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THE ROLE OF IN BOOSTING ANTIBIOTIC ACTION AGAINST IRRADIATED RESISTANT BACTERIA THROUGH NATURAL OUTER MEMBRANE PERMEABILIZERS

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Abstract:

Objective: *The objective of this research is to bring out novel approaches in order to revert pathogenic Gram-negative bacilli resistance by combining potent permeabilizers, conventional antibiotics and natural beta lactamase inhibitors increasing various antibiotics activity.*

Methods: *This research was carried out at Services Hospital, Lahore from August 2018 to June 2019. Disk diffusion along with susceptibility assays was carried out for the antibiotic susceptibility in the presence phytochemicals tested concentrations of natural non-antibacterial. Gallic acid and Thymol were potentially facilitated and permeabilizers the antibiotics passage through outer membrane with the ability of release of LPS, Triton X-100 and sensitize bacteria to SDS.*

Results: *Natural beta lactamase inhibitors and Permeabilizers combination with antibiotics initiated more resistant isolates susceptibility than antibiotic management through beta lactamase inhibitors alone. Distinct effects were also identified with 24.4 Gy in vitro gamma irradiation on beta lactamase activity, permeability barrier and outer membrane protein profiles of assessed isolates.*

Conclusions: *Synergistic effects of researched antibiotics and natural phytochemicals leads to novel clinical varieties through outer membrane destabilization or beta lactamase enzyme inactivation; it enables the use of most cost-effective and older antibiotics against resistant strains.*

Keywords: *Outer Membrane Permeability, Pathogenic, Beta Lactam Resistance, Gram-Negative Bacilli, Natural Beta Lactamase, Permeabilizers, Inhibitors and Gamma Irradiation.*

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INTRODUCTION:

Global health is affected through increased onset of antibiotic-resistant bacteria. Gram-negative bacteria are vital pathogenic bacteria that possess an outer membrane (OM) which is unique in nature. It also makes gram-negative bacteria resistant to numerous antimicrobial agents. Lipopolysaccharide (LPS) layer prevents the entrance of Hydrophilic antibacterial agents from outer membrane and basic phospholipids; whereas, Outer membrane proteins also exclude hydrophobic agents [1]. LPS is also known as endotoxins forms the major component of outer membrane leaflet of the Gram-negative bacteria. LPS also forms an infusion barrier which is essentially drug resistance [2]. For improvement of antibiotics efficacy, it is essential to search for the approaches which improve antibiotic diffusion and also bypass barrier of bacterial membrane, which plays a role in Gram-negative bacteria general antibiotic resistance. Permeabilizers weakens the outer membrane and it can also enhance bacterial cells permeability for exogenous products which includes antimicrobial agents. The use of outer membrane permeabilizers with antibiotics provides control over development of Gram-negative bacteria [3 – 6].

β-lactamases commonly cause bacterial resistance to antimicrobial agents of β-lactam [7]. β-lactamases intermingle with β-lactam antibiotics as a reaction which is an outcome of antibiotic hydrolysis forming an inactive chemical material without bacterial activity properties [8]. Enzyme inhibitors function to inactivate β-lactamases in the periplasmic space. Therefore, it protects familiar β-lactam antibiotics from hydrolysis by broad-spectrum β-lactamases or penicillinases. However, recent years have shown an increase in the bacterial resistance for such suicide inhibitors which may have an association with frequent use. Moreover, ESBL increases resistance against numerous bacterial infections. Thus, efforts are made to identify β-lactamases inhibitors in order to prevent β-lactams inactivation through β-lactamases [3, 9]. The objective of this research is to bring out novel approaches in order to revert pathogenic Gram-negative bacilli resistance by combining potent permeabilizers, conventional antibiotics and natural beta lactamase inhibitors increasing various antibiotics activity.

METHODS:

This research was carried out at Services Hospital, Lahore from August 2018 to June 2019. Disk diffusion along with susceptibility assays was carried out for the antibiotic susceptibility in the presence phytochemicals tested concentrations of natural non-antibacterial. Gallic acid and Thymol were potentially facilitated and permeabilizers the antibiotics passage through outer membrane with the ability of release of LPS, Triton X-100 and sensitize bacteria to SDS. We carried out antibacterial and isolation susceptibility test of pathogenic gram-negative bacilli Bacterial isolation for various samples through Kirby-Bauer method. Various methods were also used for the ESBL activity detection such as Double disk synergy tests, modified double-disk synergy tests, Nitrocef disks method and Phenotypic confirmatory tests [10 – 17]. Experiments were conducted on fifteen previously identified bacterial isolates which represented different species such as Escherichia coli, Pseudomonas fluorescens, Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloacae and Enterobacter sakazakii in order to differentiate between β-lactamase producer & non-producer isolates [18 – 24]. We also study source of irradiation, in vitro gamma irradiation effects on assessed multidrug-resistant isolates, minimum inhibitory concentrations determination for the selected antibiotics and lipopolysaccharide release. OM components release was assessed through silver staining through permeabilizers and radiation [gallic acid (600 µg/mL), thymol (500 µg/mL), chitosan (100 ppm) and EDTA (0.1 mM)].

Paired T-test, One-way ANOVA and Chi-Square test were used for statistical analysis. SAS software was used for the calculation of Mean and SD values.

RESULTS:

Natural beta lactamase inhibitors and Permeabilizers combination with antibiotics initiated more resistant isolates susceptibility than antibiotic management through beta lactamase inhibitors alone. Distinct effects were also identified with 24.4 Gy in vitro gamma irradiation on beta lactamase activity, permeability barrier and outer membrane protein profiles of assessed isolates. Isolates were screened for ESBL for various bacterial species. Detailed outcomes about screening for extended-spectrum β-lactamases, examination of antibacterial activity of some selected permeabilizers, evaluation of synergistic interaction, effect of in vitro gamma irradiation on MIC, evaluation of MICs of certain antibiotics alone, MICs assessment, effects of different lytic agents and evaluation of Beta-lactamase assays are given in the tabular data.

Table – I: Screening for extended-spectrum β-lactamases

Isolates No	Bacterial Species	DDST Result	MDDT Result	CFP-SCF	PRL-TZP	Nitrocefin Test	Production of Beta-Lactamase
1	Escherichia coli	-	-	-	-	-	Non producer
2	Escherichia coli	+	+	+	+	+	Producer
3	Escherichia coli	-	-	-	-	-	Non producer
4	Acinetobacter baumannii	+	+	+	+	+	Producer
5	Acinetobacter baumannii	-	-	-	-	-	Non producer
6	Acinetobacter baumannii	-	-	-	-	-	Non producer
7	Pseudomonas spp.	-	-	-	-	-	Non producer
8	Pseudomonas spp.	+	+	+	+	+	Producer
9	Pseudomonas spp.	+	+	+	+	+	Producer
10	Pseudomonas spp.	-	-	-	-	-	Non producer
11	Pseudomonas spp.	-	-	-	-	-	Non producer
12	Klebsiella pneumoniae	-	-	-	-	-	Non producer
13	Enterobacter spp.	+	+	+	+	+	Producer
14	Enterobacter spp.	-	-	-	-	-	Non producer
15	Enterobacter spp.	-	-	-	-	-	Non producer

Table – II: Examination of antibacterial activity of some selected permeabilizers

Permeabilizers conc		Escherichia coli			Acinetobacter baumannii			Pseudomonas spp.					Klebsiella pneumoniae	Enterobacter spp.		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Gallic acid	500 µg/ml	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	600 µg/ml	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	700 µg/ml	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ellagic acid	30 µM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	40 µM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	50 µM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Thymol	400 µg/ml	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	500 µg/ml	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	600 µg/ml	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
Chitosan	50 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	100 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	250 ppm	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
EDTA	0.1 mM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.5 mM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1 mM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbic acid	2 mM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5 mM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10 Mm	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+

Table – III: Evaluation of synergistic interaction

Permeabilizers a Antibiotics (Ab)		**Abalone**	**ECG**	**ECG + GA**	**ECG + T**	**Quercetin**	**Q + GA**	**Q + T**	**Permeabilizers b Antibiotics (Ab)**	**Abalone**	**Gallic acid**	**Thymol**
Escherichia coli [2]	PRL ≥21	6/R	6/R	20/M	23/S	15/R	19/M	24/S	TZP ≥21	14/R	20/M	26/S
	CFP ≥21	6/R	6/R	16/M	21/S	16/M	21/S	25/S	SCF ≥22	14/R	19/M	20/M
Acinetobacter baumannii [4]	PRL	6/R	6/R	19/M	21/S	16/R	24/S	21/S	TZP	15/R	20/M	21/S
	CFP	6/R	6/R	23/S	20/M	16/M	20/M	22/S	SCF	14/R	22/S	23/S
Pseudomonas aeruginosa [8]	PRL	20/R	12/R	16/R	25/S	18/S	22/S	20/S	TZP	17/R	23/S	26/S
	CFP	10/R	10/R	15/R	21/S	17/M	20/M	22/S	SCF	15/R	25/S	21/M
Pseudomonas aeruginosa [9]	PRL	6/R	6/R	18/S	19/S	12/R	19/S	21/S	TZP	11/R	22/S	22/S
	CFP	6/R	6/R	22/S	25/S	15/R	22/S	24/S	SCF	12/R	22/S	25/S
Enterobacter cloacae [13]	PRL	6/R	6/R	20/M	23/S	15/R	20/M	24/S	TZP	20/M	30/S	27/S
	CFP	6/R	6/R	18/M	21/S	18/M	20/M	23/S	SCF	18/M	25/S	23/S

Table – IV: Effect of in vitro gamma irradiation on MIC

Antibiotics Bacterial species		**MICs (mg/L) before irradiation**			**MICs (mg/L) after irradiation**		
		Ab-Alone	**+Gallic acid**	**+Thymol**	**Ab-Alone**	**+Gallic acid**	**+Thymol**
Escherichia coli [3]	CTX	64	8	4	64	8	4
	CFP	64	16	16	128	32	32
	E	128	32	32	256	64	64
Acinetobacter baumannii [5]	CTX	32	8	8	32	8	8
	CFP	32	8	8	64	16	16
	E	64	32	16	128	64	32
Pseudomonas aeruginosa [10]	CTX	128	32	32	128	32	32
	CFP	128	32	32	256	64	64
	E	128	128	64	256	256	128
Enterobacter sakazakii [14]	CTX	8	2	4	8	2	4
	CFP	32	16	8	32	16	8
	E	64	16	16	64	16	16

Table – V: Evaluation of MICs of certain antibiotics alone and with beta lactamase inhibitors alone

Antibiotic (Ab) Bacterial beta lactamase producer		MICs (mg/L) before irradiation					MICs (mg/L) after irradiation				
		Abal one	+E CG	ECG + Gal lic aci d	ECG+Th ymol	antibioti c disc with betalacta mase inhibitor s	Ab-Alo ne	+E CG	ECG+G arlic acid	ECG+Th ymol	antibioti c disc with betalacta mase inhibitor s
Escheric hia coli [2]	C FP	64	64	8	4	a32	64	64	8	4	a32
	P R L	128	128	16	4	b16	128	128	16	4	b16
Pseudo monas aerugino sa [8]	C FP	128	128	16	8	64	128	128	16	8	64
	P R L	128	128	8	8	64	128	128	8	8	64
Enteroba cter cloacae [13]	C FP	32	32	4	4	16	64	64	8	8	32
	P R L	64	64	4	4	0.5	128	128	8	8	1

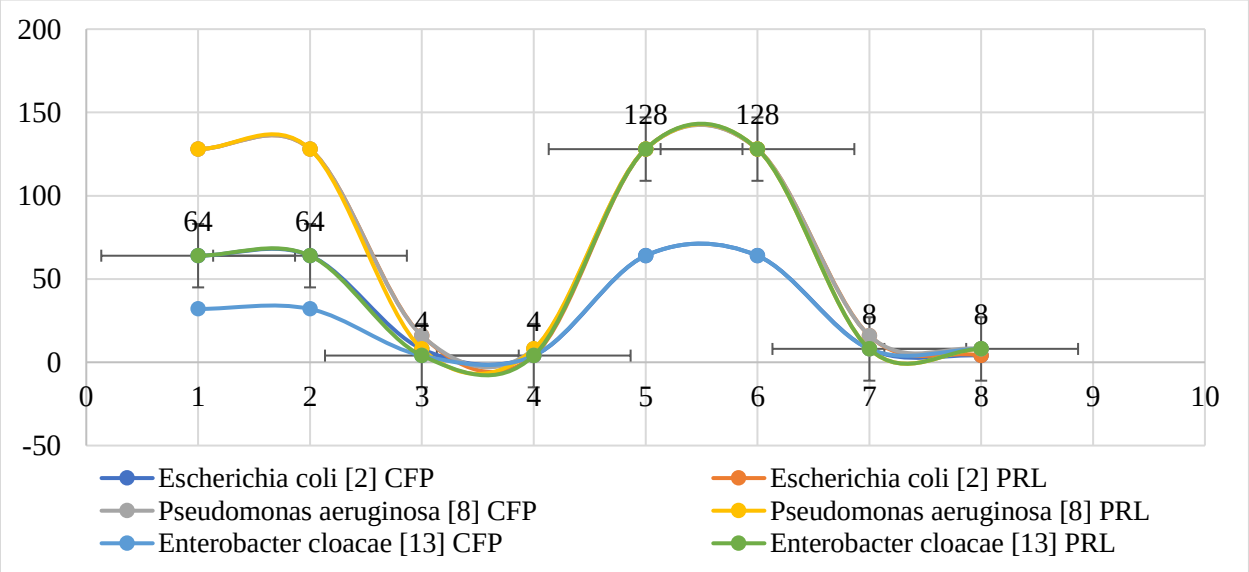

Table – VI: Evaluation of MICs

Antibiotics (Ab) Bacterial beta lactamase producer		MICs (mg/L) before irradiation					MICs (mg/L) after irradiation				
		Abalone	+Q	Q + Gallic acid	Q + Thymol	Antibiotic disc with betalactamase inhibitors	Abalone	+Q	Q + Gallic acid	Q + Thymol	antibiotic disc with betalactamase inhibitors
Escherichia coli [2]	CFP	64	32	8	2	a 32	64	32	8	2	32
	PRL	128	32	8	4	b16	128	32	8	4	16
Pseudomonas aeruginosa [8]	CFP	128	32	8	2	64	128	32	8	2	64
	PRL	128	64	8	8	64	128	64	8	8	64
Enterobacter cloacae [13]	CFP	32	16	8	4	16	64	64	32	8	32
	PRL	64	32	2	1	0.5	128	64	4	2	1

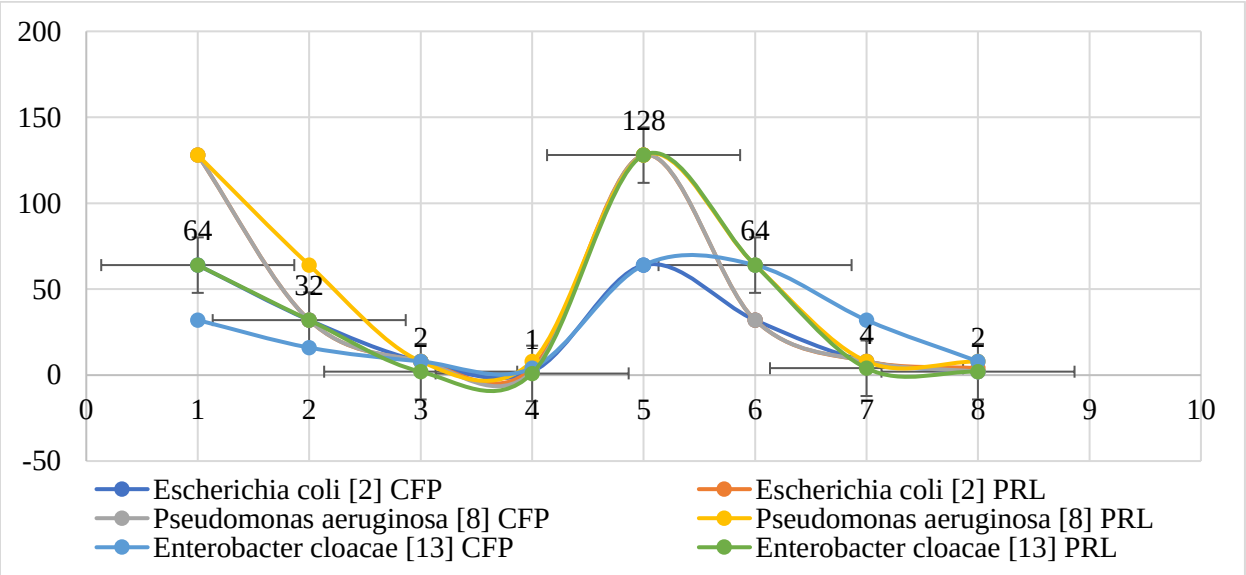

Table – VII: Effects of different lytic agents

Lytic agent	Conc.	Relative turbidity (%) P. aeruginosa$_{10}$ / E. coli$_{3}$			
		Control-	Gallic acid	Thymol	Radiation
Lysozyme	10 µg/mL	100 ± 1 /101 ± 1	99 ± 1/ 92 ± 1	100 ± 1/ 98 ± 0.6	100 ± 0.6/ 101 ± 2
Triton X-100	0.10%	100 ± 2 /103 ± 3	93 ± 3/86 ± 3	96 ± 1/ 90 ± 3	100 ± 1/ 101 ± 1.2
Triton X-100	1%	99 ± 3 /105 ± 0.6	81 ± 1/77 ± 2	84 ± 3/ 74 ± 4	99 ± 2/ 103 ± 3
SDS	0.10%	92 ± 1 /91 ± 2	58 ± 2/ 60 ± 1	60 ± 3/ 59 ± 3	92 ± 1/ 91 ± 5
SDS	1%	78 ± 0.6 / 80 ± 2	26 ± 1/ 42 ± 2	28 ± 2/ 50 ± 0.6	79 ± 2/ 82 ± 2

Table – VIII: Evaluation of Beta lactamase assays

Conc	Control	aCefoperazone		aCefoperazone + thymol		aCefoperazone+thymol+quercetin	
		Before	After	Before	After	Before	After
128/64	0.271/0.273	0.123 ± 0.001/	0.142 ± 0.001/	0.037 ± 0.002/	0.044 ± 0.0006/	0.196 ± 0.002/	0.211 ± 0.0006/
		0.148 ± 0.001	0.161 ± 0.002	0.049 ± 0.006	0.053 ± 0.002	0.215 ± 0.001	0.221 ± 0.001
64 /32	0.263 /0.263	0.119 ± 0.0006/	0.133 ± 0.003/	0.030 ± 0.001/	0.040 ± 0.001/	0.183 ± 0.003/	0.203 ± 0.001/
		0.139 ± 0.002	0.160 ± 0.001	0.048 ± 0.001	0.050 ± 0.0005	0.206 ± 0.001	0.213 ± 0.0006
32/16	0.256/0.258	0.115 ± 0.001/	0.130 ± 0.0006/	0.02 ± 0.001/	0.039 ± 0.0006/	0.179 ± 0.001/	0.20 ± 0.006/
		0.128 ± 0.0006	0.148 ± 0.002	0.045 ± 0.001	0.048 ± 0.002	0.197 ± 0.001	0.203 ± 0.001

DISCUSSION:

Gram-negative bacteria are the primary cause of nosocomial infections. As a result of acquired and intrinsic capabilities they develop resistance to antimicrobial agents and their management is also difficult [25]. We can explain the spread and emergence MDR among Gram-negative bacilli which include Pseudomonas, Enterobacteriaceae and Acinetobacter species through higher antibiotic resistance levels which generally do not attribute in intrinsic bacterial resistance alone [26]. Ability of ESBL production in the tested isolates alters as an outcome of gamma irradiation when exposed to in vitro gamma irradiation in MDDT, DDST and β-lactamase assays [27]. Radiation effects on ESBL production are significant in nature attributed to genes encoded ESBL production dominantly found in plasmids. Possibly, permeabilizers itself do not inherit bactericidal, but they may initiate related materials activity with synergetic actions [28]. Therefore, our objective of this research is to bring out novel approaches in order to revert pathogenic Gram-negative bacilli resistance by combining potent permeabilizers, conventional antibiotics and natural beta-lactamase inhibitors increasing various antibiotics activity. The combinations may also reduce drugs toxicity by avoiding the emergence of resistant variants arising in the course of treatment having synergistic effects to combat Gram-negative bacteria-induced infections [29]. Our outcomes demonstrated natural materials with increased target strains susceptibility to different antibiotics which include azithromycin, erythromycin, nitrofurantoin, novobiocin and sulphamethoxazole trimethoprim. These outcomes also highlight OM barrier which was disturbed by permeabilizers that enhanced antibiotics effects in a significant way. The test of thymol and gallic acid shows significant synergistic activities having various antibiotic classification resulting in enhanced activity and minimum antibiotic effects against species of Gram-negative bacterial. These outcomes are consistent with outcomes of another study conducted on Gallic acid [30]. Thymol is also effective for the microorganisms by lipophilic action on cellular membrane which causes polypeptide chains dispersion of cellular membrane and destabilizing cell membrane permeability. Thymol also carries outer membrane disintegrating properties with LPS release enhancing effects. With low levels of thymol, the lipid profile can be adapted for membrane in terms of structure and function [31]. In beta lactamase and MIC assays, permeabilizers combinations (Gallic acid & thymol) with beta-lactamase inhibitors and antibiotics showing high rates of permeability, higher overall bacteria susceptibility and low MIC values to antibiotics alone treatment or antibiotics with the combination of natural β-lactamase inhibitors which is also consistent with previous study conducted on the same topic [32].

Epigallocatechin gallate showed no intrinsic effect on ESBL produced by Gram-negative bacteria; however, when combined with permeabilizers facilitating the entrance across Gram-negative bacterial OM which acts as potential inhibitor. Variations in the combinations may also explain the differences which have confirmed cellular enzymes cellular locations. Reduced Gram-negative bacteria catechin susceptibility may least partially contribute to lipopolysaccharide presence which holds the role of barrier [33]. Features and presence LPS molecules in outer membrane leaflet in Gram-negative bacteria with inherited hydrophobic antibiotics resistance such as novobiocin and macrolides along with detergents such as SDS, Triton X-100 and bile salts. Thymol activity and Gallic acid contribution as confirmed membrane permeabilizers in this research through permeability assay. Both permeabilizers significantly increase OM permeability to lytic agents in assessed bacterial isolates ($P < 0.001$) as stated in other studies [34].

β-lactam antibiotics efficacy against Gram-negative bacteria depends on penetration rates across OM and

β-lactamase inactivation resistance degree. Slow penetration through OM would not suffice to build up an effective concentration of periplasm. The explanation for the reduced cefoperazone antibioticin TMB solution absorbance in selected β-lactamase presence which is attributed to extracellular enzyme availability, which was presumed initially to stem from broken and lysed cells. This extracellular activity also inactivates β-lactam antibiotic by opening the ring of β-lactam [35 – 36]. Three types of DNA damage are induced by Gamma radiation which includes single, double and nucleotide strand damage including base and sugar moiety damage. Base damage is primary in nature which is initiated through ionizing radiation. Gamma irradiation affects enzymes and protein fingerprinting. The outcomes were in the form of partially damaged plasmid DNA; this radiation may also genes expression including encoding of genes. This was also reflected with an increase in the related resistance than non-irradiated sources [37].

CONCLUSIONS:

This research recommends that gallic acid and thymol are potential outer membrane disintegrating agents with the evidence of LPS release ability and sensitize Gram-negative bacilli for various lytic agents. Different natural compounds are also active as β-lactamase inhibitors which may also develop β-lactamase inhibitors. MICs determination for cefoperazone, cefotaxime and erythromycin before and after irradiation presented an increase in the MIC of erythromycin and cefoperazone after irradiation in order to double its value with Acinetobacter baumannii, Escherichia coli and Pseudomonas aeruginosa. Synergistic effects of researched antibiotics and natural phytochemicals leads to novel clinical varieties through outer membrane destabilization or beta-lactamase enzyme inactivation; it enables use of most cost-effective and older antibiotics against resistant strains.

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