

Synergistic Interaction of Glycyrrhizin with Norfloxacin Displays ROS-Induced Bactericidal Activity against Multidrug-Resistant *Staphylococcus aureus*

Vigyasa Singh^{1,2}, Anirban Pal¹ and Mahendra P. Darokar^{1,*}

¹ Molecular Bioprospection Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, India

² Department of Pharmacology & Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ 85721, USA

* Correspondence: mp.darokar@cimap.res.in; Tel.: +91-522-2718532; Fax: +91-522-2342666

Materials and methods

Bacteriolysis assay

The effect on cell walls was evaluated using the method described previously [1]. *S. aureus* cells were inoculated in 10 mL MHB with incubation at 37°C for 18h. the bacterial cells were harvested by centrifugation at 10,000 rpm for 5 min at 4°C. Resuspension was conducted with sterile normal saline to adjust the concentration up to approximately 10⁸ CFU/mL. The inocula were treated with Gly at different concentrations (MIC, 2MIC, and 4MIC) alone and in combination with Nor. The suspensions were thoroughly vortexed and observation was carried out by taking OD at 620 nm at different intervals of time—0, 4, 8, 16, and 24 h—and then we proceeded with agitation and incubation at 37°C.

DPPH (1, 1-diphenyl 2-picryl hydrazyl) assay

The DPPH radical scavenging activity of Gly and GlyNor was determined spectrophotometrically [2]. Different concentrations of Gly and GlyNor were incubated with DPPH for 30 min at 37°C. The decrease in DPPH absorption at 517 nm was measured 10 min later. Ascorbic acid was used as a standard. The experiments were conducted in triplicate.

FRAP (Ferric ion-reducing antioxidant power) assay

A FRAP assay was conducted by following the method described earlier [3]. Freshly prepared stock solutions of acetate buffer (300 mM): TPTZ (10 mM): FeCl₃.6H₂O (20 mM) were used. The working suspension was mixed with FRAP solution and incubated at 37°C for 30 min. The percentage of reduction was observed after the treatment of Gly and GlyNor at different concentrations. The percentage reduction was measured at 593 nm. The experiment was performed in triplicate.

SOD (Superoxide dismutase) assay

The estimation of the superoxide radical scavenging activity was measured according to the method described previously [4]. A reaction mixture was prepared using 2 mM riboflavin, 12 mM EDTA solution, and 75 mM NBT and methanol. This reaction mixture was diluted in 50 mM phosphate buffer. The inhibition of NBT reduction was observed after the treatment of Gly and GlyNor. The scavenging activity was observed by measuring the absorbance at 560nm using a spectrophotometer. The assay was run in triplicate.

Results and Discussion

Bacteriolysis assay

Bacteriolysis assays are mainly performed to evaluate the effect of antibacterial agents on bacterial cell walls, wherein the decrease in absorbance at 620 nm indicates cell

wall damaging potential. Intact cells will give higher absorbance [1]. The Gly-treated bacterial cells generated high OD values up to 8 h of treatment and subsequently decreased up to 24 h of treatment in comparison to their respective untreated control (Figure S1). Although several antibacterial phytochemicals have been reported to possess bacterial cell wall damaging potential, many others either did not exhibit effects on cell wall or showed unusual observations [5, 6].

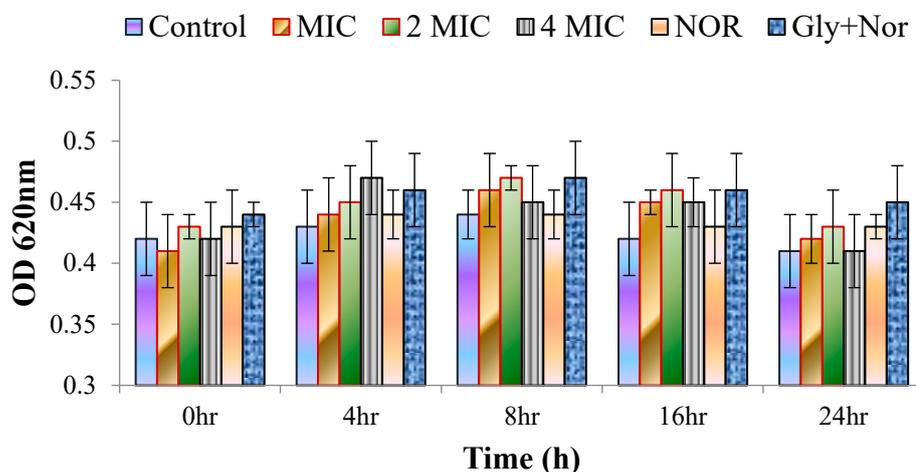


Figure S1. Effect of various concentrations of Gly on cell wall. Gly alone and in combination with Nor at different time intervals. Data are expressed as mean \pm SEM.

Antioxidant property of Glycyrrhizin

Gly was also evaluated for its antioxidant activity and it was observed that it exhibited free radical-scavenging effects at lower concentrations of 1/16MIC, 1/8MIC, 1/4MIC, and 1/2MIC. Antioxidant activity was also evaluated in combination with Nor. Gly was evaluated for its antioxidant potential using a DPPH assay. It was observed that percentage reductions of 4.21%, 17.31%, and 15.34% were observed at 1/16MIC, 1/8MIC, and 1/4MIC, respectively. GlyNor showed 19.42% percent reduction at 1/4MIC, whereas ascorbic acid, a positive control, showed 27.74% reduction at 100 μ g/ml (Table S1). In the FRAP assay, a dose-dependent effect was observed in the case of Gly's reducing ability at values of 5.78%, 17.12%, 13.26%, and 11.43% at 1/16MIC, 1/8MIC, 1/4MIC, and 1/2MIC concentrations, respectively, whereas for GlyNor, this it was found to be 17.51% at 1/4MIC. In the case of the positive control, ascorbic acid, the reducing ability was observed to be 19.68% at 100 μ g/ml (Table S1). For the SOD assay, in the case of Gly alone, the inhibition was observed to be 5.49%, 11.41%, and 9.57% at 1/16MIC, 1/8MIC, and 1/4MIC, respectively. The inhibition of 9.68% was observed in the case of GlyNor, whereas, in the case of the ascorbic acid positive control, an inhibition of 30.52% was observed at 100 μ g/ml (Table S1).

Table S1. Antioxidant activity of Gly (percent inhibition).

Concentration	DPPH assay	FRAP assay	SOD assay
1/16MIC	4.21 \pm 1.03	5.78 \pm 1.50	5.49 \pm 1.2
1/8MIC	17.31 \pm 1.0	17.12 \pm 1.20	11.41 \pm 1.0
1/4MIC	15.34 \pm 1.12	13.26 \pm 0.78	9.57 \pm 1.04
1/2MIC	6.78 \pm 1.07	11.43 \pm 0.64	7.50 \pm 1.2
GlyNor	19.42 \pm 1.0	17.51 \pm 1.20	9.68 \pm 1.0
Ascorbic acid	27.74 \pm 1.15	19.68 \pm 1.02	30.52 \pm 1.1

Data represented as mean \pm SEM.

References

74. Carson CF., Mee BJ., Riley TV. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrob. Agents Chemother.* 2002; 46, 1914-1920.
75. Bondet, V.; Brand-Williams, W.; Berset, C. Kinetics and mechanisms of antioxidant activity using the DPPH free radical method. *Food Sci. Tech.* 30: 609-615; 1997.
76. Al-Farsi, M.; Alasalvar, C.; Morris, A.; Baron, M.; Shahidi, F. Comparison of antioxidant activity, anthocyanins, carotenoids, and phenolics of three native fresh and sun-dried date (*Phoenix dactyli-fera* L.) varieties grown in Oman. *J. Agric. Food Chem.* 53: 7592-7599; 2005.
77. Siddhurajir, P.; Mohan, P.S.; Becker, K. Studies on the antioxidant activity of Indian laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. *Food Chem.* 79: 61-67; 2002.
78. Ifesan BOT, Hamtasin C., Mahabusarakam W., Voravuthikunchai SP. Inhibitory effect of *Eleutherine americana* Merr. extract on *Staphylococcus aureus* isolated from food. *J. Food Sci.* 2009; 1, 31-36.
79. Casero C, Estévez-Braun A, Ravelo AG, Demo M, Méndez-Álvarez S, Machín F. Achyrofuran is an antibacterial agent capable of killing methicillin-resistant vancomycin-intermediate *Staphylococcus aureus* in the nanomolar range. *Phytomed* 2013; 20(2), 133-138.