ANTIBACTERIAL ACTIVITY OF *GLYCYRRHIZIC ACID* AGAINST MULTI DRUG RESISTANT BACTERIA AND FUNGUS

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Glycyrrhizic acid (GA) is a kind of triterpene glycoside obtained from Liquorice (*Glycyrrhiza glabra*). The aim of this study was to search the antimicrobial activities of *Glycyrrhizic acid* against bacteria some drug resistant bacteria (*E.coli*, *A.baumanii* and *P.aeruginosa*) and fungus (*C.albicans*). To determine the non-cytotoxic concentration of GA, HEp-2 cell line was used. The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum 1% (w/v). Cells were incubated in a humidified atmosphere at 37 ºC in 5% CO_2. Antimicrobial activity of GA was screened by broth microdilution procedures and principles of the CLSI. Stock solutions of GA at the concentration of 1000 µg/ml were prepared in ethanol. GA concentration range used in the antimicrobial tests was 1.92; 3.8; 7.8; 15.6; 31.2; 62.5; 125; 250 and 500 µg/ml prepared for bacteria in Mueller-Hinton broth and for yeast in Saboraud Dextrose broth. Minimal inhibitory concentrations for GA were investigated against both standard bacterial strains; *E.coli* (ATCC 25922), *P.aeruginosa* (ATCC 27853), *A.baumanii* (ATCC 17978), and yeast-like fungus; *C.albicans* (ATCC 90028) and clinical isolates of multi drug resistant isolates. It was established that *glycyrrhizic acid* inhibited the growth of bacteria with MIC values ranging between 15.6 and 31.2 µg/ml and showed anti-yeast activity with MICs at 62.5 µg/ml. While the GA exhibited significant antibacterial activity against multidrug resistant Gram negative bacteria, it was found to be slightly less effective against *C.albicans* isolates. We think that GA may be new hope for the treatment of diseases caused by multidrug resistant bacterial isolates.

Keywords: *Glycyrrhizic acid*, resistant, bacteria, fungus.

INTRODUCTION

Globally, widespread use of antibiotics leads to the development multi-drug resistant microorganisms. Multidrug-resistant microorganims are among the major causes of hospital-acquired infections over the last 20 years (Laxminarayan and Heymann, 2012; CDCP, 2013).

Infections due to multi-drug resistant microorganisms are sources of significant medical problems in hospitals and healthcare facilities, because these kinds of infections are the major causes of both morbidity and mortality. The main multi-drug resistant Gram negative bacteria related to antimicrobial resistance are extended-spectrum beta-lactamase (ESBL)-producing gram-negative bacteria such as *Escherichia coli*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa* (Datta *et al.*, 2012; Napier *et al.*, 2013; Nordmann *et al.*, 2011).

On the other hand, another major problem in drug resistance is the resistance to antifungal drugs. Drug-resistant isolates of fungal infections have been reported to reach notable levels depending on immunocompromised patients including those who have undergone organ transplants, patients receiving chemotherapy or HIV treatments. In recent years, drug resistance in fungal infections has been reported to increase due to *C. albicans* (Goffeau, 2008; Cowen, 2008).

Because antibiotic resistance is a rapidly evolving problem in both bacteria and fungi, new drug research is proceeding fast around the World. Recently, studies especially on natural products are remarkable in this area. *Glycrrhiza glabra* is one of

the most important natural products in new drug research. It has many pharmacological activities such as antimutagenic activity (Alekperov, 2008) anti-ulcer effects (Dey *et al.*, 2009), protective action against hepatotoxicity (Wan *et al.*, 2009), antitumor promoting activity (Rafi *et al.*, 2002), etc. *Glycyrrhizic acid* is the most important component of *Glycrrhiza glabra* which have a wide range of pharmacological properties.

This study aims to search for antimicrobial activities of *Glycyrrhizic acid* against bacteria including drug resistant bacteria (*Escherichia coli*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa*) and fungus (*Candida albicans*).

MATERIALS AND METHODS

Microorganisms & *Glycyrrhizic Acid*

Escherichia coli, *Acinetobacter baumanii* and *Pseudomonas aeruginosa* and *Candida albicans* strains were obtained from Mustafa Kemal University culture collections, in Hatay province. For the culture of Gram negative bacteria (*Escherichia coli*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa)* we used EMB agar and Mueller-Hinton broth (Difco, USA).

Glycyrrhizic acid was obtained from commercially from Sigma (Sigma, USA). To determine the non-cytotoxic concentration of *Glycyrrhizic acid*, HEp-2 cell line was used. The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum 1% (w/v). Cells were incubated in a humidified atmosphere at 37 ºC in 5% CO_2.

The Effect of DMSO on the HEp-2 Cells

DMSO (dimethyl sulfoxide) was used as a solvent for *Glycyrrhizic acid.* To determine the non-toxic concentration of DMSO on HEp-2 (human larynx epidermoid carcinoma cells), 1×10^6 cells were inoculated into each well of 12-well plates containing RPMI-1640, and incubated for 48 hrs in the presence of decreasing amounts of DMSO (8%, 4%, 2%, 1%, 0.5%). The nontoxic concentration was determined up to 2%. The 0.5% and 1% concentrations of DMSO did not affect the growth of the microorganisms. Therefore, *Glycyrrhizic acid* were dissolved in 1% DMSO in experiments.

Cell Culture Tests for Cytotoxicity

To evaluate the cytotoxicity of *Glycyrrhizic acid* for human cells, (HEp-2) cell line was selected. The cells were cultured in RPMI-1640 medium with 10% (w/v) FCS. Incubation of the cells was performed at 37°**C** with 95% air and 5% carbon dioxide.

Glycyrrhizic acid was dissolved in DMSO (Sigma, USA). Stock solutions of *Glycyrrhizic acid* were prepared in DMSO at the concentration of 1%. The 250; 125; 62.5; 31.2; 15.6; 7.8; 3.9; 1.92; 0.96; 0.48 µg/ml of concentrations of *Glycyrrhizic acid* were tested.

To determine the non-cytotoxic concentration of *Glycyrrhizic acid* on HEp-2 cells, 1×10^5 cells were inoculated into each well of flat-bottomed plates, and were cultured for 8 hrs at 28°C. Next, they were allowed to grow for an additional period 96 hours. *Glycyrrhizic acid* were diluted, whereupon decreasing amounts (from 250 to 0.48 µg/ml) were placed per well. All experiments were performed in triplicate, and the results were expressed as log number cells per milliliter on the percentage of growth inhibition.

The cytotoxicity of the *Glycyrrhizic acid* was determined using a conventional hemocytometer and the trypan blue exclusion (Burlesson et al., 1992). The highest noncytocidal (on HEp-2 cells) concentration of the tested samples was determined to be 125 µg/ml. Hence, *Glycyrrhizic acid* concentrations were lower than that of non-cytotoxic concentration.

Antimicrobial Activity

Multi-drug resistant clinical isolates including both bacteria (*Escherichia coli*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa*) and fungi (*Candida albicans*) were used in the experiments. Also, the following standart microorganisms were cultured simultaneously for antimicrobial activity test: *Escherichia coli* ATCC 25922, *Acinetobacter baumannii* ATCC 17978, *Pseudomonas aeruginosa* ATCC 27853 and *C.albicans* strais (ATCC 90028). Antimicrobial activity was evaluated with broth microdilution method. Minimal inhibition concentration ranges were determined according to the CLSI (Clinical and Laboratory Standards Institute) guidelines (CLSC, 2008).

Mueller-Hinton broth (Difco, USA) and Saboraud Dextrose broth (Difco, USA) were used when testing bacterial and *Candida albicans* strains, respectively. The inoculum density was $1x10^6$ cfu/ml for bacteria and $1x10^5$ cfu/ml for fungi. *Glycyrrhizic acid* was dissolved in DMSO. Solutions in the test medium provided the required concentration ranging from 1024-0.5 µg/ml. The inoculated plates were incubated at 35°C and evaluated after 24 hours. For *Candida albicans* this incubation period was selected as 48 hours. Minimum inhibitory concentration values were determined as the lowest concentrations of the substances that had no visible turbidity. For bacteria amikacin was selected as a positive control, while, in the case of yeasts, flucanozole was used.

For antimicrobial activity of *Glycyrrhizic acid*, stock solutions of *Glycyrrhizic acid* at the concentration of 125 µg/ml were prepared in ethanol. *Glycyrrhizic acid* concentration range used in the antimicrobial tests was 0.48; 1.92; 1.56; 3.9; 7.8; 15.6 31.2; 62.5 and 125 µg/ml prepared for bacteria in Mueller-Hinton broth and for yeast in Saboraud Dextroz broth. Minimal inhibitory concentrations for *Glycyrrhizic acid* were investigated against both standard bacterial strains; *E.coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), Acinetobacter baumanii (ATCC 17978), and yeast-like fungus; *C. albicans* (ATCC 90028) and clinical isolates of multi drug resistant isolates.

Statistical Analysis

All data is represented as mean ± standard error of mean (SEM) for triplicate set of experiments. Statistical analyses were performed using Student t-test. The p value <0.05 was considered significant. All statistics in the present study were done using SPSS for Windows, version 17.5 SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Cell cultures were incubated with DMSO alone (without any supplement) in the DMSO control group. In this group, we compared the effects of DMSO with cell control group on the viability of HEp-2 cells. As given in Figure 1, DMSO at the 1% (w/v) concentration did not showed no toxic effect for the cells. This concentration of DMSO (1% w/v) did not inhibit the growth of cells. At the end of incubation, there

were no statistically significant difference in the cell number between the cell control and the DMSO ontaining groups (p>0.05). Furthermore, nor were any cytopathological changes observed in DMSO group compared with the cell control group (Figure 1).

Glycyrrhizic acid was evaluated *in vitro* for antimicrobial activity against both standart strains of *E. coli, P. aeruginosa, Acinetobacter baumanii and C. albicans* and drug-resistant clinical strains of these microorganisms. As the control drugs, flucanazole and amikacin were selected (Tables 1-4).

Antimicrobial test results performed with the standard strains (*E. coli, P. aeruginosa, Acinetobacter baumanii and C. albicans*) are given in Table 1 and 2. In Table 1 and 2, MIC values of *glycyrrhizic acid* against drug-resistant clinical isolates (*E. coli, P. aeruginosa, Acinetobacter baumanii and C. albicans*) are given. As can be seen in the table, while standart drugs inhibited the microorganisms growth between 15.6 and 31.2 µg/ml, *glycyrrhizic acid glycyrrhizic acid* proved to inhibit the growth of bacteria with MIC values ranging between 15.6 and 31.2 µg/ml and showed anti-yeast activity with MICs at 62.5 µg/ml.

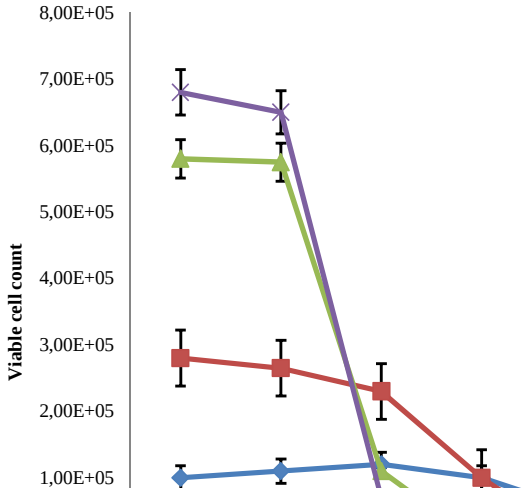

Figure 1. Effects of *Glycyrrhizic acid* on the proliferation of HEp-2 cells

Table 1. MIC (minimal inhibitory concentration) and MFC (minimal fungicidal concentration) values (µg/ml) of glycyrrhizic acid and amikacin for *E.coli*, *P. aeruginosa*, *A. baumanii* and *C. albicans*

	Drug resistant isolates		*E.coli (ATCC 25922)*	
	MIC	MFC	MIC	MCF
Glycyrrhizic acid	15.6	31.2	3.9	15.6
Amikacin	4	8	0.5	2
	Drug resistant isolates		*P. aeruginosa* (ATCC 27853)	
	MIC	MFC	MIC	MCF
Glycyrrhizic acid	15.6	62.5	7.8	31.2
Amikacin	8	16	1	2
	Drug resistant isolates		*A.baumanii* (ATCC 17978)	

	MIC	MFC	MIC	MCF
Glycyrrhizic acid	62.5	125	15.6	31.2
Amikacin	8	16	1	2

In Table 3 and 4, the synergistic effects of the standard drugs with *Glycyrrhizic acid* were investigated. As is clear in these tables, *Glycyrrhizic acid* significantly raised the effectiveness of the standart antimicrobials (amikacin and flucanazole). A synergistic activity was determined between *Glycyrrhizic acid* and amikacin, while an additional activity was found between *Glycyrrhizic acid* and flucanazole.

Table 2. MIC and MFC values (µg/ml) of *Glycyrrhizic acid* and flucanozole for *Candida albicans*

	Drug resistant isolates		*C.albicans strais (ATCC 90028)*	
	MIC	MFC	MIC	MCF
Glycyrrhizic acid	62.5	125	15.6	31.2
Flucanozole	2.5	5	2.5	5

Table 3. Synergistic effects of *Glycyrrhizic acid* and Amikacin on growth inhibition of *E.coli*, *P. aeruginosa* and *A. baumanii*

	Glycyrrhizic acid (µg/ml)					
	Drug resistant strains			*E.coli* (ATCC 25922)		
Amikacin (µg/ml)	1.56	3.12	6.24	1.56	3.12	6.24
0.5	G	G	NG	G	G	NG
1	G	NG	NG	NG	NG	NG
2	G	NG	NG	NG	NG	NG
	Glycyrrhizic acid (µg/ml)					
	Drug resistant strains			*P. aeruginosa* (ATCC 27853)		
Amikacin (µg/ml)	1.56	3.12	6.24	1.56	3.12	6.24
0.5	G	G	NG	G	G	NG
1	G	G	NG	G	G	NG
2	G	G	NG	NG	NG	NG
	Glycyrrhizic acid (µg/ml)					
	Drug resistant strains			*A.baumanii* (ATCC 17978)		
Amikacin (µg/ml)	1.56	3.12	6.24	1.56	3.12	6.24
0.5	G	G	G	G	G	NG
1	G	G	NG	NG	NG	NG
2	G	NG	NG	NG	NG	NG

Results obtained from three independent experiments; G: Growth, NG: No Growth.

Table 4. Synergistic effect of *Glycyrrhizic acid* and flucanozole on growth inhibition of *Candida albicans*

	Glycyrrhizic acid					
	Drug resistant strains			*C.albicans* strains (ATCC 90028)		
Flucanozole	1.56	3.12	6.24	1.56	3.12	6.24
1	G	G	G	NG	NG	NG
2	G	G	NG	NG	NG	NG
4	G	G	NG	NG	NG	NG

Results obtained from three independent experiments; G: Growth, NG: No Growth.

CONCLUSIONS

In conclusion, *Glycyrrhizic acid* alone was confirmed to be active against multi drug resistant isolates of *E.coli*, *P.aeruginosa, Acinetobacter baumanii* and *C.albicans*. In this study, *Glycyrrhizic acid* was shown to be more active than amikacin and flucanozole *in vitro* when combined with amikacin and flucanozole. Our results suggest a therapeutic potential of glycyrrhizic acid for the treatment caused by *E.coli*, *P. aeruginosa, Acinetobacter baumanii* and *C.albicans* infections. Further studies to be performed should evaluate to explore its therapeutic potential and the beneficial effect of glycyrrhizic acid. We think that *Glycyrrhizic acid* may be new hope for the treatment of diseases caused by drug resistant microbial isolates.

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