethal Dose (LD_{50})

The Lipinski Rule depicts the suitability of the drug molecules for oral administered. The Rule is based on the parameters such as hydrogen acceptors less than 10 and hydrogen donors less 5, molecular weight should be more than 500 daltons and partition coefficient (log P) should not be less than 5. The Ligand Properties as per Lipinski's rule and ADMET are shown in Table 5 and 6 respectively.

Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties, as derived from AdmetSAR reveal that the ligands have optimum Human Intestinal Absorption (HIA) score and significantly low carcinogenicity and BBB penetration potential directing towards safe use of combination of amoxicillin and whole black pepper extract in the management of MDR *E. coli*.[17-19] The LD_{50} doses of all the ligands indicates that Piperine, (2.7129 mol/kg) and Amoxicillin (1.7036) has greater oral toxicity compared to the other ligands.

DISCUSSION

The emergence of MDR bacteria typically, gram negative bacteria has mushroomed with discovery and development of every new class of antibiotics. In the year 2019, WHO had directed about emergence of antibiotic pandemic due to steady increase in morbidity and mortality due to MDR bacterial infections.[1,20] WHO report is also imperative to indicate that the speed at which the bacteria are showing the resistance to available antibiotics is not matching with the discovery of new antibacterial which might lead to no-antibiotic era. In order to resolve the problem of limitation to develop a new broad spectrum antibiotics, combinations of two or more antibiotics with different mechanism of action or use of antibiotic adjuvant like beta lactamase was preferred in the management of MDR infections. Though the combination therapy provides a broader antimicrobial spectrum, synergistic effects but it is equally contributing to the reoccurrence of MDR infections.[21,22]

Moreover, study of Intrinsic and extrinsic mechanism developed by bacteria indicates that overexpressed efflux pumps is one of the main survival weapon. It is apparent that the identification of antibiotic adjuvant having capability to inhibit the efflux protein without imparting any additional antibacterial activity is required.[23-27]

The results of interaction between EmrD and Phyto-compound ligands clearly depicts combination of black pepper phyto-constituents has synergistic effect

Table 3: Docking interaction between EmrD and Ligand.

Protein Name EmrD	Ligand Name	Binding Energy (kcal/mol)	No. of H Bonds	Interacting residue	Final Intermolecular Energy (kcal/mol)	vdW + Hbond + desolv Energy (kcal/mol)	Electrostatic Energy (kcal/mol)	Torsional Free Energy (kcal/mol)
2GFP	Limonene	-5.49	00	ILE100, ILE31, MET34, ILE26, TRP161	-5.79	-5.79	+0.00	+0.30
	Piperine	-6.23	01 (H1:Distance=3.19 Å)	GLN21(H1), VAL143, LEU303, TYR56, MET139	-7.04	-7.03	-0.01	+0.89
	β-Thujene	-5.42	00	ALA30, ILE26, TRP161, TYR165, MET84, ALA23	-5.70	-5.69	-0.00	+0.30
	β-Caryophyllene	-6.36	00	LEU53, LEU220, VAL299, ALA224, LEU303, TYR52, PHE249, TRP300, VAL296, VAL245, ALA228	-6.36	-6.35	+0.00	+0.00
	Linalool	-4.42	01 (H1:Distance=2.34 Å)	TYR52(H1), ILE28, PHE249, LEU53, ALA224, VAL296, VAL299, ALA221, TRP300, LEU220, LEU303, PHE249	-5.90	-5.85	-0.04	+1.49
	β-Selinene	-6.93	00	MET49, ALA224, ALA221, LEU53, ILE28, LEU220, VAL296, VAL245, ALA228	-7.23	-7.23	-0.00	+0.30
	Pinene	-5.69	00	ALA23, ALA30, TRP161	-5.69	-5.69	+0.00	+0.00
	Amoxicillin	-5.85	03 (H1:Distance=2.97 Å, H2:Distance=2.23 Å, H3:Distance=1.63 Å)	GLN60(H1), LEU61(H2), ASP190(H3)	-6.64	-4.16	-2.48	+2.09

as compared to isolated piperine. Further, the ADMET analysis shows that these phyto-compounds of black pepper are safe for oral administration especially with reference to human Intestinal absorptivity, possibility of crossing BBB and toxicity (LD_{50}).

CONCLUSION

The present study of *in-silico* molecular docking provides evidence that phyto-compounds of black pepper binds to efflux pump EmrD of *E. coli* responsible for reducing the antibacterial potential of amoxicillin.[28-30] Further, *present study* provide additional properties such as drug-likeness, ADMET prediction and toxicity analysis, which could help in developing non-toxic and effective combination formulation of phyto-compounds and amoxicillin.[31,32]

Accordingly, in the present paper *in silico* molecular docking approaches is used to provide reliable predictions as well as new knowledge on the Emrd inhibition potential of phyto-constituents of black pepper and using these phyto-constituents as adjuvants[33,34] for amoxicillin.

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CONFLICT OF INTERST

The authors declare no conflict of interest

ABBREVIATIONS

MDR: Multi Drug Resistance; **RND:** Resistance nodulation-cell division; **MFS:** Major facilitator superfamily; **ADMET:** Absorption, Distribution, Metabolism, Excretion and Toxicity; **LGA:** Lamarckian Genetic Algorithm.

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Table 4: Multidrug resistance associate protein (PDB ID: 2GFP) docking with Legand.

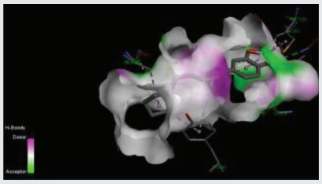
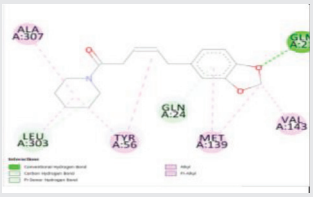
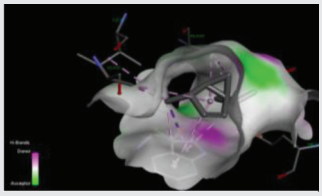
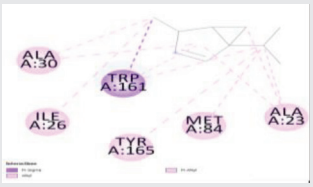
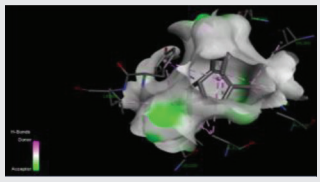
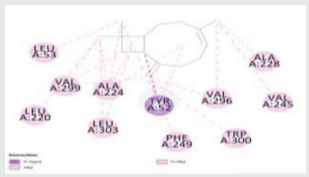
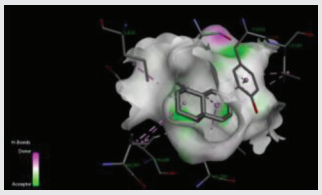
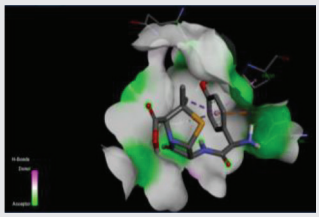
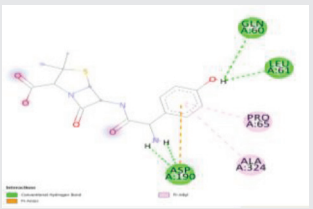
Name of the protein	Name of the Ligand	Ligand Protein Binding (H-bondinteractions)	Ligand- Amino Acid Binding 2D interaction patterns
Multidrug resistance associate protein (PDB ID: 2GFP)	Piperine		
	β-Thujene		
	β-Caryophyllene		
	β-Selinene		
	Amoxicillin		

Table 5: Ligand Properties as per Lipinski's rule.

Sr. No.	Ligand Name	Molecular Weight in g/mol (<500)	LogP (≤5)	Hydrogen Bond Donor (≤5)	Hydrogen Bond Acceptor (≤10)	Rotatable Bond Count
1	Piperine	285.34 g/mol	3.5	0	3	3
2	limonene	136.23 g/mol	3.4	0	0	1
3	β-Thujene	136.23 g/mol	3.4	0	0	1
4	β-Caryophyllene	204.35 g/mol	4.4	0	0	0
5	Linalool	154.25 g/mol	2.7	1	1	4
6	β-selinene	204.35 g/mol	5.4	0	0	1
7	Amoxicillin	365.4 g/mol	-2	4	7	4
8	Pinene	136.23 g/mol	3.1	0	0	0

Table 6: ADMET properties of the Ligands.

SN	Compound	HIA	BBB	Carcinogenicity	LD_{50} in Rat (mol/kg)
1	Piperine	0.9964	1.0000	Non-carcinogens	2.7129
2	Limonene	0.9444	0.9887	Non-carcinogens	1.4819
3	B-thujene	0.9804	0.9973	Non-carcinogens	1.6406
4	B-caryophyllen	0.9564	0.9923	Non-carcinogens	1.4345
5	B-selinene	0.9732	0.9947	Non-carcinogens	1.4979
6	Pinene	0.9628	0.9942	Non-carcinogens	1.4350
7	Amoxicillin	0.9967	0.9008	Non-carcinogens	1.7036

susceptibility profiles of Escherichia coli Strains lacking multidrug efflux pump genes. Antimicrob Agents Chemother. 2001;45(4):1126-36. doi: 10.1128/AAC.45.4.1126-1136.2001, PMID 11257026.

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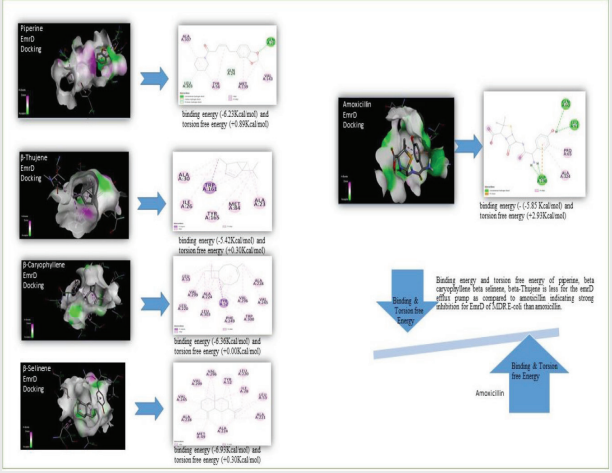
PICTORIAL ABSTRACT



SUMMARY

The present paper is aimed to evaluate the potential of various phenolic phyto-compounds of black pepper as the amoxicillin adjuvants in comparison with isolated piperine against the MDR *E. coli*. using *in-silico* molecular docking. The result indicates that binding energy (Kcal/mol) and torsion free energy (Kcal/mol) of piperine (-6.23, +0.89), beta caryophyllene (-6.36, +0.00), beta selinene (-6.93, +0.30), beta-Thujene (-5.42, +0.30) is less for the emrD efflux pump as compared to amoxicillin (-5.85, +2.93) respectively indicating strong inhibition for EmrD of MDR *E. coli* than amoxicillin. The results are also indicating that black pepper extract containing all aforementioned phyto-constituents has synergistic effect in comparison with isolated piperine against the MDR *E. coli*.

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