

Antibacterial and Antioxidant Activity of extract and fractions of *Lonicera nummularifolia* Jaub & Spach

Mohammad Ali Farboodniay Jahromi^{1,*}, Amir Emami^{2,4}, Raha Nazeri³, Neda Pirbonyeh^{2,4}

¹Medicinal plants processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

²Department of Bacteriology & Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

³Department of pharmacognosy, School of pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

⁴Burn & Wound Healing Research Center, Department of Microbiology, Amir-Al-Momenin Burn Hospital, Shiraz University of Medical Sciences, Shiraz, Iran.

Abstract

The present study deals with antibacterial and antioxidant screening of ethanolic leaf extract and various fractions of *Lonicera nummularifolia*, an Iranian endemic plant of Caprifoliaceae family. The ethanolic extract was prepared using soxhlet extraction procedure and fractionation of the extract was performed by a continuous liquid-liquid extraction method. Total phenol, flavonoid and triterpenoid content of the extract were determined, using standard spectrophotometric methods. DPPH and FRAP assays were conducted to evaluate the antioxidant capacity. Antibacterial activity of total ethanolic extract and fractions were evaluated by measuring MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) against four standard bacterial strains, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosae* and *Acinetobacter baumannii*, using broth microdilution method. Total phenol content of hydroalcoholic extract was found to be higher than flavonoid. Results of antioxidant assays indicated that ferric reducing antioxidant power of the extract was promising. Results of antibacterial screening declared the lowest MIC value for ethanol extract and therefore higher bacteriostatic activity against the tested bacterial strains compare to chloroform and ethyl acetate fractions. Ethanolic extract also revealed significant bactericidal activity among all the tested extracts. Overall inspection of the results declared moderate DPPH free radical scavenging while promising ferric reducing activity and antibacterial properties of *L. nummularifolia* which is worthy of further detailed investigations. Isolation and characterisation of active substances of the extract are interesting approaches in order to validate antibacterial and ferric reducing properties of the plant.

Keywords: Antimicrobial activity, Antioxidant, *Lonicera nummularifolia*

1. Introduction

Lonicera nummularifolia Jaub. & Spach is a thorny shrub belonging to the family Caprifoliaceae, which is native for arid, semi-arid and steppe areas. This plant is one of the forest species of Fars province which is seen along with oak

species in Zagros forests especially in Sepidan and Arjan plain. Six species of the genus have been reported as endemic in Iran, including *L. nummularifolia*, *L. periclymenum*, *L. persica* Jaub. & Spach (European honeysuckle) and *L. japonica* (1). The tincture of *L. japonica* has been reported useful in the treatment of asthma, urinary tract diseases, fever, inflammation, muscle cramps, and bacterial infections. Polyphenolic compounds isolated from

Corresponding Author: Mohammad Ali Farboodniay Jahromi, Medicinal plants processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
Email: farboodnia@sums.ac.ir

this species inhibit platelet activity and cause cell resistance to hydrogen peroxide production (2). Evaluation of antioxidant and acetylcholinesterase inhibitory activities of *L. nummularifolia* from Kohgiluyeh and Boyerahmad has been reported earlier (3).

Antimicrobial effects of *L. japonica* against gram positive and gram negative anaerobic bacteria has been reported earlier. In particular, the *in vitro* activity of n-butanol extract against 104 clinical strains of an aerobic bacteria was evaluated using agar dilution method and the results were compared with erythromycin, cefotaxime, imipenem, clindamycin and metronidazole. It was found that *L. japonica* and imipenem have been the most active antimicrobial agents tested (4). Antibacterial analysis of *L. japonica* chloroform and ethanol leaf extracts have shown significant degree of inhibition against the tested pathogenic organisms. Ethanolic extract of leaves and flowers were prepared and phytochemical analysis was performed to detect the presence of alkaloids, phenols, terpenoids and flavonoids (5). Antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Vibrio cholerae* and *Salmonella typhi* was performed. Extracts of leaf and flower in different dilutions demonstrated antibacterial activity against the tested microorganisms (5). Investigation of antimicrobial properties of a combination of *L. japonica* extract-silver nanoparticle was performed against *E. coli* and the results showed more significant antibacterial activity compared to single extract (6). Antibacterial and ferric reducing properties of *L. nummularifolia* has not been studied previously. Our review of the literature indicated that phytochemical and pharmacological studies, especially the assessment of phenolic and flavonoid content as well as the reducing activity of the plant, have not been performed so far. The plant is expected to contain significant amounts of active phenolic acids, iridoid glycosides and triterpene saponins as marker secondary metabolites (7). Based on the chemical composition and earlier studies and in order to locate the active fractions, evaluation of antibacterial and antioxidant capacity of the plant was undertaken in the present study.

2. Material and Methods

2.1. Plant material

Leaves of *L. nummularifolia* were collected from Sepidar mountain at Maymand, and authenticated by the plant taxonomist of the Dept. of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran. A voucher specimen of the plant, MPPRC-96-1, has been deposited in the herbarium of Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

2.2. Chemicals and Reagents

Methanol, aluminum chloride, sodium carbonate, acetate buffer, ferric chloride, hydrochloric acid, ammonium persulphate, Folin-Ciocalteu reagent and TLC plates were purchased from Merck (Darmstadt, Germany). Ethanol % 96 was purchased from Zakaria Co., Jahrom, Iran. Quercetin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich, USA. Bacterial strains were obtained from Persian Type Culture Collection (PTCC), IROST, Tehran, Iran.

2.3. Preparation of extract and fractions

Dried aerial parts of *L. nummularifolia* were powdered and exhaustively extracted (100g) with ethanol 96% for 4 h using a Soxhlet apparatus. The extract was filtered and the solvent was removed under reduced pressure to afford a light brown gummy residue as ethanolic extract. The extract was further suspended in water and fractionated successively with n-hexane, chloroform, ethyl acetate and n-butanol in a liquid-liquid extractor. The individual fractions washed with water in a separatory funnel and the solvent was evaporated to dryness at 50 °C under reduced pressure. The dried extract and fractions were further concentrated in a speed vacuum and freeze dried and finally stored at -20 °C prior to pharmacological screening.

2.4. Preliminary Phytochemical Screening

The methanolic solution of *L. nummularifolia* ethanol extract was assessed for the presence of phytochemical components, using the standard reagent methods (8,9). The results of preliminary phytochemical and DPPH screening

Table 1. Reagent tests with hydroalcoholic extract of *L. nummularifolia*.

Types of Tests	Phytochemical constituents	EtOH Extract
Ferric Chloride	Phenols	+
Vanillin HCl	Flavonols	+
Frothing	Saponins	-
Anisaldehyde /H ₂ SO ₄	Phenylpropanoids	+
Shinoda	Flavonoids	+
Zinc HCl	Flavonoids	+
Draggendorff	Alkaloids	-
Meyer	Alkaloids	-
Wagner	Alkaloids	-
Salkowski	Terpenoids	+
Liebermann	Steroids	+
Vanillin/ H ₂ SO ₄	Terpenoids	+
DPPH	Antioxidant Activity	+

are presented in table 1.

2.5. Determination of total phenol

Total phenolic content of *L. nummularifolia* ethanolic extract (LNE) was measured using Folin-Ciocalteu spectrophotometric method (10). To develop a standard calibration curve, 0.5 mL of gallic acid solutions of increasing concentration ranging from 6.25-200 mg/L were mixed separately with 5 mL of 10% Folin-Ciocalteu reagent and 4 mL of 1.0 M solution of sodium carbonate. To determine the total phenolic content of the hydroalcoholic extract, a stock solution of 800 µg/mL of extract was used. Proper dilution of the stock solution was oxidized with Folin-Ciocalteu reagent and neutralized by sodium carbonate solution as given for standard. The absorbance of mixture was recorded at 765 nm after 15 min against Folin-Ciocalteu reagent as blank using a PG instrument T90 UV spectrophotometer. Total phenolic content was expressed as mg of gallic acid equivalent (GAE)/g of dry plant material using the expression from the calibration curve ($Y=0.00571x-0.0843$, $r^2=0.9985$). Where Y is the absorbance and x is the gallic acid equivalent. This assay was conducted in triplicate and the standard deviation was calculated.

2.6. Determination of total flavonoid content

Total flavonoid content of the extracts was determined by an aluminum chloride colo-

rimetric assay as described previously (11). This colorimetric procedure is based on an aluminum (III) complex formation reaction. For preparation of calibration curve 5 mL of 2% aluminum chloride solution in methanol were mixed with various concentration of quercetin solutions of different concentrations ranging from 0-80 µg/mL. Absorbance was measured at 415 nm on a PG-T90 UV spectrophotometer after 10 min. For quantitative determination of total flavonoid content of LNE, a solution of 300 µg/mL of each extract in methanol was prepared and the analysis was performed using the above procedure. Pure methanol was used as control while a mixture of 2 mL of extract (300 µg/mL) and 2 mL of methanol served as the blank. Total flavonoid content was expressed as mg of quercetin equivalent (QE)/g of dry plant material using the equation obtained from the calibration curve ($Y=0.02985x+0.04811$, $r^2=0.9978$). Where Y is the absorbance and x is the quercetin equivalent.

2.7. Determination of total triterpene content

The five-membered ring triterpenoids are the major constituents of *L. nummularifolia* leaf extract. Total triterpenoid content of ethanolic extract was measured according to a standard spectrophotometric method reported earlier with a minor modification (12, 13). Based on this procedure, vanillin-acetic acid solution (5%) was used. Ursolic acid (Sigma-Aldrich) was used as a triterpene

standard and the calibration curve was plotted with various concentrations of ursolic acid 20, 40, 60, 80 and 200 µg/mL. To prepare 25 mL of this solution, carefully weighed 1.25 mg of vanillin powder dissolved in 25 mL of acetic acid. Perchloric acid solution (70%) was prepared by diluting conc. perchloric acid in a ratio of 7: 3 using double distilled water. To prepare standard solution of triterpenoid, 1.0 mg ursolic acid in absolute ethanol was diluted to 5 mL in a volumetric flask and then aliquots of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mL of this solution were transferred to separate tubes and the volume of each sample tube was then adjusted, to 1.0 ml with absolute ethanol, to obtain concentrations of 20, 40, 80, 160 and 200 µg/mL, respectively. Then 0.4 mL of vanillin-acetic acid solution and 1.0 mL of perchloric acid were added and the tubes were incubated at 60 °C for 15 min and then cooled in an ice bath for 10 min. The samples were then removed from the ice bath and 5 mL of glacial acetic acid was added to each sample. A mixture of absolute ethanol, vanillin-acetic acid and perchloric acid, free from extract served as blank. All samples were allowed to reach room temperature prior to measurement of the absorbance at 548 nm. Based on the above described method, a solution of 2.0 mg/mL of *L. nummularifolia* ethanol extract was used for measurement of total triterpenoids. Each assay was performed in triplicate.

2.8. DPPH Free radical scavenging assay

In this study, two assays were used for assessment of antioxidant characteristics of the plant extract. DPPH radical scavenging activity of the extract and quercetin as standard were determined using a previously reported procedure (14). Twenty microliters of various concentration of the methanolic solution of extract (6.25-200 µg/mL) were mixed with 200 µL solution of 100 mM DPPH in methanol. The mixture was left in the dark for 30 min and the absorbance was measured at 490 nm using a Biotek, ELX800 microplate reader. A sample containing 20 µL of methanol and 200 µL of DPPH solution served as control while a solution of equal amount of extract in methanol was used as the blank. All tests were conducted in triplicate. Percentage inhibition of extract against DPPH was calculated using the following equation:

$$\% \text{Inhibition} = 100 - [(A_{\text{sample}} - A_{\text{blank}} / A_{\text{control}}) \times 100] \quad \text{Eq. 1}$$

2.9. Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was performed according to a procedure described by Benzie and Strain (15). Solutions of acetate buffer 300 mM pH 3.6, Ferric chloride (III) 20 mM and TPTZ 10 mM (in 40 mM HCl) were separately prepared. The FRAP reagent was freshly prepared by mixing the above solutions including acetate buffer, TPTZ and FeCl₃ at the ratio of 10:1:1 (v/v/v) respectively. Briefly 20 µL of extract was mixed with 180 µL of FRAP reagent and the absorbance was measured at 593 nm after 10 min incubation at 37 °C using an Epoch microplate spectrophotometer. Control contained 20 µL of methanol and 180 µL of FRAP reagent without extract. A sample containing extract and the solvent served as blank. Quercetin was used as antioxidant standard and positive control. The absorbance of tested samples were compared to a FeSO₄ standard curve and the values were expressed as percentage of Fe³⁺ reduced by the extract. An antioxidant compound or extract causes, reduction of Fe³⁺ to Fe²⁺ ion, leading to an increase in the absorption at 593 nm due to the formation of a blue Fe²⁺/TPTZ complex.

2.10. Antibacterial Screening

Antibacterial activity of total extract of *L. nummularifolia* extract and fractions were evaluated against 4 standard bacterial strains including *Pseudomonas aeruginosa* (PTCC-27853), *Escherichia coli* (PTCC-25922), *Staphylococcus aureus* (PTCC-25923) and *Acinetobacter baumannii* (PTCC-17978).

2.11. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration for the standard bacterial isolates (*P. aeruginosa*, *E. coli*, *S. aureus* and *A. baumannii*) was performed by microdilution method, recommended by Clinical and Laboratory Standards Institute, CLSI, 2018 (16). In brief, all wells in a row were filled with 100 µL of nutrient broth medium. For serial dilution of test solutions, a total of 100 µL of each solution at a concentration of 128 µg/mL was added to the first

well, and 7 dilutions were prepared serially. Then 5 μ L of test bacteria with concentration of 5×10^5 CFU/mL was added to all inoculated wells. This was performed for all test bacteria and solutions. For contamination control, two wells were just filled with broth medium and a broth medium with added extract, respectively. The growth activity of the test strains were checked prior to screening.

2.12. Minimum bactericidal concentration (MBC)

In order to determine bactericidal activity of extract and fractions, the minimum bactericidal concentration (MBC) was determined. A 0.1 mL solution from each well, which was determined as MIC, was sub-cultured on fresh Mueller-Hinton Agar plates with no inhibitors. At the same time, one well with a lower concentration, and the two wells with higher concentrations than MIC were tested for bactericidal activity. Inoculated plates were incubated for 18 to 20 hours at 37 °C and the growth activity was evaluated. No bacterial growth on agar plates was recorded as bactericidal concentration, while the minimum concentration was considered as the MBC of the test solutions (17).

3. Result

3.1. Quantification of total phenolics

Total phenol content of LNE was measured and expressed as mg gallic acid equivalent GAE/gram of extract. The standard curve plotted for various concentrations of gallic acid standard, using UWin5.0 software and the calibration curve equation of line obtained are as follows:

$$\text{Abs} = K_1 (\text{Conc}) + K_0,$$

where as $K_0 = 0.02442$, $K_1 = 0.00511$ ($r^2 = 0.9990$).

According to the results, the phenol content of ethanol extract was calculated as 52.55 ± 0.37 mg GAE/g of extract.

3.2. Total flavonoid content

Determination of flavonoid content of LNE was performed using aluminum chloride method which was performed through formation of a yellow complex with flavonoid compounds in the extract and measuring the intensity. According-

ly, the total flavonoid content of LNE was found to be 43.98 ± 2.68 mg quercetin equivalent, QE/g of extract. The equation of line obtained for various concentrations of standard solution of quercetin was found to be as follows:

$$\text{Abs} = K_1 (\text{Conc}) + K_0,$$

whereas $K_0 = 0.04811$, $K_1 = 0.02985$ ($r^2 = 0.9978$).

3.3. Determination of total triterpenoid

The absorbance calculated for various concentrations of ursolic acid standard. The standard curve for ursolic acid was plotted and the line equation is as follows: $\text{Abs} = K_1 (\text{Conc}) + K_0$, whereas $K_0 = 0.00961$, $K_1 = 0.00069$ ($r^2 = 0.9996$).

Using the above equation, ursolic acid content of the standard solutions were calculated. According to the results, the total triterpenoids of ethanolic extract was found to be 51.89 ± 2.6 mg/g of extract. The amount of triterpenoid of various concentrations of the extract stock solution were measured. All assays were conducted in triplicate.

3.4. DPPH radical scavenging activity

Based on the results, the total ethanolic extract declared. As evidenced by the results, *L. nummularifolia* extracts were found to be active against DPPH free radical and compared with those of quercetin. According to the results in table 2, from 100 to 800 μ g/mL, the reproducibility is almost constant and the curve shows a linear trend. IC_{50} values of quercetin in DPPH radical scavenging was determined (Table 2). The percentage of DPPH free radical scavenging for total extract was measured at concentrations ranging from 6.25 to 800 μ g/mL and the results are presented in table 2. According to the results, the lowest percentage of inhibition was related to the concentration of 6.25 μ g/mL with 4.98% and the highest percentage of inhibition by LNE, attributed to the concentration of 800 μ g/mL with 81.19% (Figure 1). The results indicated a weak inhibitory activity of this extract against DPPH free radicals. As illustrated in figure 1, the DPPH free radical scavenging capacity of *L. nummularifolia* extract reaches maximum level at the concentration of 800 μ g/mL.

Table 2. DPPH Radical scavenging activity of various concentration of *L. nummularifolia* ethanolic extract.

EtOH Ext.(µg/mL)	% DPPH Scavenging
6.25	4.98±0.17
12.5	6.20±0.51
25	7.78±0.17
50	8.51±0.62
100	12.28±1.34
200	18.73±2.27
400	26.64±1.86
800	61.19±1.98
Que. 100	83.25±.64

3.5. Ferric Reducing Antioxidant Power

Ferric reducing antioxidant power, is an evaluation of the oxidation-reduction potential of the testing samples. The ethanolic leaf extract of *L. nummularifolia* displayed reducing abilities in comparison to quercetin, indicating the presence of compounds with potential reducing capacity. According to the results, *L. nummularifolia* extract exhibited a significant reducing ability (Table 3).

Evaluation of the reducing power of LNE revealed reduction of Fe³⁺ to Fe²⁺ by the extract. The FRAP value curve was plotted against the concentrations of extracts and the percentage of reduction was calculated accordingly. Table 3 shows the percentage reduction of Fe³⁺ versus concentrations of LNE, with a maximum reducing power at 200 µg/mL (Figure 2). According to the results, the degree of ferric reducing power, observed for the ethanol extract was found to be 71.01%, while quercetin standard exerted more than 85% ferric reduction at this concentration.

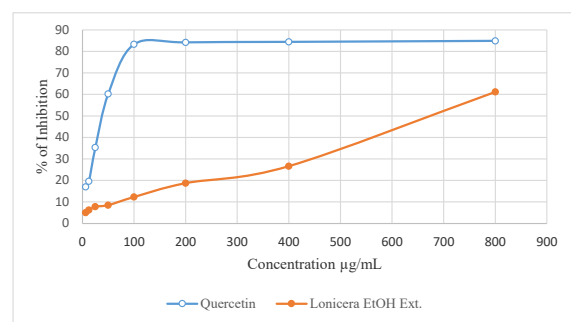


Figure 1. Comparison of DPPH free radical inhibition of quercetin and *L. nummularifolia* ethanolic extract.

Table 3. Percentage reduction of Fe³⁺ at various concentration of *L. nummularifolia* extract.

EtOH Ext.µg/ml	% Reduction
6.25	4.69±0.45
12.5	7.25±0.74
25	34.25±0.78
50	49.13±1.29
100	55.85±2.28
200	71.07±0.50
Que. 88.0	88.42±0.82

3.6. Antibacterial Activity

Antibacterial activity of hydroalcoholic extract and some fractions of *L. nummularifolia* against *A. baumannii*, *E. coli*, *S. aureus* and *P. aeruginosa* were evaluated, both qualitatively and quantitatively by standard methods for bacterial growth and the zone of inhibition test was done by agar well diffusion method. The results of evaluation of antibacterial activity of the extracts and fractions were recorded in table 4.

The results revealed that all extracts and fractions were potentially effective in suppressing microbial growth of bacteria with variable potencies. Ethanolic extract was the most effective in retarding microbial growth at the concentration of 10 mg/mL. All other fractions showed inhibitory activity against the tested microorganisms.

Results of antibacterial activity revealed that, *E.coli* was the most resistant strain to *L.nummularifolia* extract followed by *S. aureus*, while *A. baumannii* and *P. aeruginosa* were the most susceptible bacterial strains to the plant extract respectively.

In order to evaluate antibacterial properties of ethanolic extract, MIC and MBC values of the most effective extracts were determined

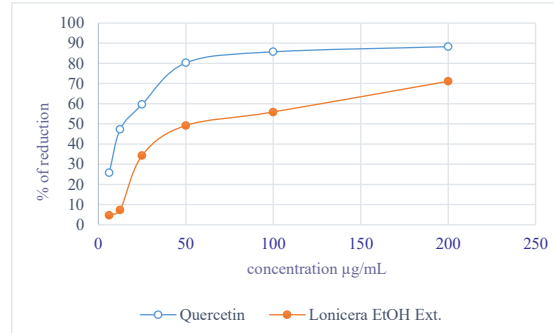


Figure 2. Percentage of FRAP of *L. nummularifolia* ethanolic extract.

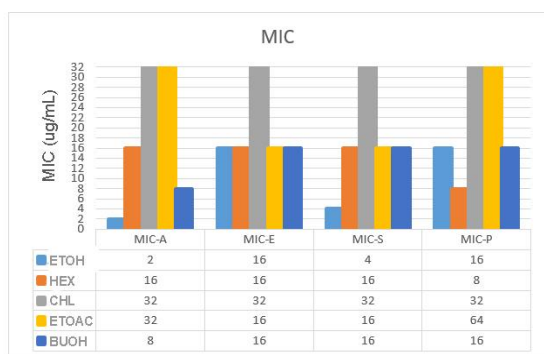


Figure 3. MIC values of ethanolic extract and fractions of *L. nummularifolia* against the tested bacterial strains A=*A. baumannii*, E=*E. coli*, S=*S. aureus*, P=*P. aeruginosa*.

by broth microdilution method. The minimum inhibitory and bactericidal concentrations of plant extract and fractions were given in Table 5, Figure 3, 4.

As presented in table 5, the ethanolic extract was the most effective extract against *A. baumannii* followed by *S. aureus* with MIC values of 8.0 and 16 $\mu\text{g}/\text{mL}$ respectively while it showed bactericidal effect against both *E. coli* and *P. aeruginosa* with MIC values 32 $\mu\text{g}/\text{mL}$. n-Hexane fraction demonstrated higher degree of bactericidal properties against *S. aureus* (MIC=16 $\mu\text{g}/\text{mL}$) compare to other tested microorganisms. The MIC values obtained for this fraction against both *E. coli* and *P. aeruginosa* were found to be 32 $\mu\text{g}/\text{mL}$. As depicted in table 2, chloroform extract showed the same MIC and MBC values against *A. baumannii* which were recorded at the concentration of 32 $\mu\text{g}/\text{mL}$. *P. aeruginosa* revealed lowest degree of susceptibility against *L. nummularifolia* chloroform fraction (MIC=128 $\mu\text{g}/\text{mL}$). According to the values presented in Table 5, ethyl acetate

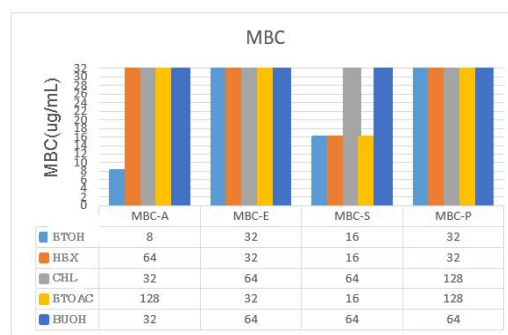


Figure 4. MBC values of *L. nummularifolia* ethanolic extract and fractions against the tested bacterial strains. A=*A. baumannii*, E=*E. coli*, S=*S. aureus*, P=*P. aeruginosa*.

fraction showed higher degree of bactericidal effect against *S. aureus* (MBC=16 $\mu\text{g}/\text{mL}$) followed by *E. coli* (MBC=32 $\mu\text{g}/\text{mL}$), while both *P. aeruginosa* and *A. baumannii* showed lower levels of susceptibilities to ethyl acetate fraction (MBC=128 $\mu\text{g}/\text{mL}$).

n-Butanol fraction displayed bacteriostatic and bactericidal characteristics at MIC and MBC values of 8.0 and 32 $\mu\text{g}/\text{mL}$ respectively whereas the other three tested bacterial strains showed the same and lower degrees of bacteriostatic and bactericidal effects (MIC=16, MBC=64). Among fractions, n-hexane declared lowest inhibitory concentration (8.0 $\mu\text{g}/\text{mL}$) but far higher values of inhibitory concentrations were recorded for other fractions (Table 5, Figure 3, 4).

4. Discussion

Infectious diseases are one of the most common disorders that impose a huge health and financial burden on human societies worldwide. Despite extensive efforts and widespread

Table 4. Antibacterial activity of hydroalcoholic extract and fractions of *L. nummularifolia* (10 mg/mL).

Extract / Fractions	Zone of inhibition (mm) (n=3)			
	Gram (-ve)		Gram (+ve)	
	Bacterial Species			
	<i>P. aeruginosa</i>	<i>A. baumannii</i>	<i>E. coli</i>	<i>S. aureus</i>
Ethanol	24.00±0.00	26.08±0.20	19.03±0.45	25.87±0.54
n-Hexane	25.20±0.32	21.00±0.00	19.42±0.34	20.00±0.78
Chloroform	19.50±0.02	19.40±0.45	17.98±0.08	17.54±0.46
Ethyl acetate	12.00±0.00	16.00±0.00	18.07±0.04	19.93±0.15
n-butanol	22.01±0.09	23.05±0.78	18.86±0.34	18.23±0.00

Data are means of three replicates (n=3)±standard error.

Table 5. MIC and MBC values of *L. nummularifolia* extract against *A. baumannii*, *E. coli*, *S. aureus* and *P. aeruginosa*.

Extract / Fractions	Gram (-ve)						Gram (+ve)	
	Microorganisms							
	<i>P. aeruginosa</i>		<i>A. baumannii</i>		<i>E. coli</i>		<i>S. aureus</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)	($\mu\text{g/mL}$)
Ethanol	16.00	32.00	2.00	8.00	16.00	32.00	4.00	16.00
n-Hexane	8.00	32.00	16.00	64.00	16.00	32.00	16.00	16.00
Chloroform	32.00	128.00	32.00	32.00	32.00	64.00	32.00	64.00
Ethyl acetate	64.00	128.00	32.00	128.00	16.00	32.00	16.00	16.00
n-butanol	16.00	64.00	8.00	32.00	16.00	64.00	16.00	64.00

Data are means of three replicates (n=3).

research, the infectious microorganisms are still needed to remain under rigorous controls. The emergence of microbial resistance to antibiotics together with the change in diseases patterns have led to increased tendency towards herbal remedies. (18-20).

Antibacterial screening of the extracts and fractions was performed on three Gram-positive and a Gram-negative bacterial species. *L. nummularifolia*, is a known endemic shrub to the southern part of Fars province in Iran. Literature search through scientific databases, indicated just an antioxidant report but no report was found on antimicrobial effect as well as the total triterpenoid quantification of this plant species. The present study is therefore considered as the first report on antibacterial screening of extract and fractions and quantification of total triterpene content of *L. nummularifolia*. A diet rich in polyphenols is of great importance in the prevention of chronic diseases such as hypertension, diabetes and heart diseases (21).

The current study also focused on determination of total phenolic, flavonoid and triterpenoid composition of the leaves of *L. nummularifolia*, which indicated higher content of phenolics and triterpenoids as the major chemical markers compare to flavonoids. Phenolic compounds as one of the important group of phytochemicals are responsible for demonstration of wide range of biological activities. They are highly effective in preventing destructive effects of free radicals in the body (22). Antimicrobial activity of many pheno-

lic compounds with diverse structural features (23) have been reported in earlier studies. The wealth of hydroxyl groups on a single or different ring in their structures can initiate the reactions with free radicals, transferring electrons, which neutralize and inhibit the oxidative effect of free radicals (24). Flavonoids play a significant contribution in manifestation of antioxidant and antibacterial effects and various other pharmacological properties. Both catechin and epicatechin in green tea are responsible for antioxidant effect of this plant (25). The lower content of flavonoid compare to phenol in *L. nummularifolia* might be attributed to the diversity of polyphenolic compounds in the plant extract. Flavonoids as a major class of the phenolic compounds of LNE, are able to demonstrate a variety of interesting *in vitro* and *in vivo* pharmacological effects (26).

The level of triterpene content is almost similar to that of phenol in LNE. Numerous studies have shown that many triterpenoids are involved in preventing cancers and other diseases. They have also been reported to exhibit anti-inflammatory effect (27, 28). The results of DPPH free radical scavenging assay showed that the IC₅₀ value of ethanolic extract was 668.88±15.81. Comparison of IC₅₀ value obtained for the ethanolic extract and quercetin standard indicated a weak DPPH radical scavenging effect of *L. nummularifolia*. Unlike radical scavenging effect, the FRAP value for this extract was found to be prominent, which imply a significant reducing character of the chemical components of ethanolic extract. In FRAP as-

say, antioxidant agents or extracts can reduce the ferric-tripyridyl triazine (Fe^{+3} -TPTZ) complex formed during this test to an intense blue ferrous form (Fe^{+2} -TPTZ) at low pH conditions. Fe^{2+} is capable of generating free radicals from peroxide, which is implicated in many diseases. Therefore, reduction of Fe^{2+} levels in the Fenton reaction would protect against oxidative damage and hence the reducing ability of an extract or compound may serve as a significant indicator of its potential antioxidant activity (29). Comparison of data obtained from other antioxidant assays showed that the free radical scavenging ability of extracts may be partly due to their iron chelating capacity. This interpretation gained support from the previous studies reporting the second position for *L. nummularifolia* fruit extract in demonstration of antioxidant activity among 28 fruits using FRAP assay (30). Numerous studies have shown that reducing ability and the consequent antioxidant effect have an important role in preventing diseases such as coronary artery disease and allergic diseases (31). In the plasma of heart patients, FRAP levels are significantly lower than in normal subjects. There was also a negative association between FRAP values with disease formation and prolongation. Therefore, with regard to the FRAP value obtained, it can be concluded that ethanol extract of *L. nummularifolia* insert its antioxidant character through the reducing effect. Studies show that antioxidant activity is directly related to the amount of phenol content in the sample (32). However, in the present study, it may be inferred that the major part of phenolic structures of the ethanolic extract, are in glycosylated or esterified form with sugars, or the chemical forms that inhibit their reactivity and interference with free radicals (33). It should be noted that, with regard to the triterpenes of extract, this group of compounds may contribute totally or partially to the manifestation of reducing properties and therefore antioxidant activity of the ethanolic extract (34, 35).

The flavonoid content of the extract can contribute to the declaration of reducing activity.

Antibacterial activity of total extract, n-hexane, chloroform, ethyl acetate and n-butanol fractions *L. nummularifolia* were investigated

against four bacterial strains, including *E. coli*, *A. baumannii*, *S. aureus* and *P. aeruginosa*. On the basis of the MIC and MBC values obtained, total ethanol extract and all fractions of *L. nummularifolia* were effective against *A. baumannii* but ethanol extract and butanol fraction were found to be the most active among the tested bacterial strains. The highest MIC value was displayed by chloroform and ethyl acetate fractions, whereas the lowest MBC was detected for ethanol fraction (Table 5, Figure 3, 4). Based on the results, it seems that, the bacterial inhibitory ability of ethanolic extract against *Acenitobacter* is due to the presence of various classes of chemical constituents, bearing diverse structural functionalities and their possible synergistic effects.

Antibacterial screening declared the efficacy of the extract and fractions against *E. coli*, and the MIC and MBC values showed the same trend as the extract and the fractions showed inhibitory effect while lacking significant lethal effect against *E. coli*. Evaluation of MIC and MBC values of chloroform fraction, showed no sign of effectiveness against *E. coli*. Evaluation of the antibacterial effects of total extract and fractions of LNE against *S. aureus* showed the lowest MIC value for total extract, followed by n-hexane, ethyl acetate and butanol fractions. In the study of bactericidal activity of extract against *S. aureus*, the lowest MBC value was determined for ethanol extract, n-hexane and ethyl acetate fractions. In case of *P. aeruginosa*, all fractions had bactericidal potency at the concentration of 32 mg/mL. The highest MIC or inhibition was recorded for chloroform and ethyl acetate fractions, while the lowest inhibition (MIC) was for n-hexane fraction. The study of antibacterial results on the plant showed that the chloroform and ethyl acetate fractions had the highest inhibitory effect, respectively.

Antibacterial screening of the extracts and fractions were performed on three Gram-positive and a Gram-negative bacterial species. The data procured in the present study declare that the growth of both Gram-positive and Gram-negative bacteria were influenced by the *L. nummularifolia* extract and fractions. Ethanol extract displayed antibacterial activity against four bacterial strains. However it showed higher degree of activity

against gram-positive bacteria. Both n-hexane and ethyl acetate declared same pattern of antibacterial activity against gram-positive bacteria, while exhibited a lower degree of effectiveness against gram-negative among the tested strains. The chloroform and n-BuOH fractions showed lower extent of antibacterial activity comparison to the total extracts and other fractions. *A. baumannii* and *S. aureus* were found to be the most sensitive strains to *L. nummularifolia* extract and fractions.

Thus, it may be concluded that the antibacterial effect detected for *L. nummularifolia* is partially due to the structural characteristics of phenolic constituents which enable them to bind to the bacterial membranes leading to subsequent disruption and leakage of cellular contents (36).

The chemical composition, antibacterial and antioxidant effects of *L. caerulea* fruit has been studied previously (37). The results of present study are in agreement with an earlier investigation that declared antimicrobial activity of *L. caerulea* against *Listeria monocytogenes*, *Ko curia rhizophila*, *Bacillus subtilis* and *Campylobacter jejuni* and *E. coli* (38). Comparison of the results from the present study with the previous research with respect to the phenolic and flavonoid content may indicates the close profile of secondary metabolites with that of *L. japonica* (39). However, the measurement of total triterpene levels and evaluation of antibacterial potential of *L. nummularifolia* performed in this study have not been reported earlier.

5. Conclusion

Within the limitations of this study, it can be concluded that, *L. nummularifolia* extract

References

1. Jalali P, Saadat YA, Sadeghi H. Micro-propagation of *Lonicera nummularifolia* Jaub. & Spach. *Iranian Journal of Rangelands and Forest Plants Breeding and Genetic Research*. 2011;19:71-84.
2. Park HS, Park KI, Lee DH, et al. Polyphenolic extract isolated from Korean *Lonicera japonica* Thunb. induce G2/M cell cycle arrest and apoptosis in HepG2 cells: involvements of PI3K/Akt and MAPKs. *Food Chem Toxicol*. 2012;50(7):2407-

contains active constituents, which exhibit antibacterial effects. Among different extracts, the highest contribution to antibacterial activity, by far made by the ethanolic extract and n-hexane fraction, particularly against *S. aureus* and *A. baumannii*. However, ethanol extract shows wider spectrum of antibacterial characteristics at lower concentrations. So, further studies are required to verify bactericidal behaviour of the individual chemical components on various bacterial and fungal pathogens. The plant demonstrated a prominent ferric reducing activity and probably exert antioxidant effect through the reducing character. Presence of phenols, flavonoids and triterpenoids can clearly justify the manifested biological activities of *L. nummularifolia* and thus its usage in traditional herbal medicine.

Statistical Analysis

The results obtained were expressed as mean values \pm SD. Statistical comparison of data was performed using one way analysis of variance (ANOVA) followed by Tukey's range test for multiple comparisons. All levels of significance were set at $P < 0.05$.

Acknowledgments

Authors are grateful to the Deputy of Research and Technology, Shiraz University of Medical Sciences for financial support of this project. This work is a part of the research done by Raha Nazeri for the preparation of Pharm D. thesis, filed under the registration No.1397-989.

Conflict of Interest

None declared.

2416. doi:10.1016/j.fct.2012.04.034
3. Hajimehdipoor H, Ara L, Moazzeni H, Esmaeili S. Evaluating the antioxidant and acetylcholinesterase inhibitory activities of some plants from Kohgiluyeh va Boyerahmad province. *Iran. Res J Pharmacog*. 2016;3(4):1-7.
4. Rhee KH, Lee K-H. Antimicrobial effects of *Lonicera japonica* against Gram positive and Gram negative anaerobic bacteria. *Nat Prod Sci*. 2011;17:23-5.
5. Sandigawad A. Analysis of phytochemi-

cals and antibacterial potential of *Lonicera japonica* Thunb. *Int J Pharm Bio Sci.* 2015;6:571-83.

6. Yang L, Aguilar ZP, Qu F, Xu H, Xu H, Wei H. Enhanced antimicrobial activity of silver nanoparticles-*Lonicera Japonica* Thunb combo. *IET Nanobiotechnol.* 2016;10(1):28-32. doi:10.1049/iet-nbt.2015.0027

7. Miliuskas G, Venskutonis P, Van Beek T. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food chem.* 2004;85:231-37.

8. Roopashree TS, Dang R, Rani RHS, Narendra C. Antibacterial activity of antipsoriatic herbs: *Cassia tora*, *Momordica charantia* and *Calendula officinalis*. *Int J Appl Res Nat Prod.* 2008;1:20-8.

9. Sofowora A. Phytochemical Screening of Medicinal Plants and Traditional Medicine in Africa. Spectrum Books Ltd, Ibadan. Nigeria. 1993:150-6.

10. Pourmorad F, Hosseinimehr S, Shahabimajd N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *Afr J Biotechnol.* 2006;5:1142-5.

11. Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chem.* 2005;91:571-7.

12. Liang C, Liu Z, Liu X, Geng Y, Song H, Ding S, et al. The extract optimization and identification study of bioactive total triterpenoids from the rare traditional Chinese medicine *Qinling Polyporus umbellatus*. *J Chem Pharm Res.* 2014;6:1283-9.

13. Smina TP, Mathew J, Janardhanan KK, Devasagayam TP. Antioxidant activity and toxicity profile of total triterpenes isolated from *Ganoderma lucidum* (Fr.) P. Karst occurring in South India. *Environ Toxicol Pharmacol.* 2011;32(3):438-446. doi:10.1016/j.etap.2011.08.011

14. Moein S, Farzami B, Khaghani S, Moein M, Larijani B. Antioxidant properties and prevention of cell cytotoxicity of *Phlomis persica* Boiss. *Daru.* 2007;15(2):83-8.

15. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Anal Biochem.* 1996;239(1):70-6.

16. CLSI. Performance Standards for Antimi-

crobial Susceptibility Testing. CLSI Supplement M100. 28th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.

17. Moazamian E, Emami A, Mehdizadeh H, Pirbonyeh N. Bactericidal activity of pigments isolated from Fars province (Iran) environmental bacteria on MDR clinical isolates of *Acinetobacter*. *Int J Mol Clean Microb.* 2018;8:922-30.

18. Cowan MM. Plant products as antimicrobial agents. *Clin Microbiol Rev.* 1999;12(4):564-582.

19. Xu HX, Lee SF. Activity of plant flavonoids against antibiotic-resistant bacteria. *Phytother Res.* 2001;15(1):39-43. doi:10.1002/1099-1573(200102)15:1<39::aid-ptr684>3.0.co;2-r

20. Zheng WF, Tan RX, Yang L, Liu ZL. Two flavones from *Artemisia giraldii* and their antimicrobial activity. *Planta Med.* 1996;62(2):160-162. doi:10.1055/s-2006-957841

21. Kula M, Majdan M, Radwańska A, Nasal A, Hałasa R, Głód D, et al. Chemical composition and biological activity of the fruits from *Lonicera caerulea* var. *edulis* 'Wojtek'. *Academia J Med Plants.* 2013;1:141-8. doi: 10.15413/ajmp.2013.0134.

22. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev.* 2009;2(5):270-278. doi:10.4161/oxim.2.5.9498

23. Bouarab-Chibane L, Forquet V, Lanteri P, et al. Antibacterial Properties of Polyphenols: Characterization and QSAR (Quantitative Structure-Activity Relationship) Models. *Front Microbiol.* 2019;10:829. Published 2019 Apr 18. doi:10.3389/fmicb.2019.00829

24. Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.* 2004;74(17):2157-2184. doi:10.1016/j.lfs.2003.09.047

25. Graham HN. Green tea composition, consumption, and polyphenol chemistry. *Prev Med.* 1992;21(3):334-350. doi:10.1016/0091-7435(92)90041-f.

26. Jaiswal R, Müller H, Müller A, Karar MG, Kuhnert N. Identification and characterization of chlorogenic acids, chlorogenic acid glycosides and flavonoids from *Lonicera henryi* L. (Caprifoliaceae) leaves by LC-MSn. *Phytochem-*

istry. 2014;108:252-263. doi:10.1016/j.phytochem.2014.08.023

27. Liby KT, Yore MM, Sporn MB. Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. *Nat Rev Cancer*. 2007;7(5):357-369. doi:10.1038/nrc2129

28. Patlolla JM, Rao CV. Triterpenoids for cancer prevention and treatment: current status and future prospects. *Curr Pharm Biotechnol*. 2012;13(1):147-155. doi:10.2174/138920112798868719

29. Kumari S, Deori M, Elancheran R, Kotoky J, Devi R. In vitro and In vivo Antioxidant, Anti-hyperlipidemic Properties and Chemical Characterization of Centella asiatica (L.) Extract. *Front Pharmacol*. 2016;7:400. Published 2016 Oct 28. doi:10.3389/fphar.2016.00400

30. Guo C, Yang J, Wei J, Li Y, Xu J, Jiang Y. Antioxidant Activities of Peel, Pulp and Seed Fractions of Common Fruits as Determined by FRAP Assay. *Nutr Res*. 2003;23:1719-26.

31. Liguori I, Russo G, Curcio F, et al. Oxidative stress, aging, and diseases. *Clin Interv Aging*. 2018;13:757-772. Published 2018 Apr 26. doi:10.2147/CIA.S158513

32. Verzelloni E, Tagliazucchi D, Conte A. Relationship between the antioxidant properties and the phenolic and flavonoid content in traditional balsamic vinegar. *Food chem*. 2007;105:564-71.

33. Marie D, Ateba G, Felicite K, Fernando K, Chia A. Oxidative Stress in Patients with Chronic Inflammatory Diseases in a Tertiary Health Care Setting in Africa. *J Autoimmune Disord*.

2017;3:47.

34. Grace-Lynn C, Darah I, Chen Y, Latha LY, Jothy SL, Sasidharan S. In vitro antioxidant activity potential of lantadene A, a pentacyclic triterpenoid of Lantana plants. *Molecules*. 2012;17(9):11185-11198. Published 2012 Sep 19. doi:10.3390/molecules170911185

35. Santiago LA, Dayrit KC, Correa P, Mayor AB. Comparison of antioxidant and free radical scavenging activity of triterpenes α -amyrin, oleanolic acid and ursolic acid. *J Nat Prod*. 2014;7:29-36.

36. Lopez-Romero JC, González-Ríos H, Borges A, Simões M. Antibacterial Effects and Mode of Action of Selected Essential Oils Components against Escherichia coli and Staphylococcus aureus. *Evid Based Complement Alternat Med*. 2015;2015:795435. doi:10.1155/2015/795435

37. Caprioli G, Iannarelli R, Innocenti M, et al. Blue honeysuckle fruit (*Lonicera caerulea* L.) from eastern Russia: phenolic composition, nutritional value and biological activities of its polar extracts. *Food Funct*. 2016;7(4):1892-1903. doi:10.1039/c6fo00203j

38. Raudsepp P, Anton D, Roasto M, Meremäe K, Pedastsaar P, Mäesaar M, et al. The antioxidative and antimicrobial properties of the blue honeysuckle (*Lonicera caerulea* L., Siberian rhubarb (*Rheum rhaponticum* L.) and some other plants, compared to ascorbic acid and sodium nitrite. *Food control*, 2013;3:129-35.

39. Sandigawad AM. Analysis of Phytochemicals and Antibacterial Potential of *Lonicera Japonica* Thunb. *Int J Pharm Bio Sci*. 2015;6:571-83.