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Original Article

The Extract Analysis and Antibacterial Survey of Different Parts of Gilan Native *Clerodendrum bungei* on Clinical Isolates

Running title: The Antibacterial Effect of the Extract of *Clerodendrum Bungei*

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Abstract

Medicinal uses of plants to treat of diseases has increased in recent years. *Clerodendrum* is a genus of plants belonging to Verbenaceae family. *Clerodendrum bungei* Steud. is native to the Gilan province, but its antibacterial effects have not been studied. For this reason, the aim of the present study was to investigate the phytochemical analysis and antibacterial properties of the alcoholic extracts of *C. bungei*. was collected from Sowme' eh Sara city located in the northwest of the Gilan province, from May to August 2018 with equal amount. Extracts were prepared from different parts of *C. bungei* using 70% ethanol by the maceration method and were analysed using GC/MS. Seven bacterial isolates from clinical samples were collected from patients referred to the social welfare polyclinic in Rasht. After confirmatory diagnostic tests for all specimens, an antibiogram test (disk diffusion method) was performed and the antimicrobial activities of the extracts were evaluated using disk diffusion and broth dilution methods on 7 isolates of *Escherichia coli*, *Enterobacter*, *Shigella*, *Klebsiella*, *Pseudomonas*, *Staphylococcus aureus*, *coagulase negative Staphylococcus*. The results were showed that the alcoholic extract of the leaves before flowering was more effective on *Pseudomonas* than other bacteria. Also, the alcoholic extract of the leaves during flowering was effective on *S. aureus*. The extract of the flower was effective on *E. coli* only at a concentration of 250 mg/ml. The MIC values of the different alcoholic extracts of *C. bungei* on all isolates were 8.06 to 500 mg/ml. The MBC values of the different extracts of *C. bungei* on isolates were 500 mg/ml. The phytochemical compound, phytol was identified as the main component in the leaf extracts of *C. bungei* before (9.52%) and during (3.6%) flowering and therefore it seems to be the compound responsible for the antimicrobial properties of *C. bungei* leaves. The main effective components in the flowers, stems and roots of *C. bungei* were identified to be linalool (7.8%), aziridine (3.53%), thymol (21.84%), respectively. Linalol prevents dental cavity and has antiallergic, antiviral and antibacterial effects. Thymol has a very strong antimicrobial properties and can destroy outer membrane (OM) of gram-negative bacteria and remove the lipopolysaccharides and increase the permeability of the plasma membrane. Among the chemical compounds identified in the extracts, compounds with functional groups and acircular structure seems to have antibacterial properties. It is concluded from this study that *C. bungei* possesses antibacterial properties.

Keywords: *Clerodendrum bungei*, Disc Diffusion, Antimicrobial test, Phytochemical compounds, GC-MS

Introduction

Medicinal uses of plants to treat of diseases has increased in recent years. The overuse of chemical drugs for the treatment of diseases has led to the increasing outbreak of microbial resistant isolates. Resistant to chemical drugs is has encouraged attempts to find alternative antimicrobial agents. Plants and their compounds, including different essential oils and extracts, have the potential to be substituted for chemical drugs, while they impose less side effects compared to chemical drugs [1].

Medicinal plants are rich in secondary metabolites and, in fact, are the main sources of many medicinal substances with one or more of their tissues containing an effective ingredient. This ingredient usually composes less than 1% of the dry weight of the plant. It is unclear exactly since when the plants have been used as drugs. Certainly, information about the effects and properties of medicinal plants have existed from a very long time ago and have

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finally reached us today. These efforts have continued to this day and are now proceeding as at a fast rate [2].

Clerodendrum spp. is a big genus of plants from the Verbenaceae family. 580 species of this genus has already been identified around the world [3] which are widely distributed in the tropical and subtropical regions of the world and exist in the form of trees and small shrubs. Many species of Clerodendrum are used in traditional medicine in countries such as India, China, Japan and Thailand [4]. *C. bungei* Steud. has been used in traditional medicine for treating boils, hemorrhoids, eczema and its roots are used to reduce hypertension (high blood pressure) and treat rheumatism, Thiamine deficiency (beriberi), and uterine prolapse [5].

This plant was named by one of nineteenth-century botanists, Professor Alexander von Bang. This native Chinese plant is a shrub that grows up to 180 to 200 centimeters. The leaves are reciprocal and ovoid and very broad and reach 30 cm in length. The color of the surface of the leaves is dark green and the color of the underlying leaf is reddish. The flowers are pink and 8 mm in diameter in bulk in a muddy inflorescence at a width of 20 to 10 cm. The small buds of these flowers are spiky in color. When they open they are mildly pink. The flowers have a sharp smell. The flowering time is from mid-summer to early fall. The chemical components of this genus are phenylpropanoids and phenylethanoid glycosides, flavonoids, diterpenoids and iridoids, glycosylated and steroid compounds. Shan-Shan Liu *et al*., (2009), extracted various glycoside and ethanoid compounds from the root (*C. bungei*) using spectroscopic methods. The results of this study showed that biochemical compounds in the root have an inhibitory effect on cell proliferation of human cervical cancer (CCL-2) [5].

C. bungei is one of the species of this genus that in traditional medicine, in China, leaves and stems are used for detoxification. *C. bungei* is a plant of this genus that is native to the Gilan province, but its antibacterial effects have not been studied. For this reason, the aim of the present study was to investigate the antibacterial properties of the alcoholic extracts of *C. bungei*.

Material and Methods

Collecting Plants and Preparation of Ethanolic Extract

C. bungei Steud. was collected from Sowme' eh Sara city located in the northwest of the Gilan province, from May to August 2018. Extracts were prepared from different parts of *C. bungei* (leaves before and during flowering, flower, stem and root) using 70% ethanol by the maceration method.

Fig. 1 *C. bunge*i Steud.before flowering

At first,different parts of the *C. bungei*, including leaf before flowering, flower, leaf during flowering, root and stem were collected. After confirmation of the genus and species in the Herbarium lab of Research Institute of Forests and Rangelands (herbarium code: 107149) by botanist, they were dried in shade and powdered.

Maceration method was used for extraction. In this method, 10 grams of powdered *C. bungei* were poured for 100 mL of solvent (70% ethanol) and placed on a shaker for 24-48 hours and after this time, the extract was passed through whatman filter paper. The solvent was then removed using a distillation apparatus and kept at a refrigerated temperature and in dark until used.

Collecting Samples and Isolating the Bacteria

This study was conducted to investigate the antimicrobial effect of the alcoholic extract of *C. bungei* on clinical isolates. The isolates were collected from the Social Security Laboratory of Rasht during the three months (April to July 2017). The effect of alcoholic extracts of the leaf - before and during flowering- flower, stem, and root of the plant was investigated on seven bacterial isolates including *E. coli, Enterobacter, Shigella, Klebsiella, Pseudomonas, S. aureus,.coagulase negative Staphylococcus*. The isolates were cultured on nutrient agar medium for accurate identification and confirmation of bacterial genomicity in aseptic conditions and incubated at 37 °C for 24 hours. Then, with the appearance and growth of colonies in the culture medium, the specimens were kept in the refrigerator for subsequent diagnostic tests. In order to identify the bacteria, direct microscopic observation of Gram staining slide were used followed by culture on differential culture medium including EMB, TSI, Simmons' Citrate Agar (SCA), Urea, *Salmonella*, *Shigella* Agar (SS) and Mannitol Salt Agar (MSA) as well as Oxidase Test.

Determining the Antibiotic Susceptibilities of the Isolates

In this study, disk diffusion method was used to measure the susceptibility of isolates to 10 antibiotics including amoxicillin (20µg), cefalotin (30µg), ciprofloxacin(5µg), gentamicin(10µg),nitrofurantoin(300µg), ceftriaxone (30µg), Imipenem (10µg), nalidixic acid (30µg),

chloramphenicol (30µg), tetracycline (30µg)antibiotic disc which is manufactured by Padtan Teb company. From a 24-hour culture, the isolates with turbidity condition equal to 0.5 McFarland's turbidity (1.5×10^8 CFU / ml) were cultured on Muller-Hinton Agar (MHA). The antibiotic discs were placed on the MHA plates using a pair of sterile forceps. It was then incubated for 24 hours at 37 °C and then the diameter of the inhibition zone of bacteria was measured using a ruler. The susceptibility or resistance of each of the bacteria to the examined antibiotics was determined using the CLSI (Clinical and Laboratory Standards Institute) 2018.

Determining the Susceptibilities of the Isolates to the Alcoholic Extract by Disk Diffusion Method

Three concentrations of 500, 250 and 125 mg/ml of the alcoholic extracts were used to prepare the disks. First, blank disks were placed inside the plate next to the flame and under sterile conditions using a sterile clamp and were placed in an incubator at 37 °C until it dried up. This was repeated several times till the amount of extract in each disk reached 200 µl. After drying, the disks were kept in the refrigerator for later use. First, the Muller Hinton Agar (Quelab) was prepared according to the manufacturer's instructions and after the 24 hour, cultivation of isolates in nutrient agar culture medium (Which had the turbidity, equivalent to 0.5 MacFarlend), the lawn culture was done on it. The disks impregnated with the extracts were placed at the right distance from each other on the MHA medium and then incubated for 24 hours at 37 °C. Then the diameter of the inhibition zone was measured using a ruler.

Determining the MIC and MBC of the Extracts Using Broth Dilution Method

To determine the MIC, first, the Muller Hinton Broth (Quelab) culture was prepared according to the manufacturer's instructions. For each bacterium, 10 tubes were considered; all tubes were fed 0.5 ml of normal saline and 0.5 ml of Sterile Muller Hinton Broth culture medium. Then, 0.5 ml of the extract was poured into the first tube at the concentration of 500 mg/ml and the extract was diluted by serial dilution method. Then 0.5 ml of 24-hour culture of bacteria with 0.5-MacFarland turbidity was poured into all tubes and the content of each tube was well mixed. Two tubes were considered as positive and negative controls. In the negative control tube, only 0.5 ml of 0.5 McFarland turbidity of microbial suspension was added to 0.5 ml of the Muller Hinton broth medium. In the positive control tube, only 0.5 ml of the extract and 0.5 ml of the Muller Hinton broth medium were added. The tubes were then incubated for 24 hours at 37 °C and after that, the results were observed. The last tube that was transparent was considered as MIC. This was done for all 5 extracts and the effect of each extract was tested on 7 bacterial isolates including *S. aureus, negative coagulase Staphylococcus, Shigella, Klebsiella, Enterobacter, Pseudomonas and E. coli*. According to the results (MIC), all of the tubes that were transparent and in which the bacterial growth was not observed, were cultured on a culture medium of the MHA (Quelab) and incubated for 24 hours at 37 °C. After that, the plate that did not show any bacterial growth on it and had the lowest concentration of the extract was considered as MBC.

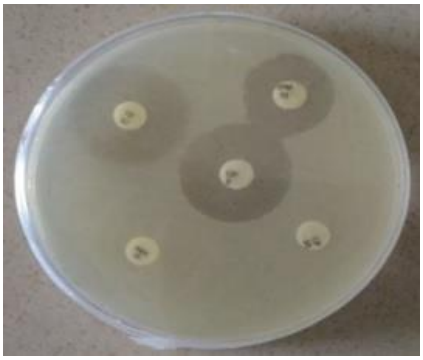


Fig. 2 Antibiogram in Muller Hinton Agar

Fig. 3 The disc diffusion of the extract of the leaves before flowering, Right: *S. aureus*, left: *Klebsiella*.

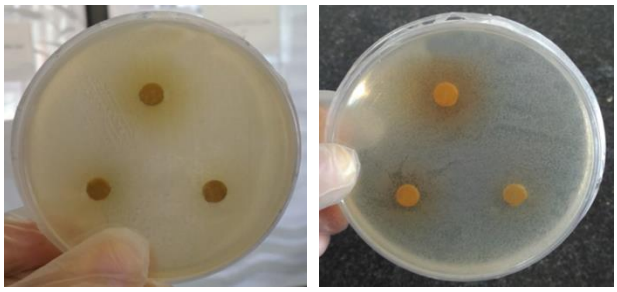


Fig. 4 Determination of susceptibility of *E. coli* isolates (right) and *klebsiella* (left) to different concentrations of flower extract.

Determining the Phytochemical Composition of the Extracts

Phytochemical composition of the alcoholic extract from different parts of *C. bungei* was analyzed using gas chromatography – mass spectrometry (GC-MS) (The Agilent 5977 Series MSD and Agilent 7890 Series GC, USA) in the laboratory of assay and material evaluation at the Islamic Azad University- Branch of Rasht. 1 µl

aliquot from each sample was injected into the GC/MS system. Analysis conditions were 3 min at 40 °C, 3 min at 300 °C for sult column temperature, 260 °C for injector temperature, with helium as the carrier gas and split ratio of 1:30. The sample (1 µl) was evaporated in a split less injector at 260 °C. Run time was 2 min [6]. The phytochemical compounds were identified by comparison of their mass spectra with NIST and mass spectral library.

Statistical Analysis

The results of the study were analyzed using Statistical Package for Social Sciences, version 16.

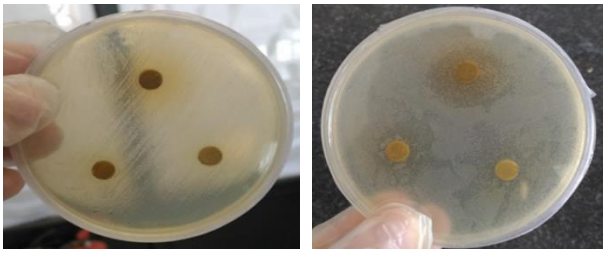


Fig. 5 Determination of susceptibility of *E. coli* (right) and *klebsiella* (left) to different concentrations of stem extract.

Table 1 Results of the disk diffusion method based on CLSI (2013) on clinical isolates

Type of pathogen Antibiotics	*E. coli*	*Enterobacter*	*Shigella*	*Klebsiella*	*Pseudomonas*	*S. aureus*	*Negative Coagulase Staphylococcus*
Nalidixic acid	I*	R**	R	I	-	-	-
Chloramphenicol	S***	R	S	I	R	S	S
Gentamicin	S	S	S	S	S	S	S
Ceftriaxone	S	S	I	I	I	I	S
Nitrofurantoin	S	R	S	R	R	S	S
Imipenem	I	S	R	I	S	S	S
Tetracycline	S	R	S	S	I	S	S
Amoxicillin	S	R	I	R	R	R	R
Cephalothin	I	S	I	S	R	S	S
Ciprofloxacin	I	S	R	S	S	S	S

*Intermediate ** Resistant *** Sensitive

Table 2 Comparison of the diameter of the inhibition zone (in millimeters) of different antibiotics on the 7 clinical isolates

Type of pathogen Antibiotics	*E. coli*	*Enterobacter*	*Shigella*	*Klebsiella*	*Pseudomonas*	*Staphylococcus aureus*	*Negative Coagulase Staphylococcus*
Nalidixic acid	16	9	0	17	-	-	-
Chloramphenicol	19	8	25	16	0	28	28
Gentamicin	20	22	24	19	22	22	15
Ceftriaxone	30	18	22	20	19	16	25
Nitrofurantoin	20	7	23	7	7	19	26
Imipenem	20	28	19	22	27	25	28
Tetracycline	15	8	24	22	17	22	27
Amoxicillin	17	8	12	7	7	12	20
Cephalothin	11	19	14	7	7	19	32
Ciprofloxacin	19	35	7	22	32	25	30

Table 3 Comparison of the diameter of the inhibition zone (mm).

Type of pathogen Alcoholic Extract Concentration (mg/ml)	*E. coli*	*Enterobacter*	*Shigella*	*Klebsiella*	*Pseudomonas*	*S. aureus*	*Negative Coagulase Staphylococcus*
Leaves before flowering /500	0	0	0	15	18	10	12
Leaves during flowering/500	0	7	0	0	0	10	0
Flower extract/500	0	0	0	0	0	0	0
Stem extract/500	20	0	0	7	0	0	0
Root Extract/500	0	0	0	0	8	0	0
Leaves before flowering/250	9	0	9	10	18	8	12
Leaves during flowering/250	0	0	0	0	0	8	0
Flower extract/250	12	0	0	0	0	0	0
Stem extract/250	15	7	0	0	7	7	0
Root Extract/250	0	0	0	0	7	0	0
Leaves before flowering/125	8	8	8	8	8	7	8
Leaves during flowering/125	0	0	0	7	0	0	7
Flower extract/125	0	0	0	0	0	0	0
Stem extract/125	13	0	0	0	7	0	0
Root Extract/125	8	9	0	0	8	0	0
Leaves before flowering/62.5	7	8	8	8	8	7	9
Leaves during flowering/62.5	0	0	0	0	0	0	0
Leaves before flowering/32.25	7	9	8	7	8	7	8
Leaves during flowering/32.25	0	0	0	7	0	0	7

Different concentrations of the alcoholic extracts of different parts of the *C. bungei* Steud. plant on the 7 clinical isolates.

Table 4 Evaluation of the Minimum Inhibitory Concentration (MIC) of the alcoholic extracts of *C. bungei* Steud. (mg/ml) on clinical isolates.

Plant organs Pathogen	Leaves before flowering	Leaves during flowering	Flower	Stem	Root
E. coli	62.5	125	32.25	32.25	32.25
Enterobacter	250	250	62.5	250	250
Shigella	125	62.5	32.25	125	125
Klebsiella	250	250	125	500	250
Pseudomonas	250	125	250	250	250
S. aureus	125	16.12	62.5	8.06	125
Negative Coagulase Staphylococcus	62.5	62.5	62.5	16.12	16.12

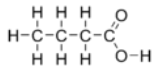
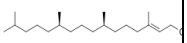
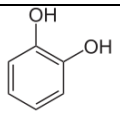
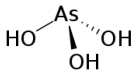
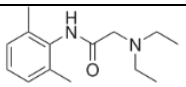
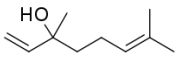
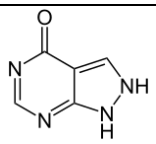
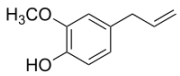
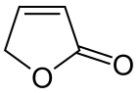
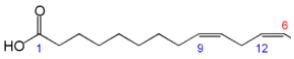
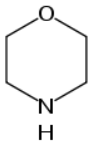
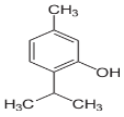
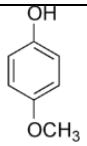
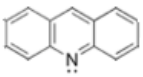
Table 5 Evaluation of results of Minimum Bactericidal Concentration (MBC) of the alcoholic extracts of *C. bungei* Steud. (mg/ml) on clinical isolates.

Plant organs Pathogen	Leaves before flowering	Leaves during flowering	Flower	Stem	Root
E. coli	0	500	500	500	500
Enterobacter	500	500	500	500	500
Shigella	0	500	500	500	500
Klebsiella	500	500	500	500	500
Pseudomonas	0	500	500	500	500
S. aureus	0	500	500	0	500
Negative Coagulase Staphylococcus	0	500	500	500	500

Table 6 Amount and components of the alcoholic extracts of the different parts of *C. bungei* Steud. were measured and determined using gas chromatography – Mass spectrometry(GC-MS)

Plant Organ	No. / Component	RT (min)	Peak area (%)
Leaves before flowering	1. Benzoic acid	22.89	4.67
	2. Phytol	29.2	9.52
	3. Gamolenic acid	29.5	9.29
Leaves after flowering	1.Succinic acid	20.5	1.92
	2. Silane	22.8	2.64
	3.Morphinan	25.35	0.86
	4. Phytol	29.2	3.6
	5. Silicic acid	29.5	1.5
	6. Arsenous acid	35.7	0.15
Flower	1. Linalool	18.01	7.8
	2. Maleamic acid	19.9	5.15
	3. Allopurinol	19.02	4.91
	4. Furanone	7.8	3.08
	5. Benzene	19.2	2.78
	6. Ethanol	18.7	2.53
	7. Morpholine	34.2	0.43
	8. Benzoic acid	5.53	1.78
	9. Triethylene diamine	9.59	1.23
	10. Mequinol	11.8	1.2
	11. Butanoic acid	14.09	1.76
	12. Catechol	14.31	1.39
	13. Silane	22.5	1.36
	14. 2- methoxy – 4- vinyl- 1-phenol	16.6	0.53
	15. Lidocaine	24.6	0.42
	16. Ethane	39.1	1.06
	17. Lethane	19.8	1.6
Stem	1. 2- methoxy -4- vinyl-phenol	16.8	1.84
	2. Aziridine	20.3	3.53
	3 Acetic acid	20.7	3.18
	4. Acridine	21.99	0.65
	5. Butanoic acid	22.8	2.72
	6. Phytol	29.2	1.27
Root	1. 2- methoxy -4- vinyl-phenol	16.44	2.24
	2. Eugenol	17.48	1.55
	3. Acridine	20.7	1.97
	4. Silane	22.89	2.80
	5. Phthalic acid	25.94	1.02
	6. Hexadecanoic acid	27.66	9.89
	7. Linoleic acid	29.8	4.64
	8. Eicosane	29.9	4.30
	9. Thymol	16.8	21.84
	10. Arsenous acid	18.42	2.84
	11. Silicic acid	20.06	8.67

Table 7 Indicator components of *C. bungei* Steud. extract in different stages of growth and their characteristics

No.	Compound	Molecular formula	Chemical structure	No.	Compound	Molecular formula	Chemical structure
1	Benzoic acid	$C_7H_6O_2$		10	Butanoic acid	$C_4H_8O_2$	
2	Phytol	$C_{20}H_{40}O_1$		11	Catechol	$C_4H_4O_2$	
3	Silicic acid	$H_4O_4Si_1$		12	Silane	Si_1H_4	
4	Arsenous acid	$H_3As_1O_4$		13	Lidocaine	$C_{14}H_{22}N_2O_1$	
5	Linalool	$C_{10}H_{18}O_1$		14	Acetic acid	CH_3COOH	
6	Allopurinol	$C_5H_4N_4O_1$		15	Eugenol	$C_{10}H_{12}O_2$	
7	Furanone	$C_4H_4O_2$		16	Linoleic acid	$C_{18}H_{32}O_2$	
8	Morpholine	$C_4H_9N_1O_1$		17	Thymol	$C_{10}H_{14}O_1$	
9	Mequinol	$C_7H_8O_2$		18	Acridine	$C_{13}H_9N_1$	

Results

Antibiotic Sensitivity Test Results

For this study, 10 types of antibiotic disks were used including Nalidixic Acid, Chloramphenicol, Gentamicin, Ceftriaxone, Nitrofurantoin, and Imipenem, Tetracycline, Amoxicillin, Cephalothin and Ciprofloxacin (Fig. 3). Antibiogram results are shown in Tables 1 and 2. Results of determining the susceptibility of the alcoholic extracts of *C. bungei* on clinical isolates

In this study, disks prepared with alcoholic extracts from different parts of the plant were tested on clinical isolates (Fig. 4 to 6), the results are shown in Table 3.

According to table 4, the MIC range of the leaf extract before flowering, the leaf extract during flowering, flower, stem, and root was measured as 62.5 -250 mg/ml, 16.12-250 mg/ml, 32.25-250 mg/ml, 8.06-500 mg/ml, and 16.12-250 mg/ml, respectively. Results of MIC of the Extracts on Clinical Isolates

Results of MIC of the alcoholic extracts of *C. bungei* Steud. are shown in Table 4. Minimum Bactericidal Concentration (MBC) results for the alcoholic extracts of *C. bungei* on clinical isolates

Minimum bactericidal concentration of 7 bacteria including *E. coli*, *Enterobacter*, *Shigella*, *Klebsiella*, *Pseudomonas*, *S. aureus*, *Negative coagulase Staphylococcus* for the alcoholic extracts of the leaf before and during flowering, flower, stem and root of *C. bungei* (Fig. 7) are shown in Table 5. 6 and 11 distinct peaks, respectively, which indicate the different phytochemical compounds in the extracts (Table 6 and Fig. 7).

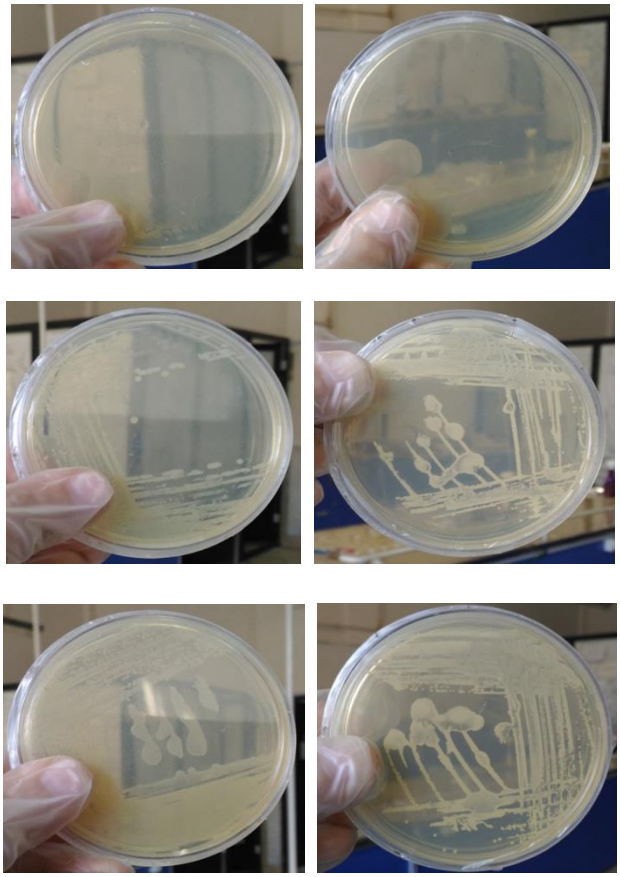


Fig. 6 MBC results from top to bottom, respectively, at concentrations of 500, 250 and 125 mg/ml of extract (The right side is related to the effect of leaf extract before flowering on *Klebsiella* and the left side is related to the effect of flower extract on *S. aureus*).

Results of GC/MS Chromatogram Analysis of Extracts

GC/MS chromatogram analysis of extracts of different parts of *C. bungei* is shown in Table 6. The chromatograms of the extracts of the leaves before and during flowering, flowers, stems and roots show 3, 6, 16,
The compounds present in the extracts and their characteristics are listed in Table 7.
The phytochemical compound, phytol was identified as the most abundant compound in the extract of leaves before (9.52%) and during (3.6%) flowering by comparing their mass spectra with NIST and mass spectral library. The most abundant components in the flowers, stems and roots were identified to be linalool (7.8%), aziridine (3.53%), thymol (21.84%), respectively.

Discussion and Conclusion

Plants can be considered as a source of potential chemical substances that have not been fully exploited. These potentially useful chemicals can be used not only as a medicine, but as a unique model as a starting point for the development of pharmaceutical analogues as well as an interesting tool in order to better understand the biological phenomena [7]. Infection of several viral diseases could be prevented by the application of extracts compounds from *Clerodendrum* spp. [8].
C. bungei is a plant of this genus that is native to the Gilan province, but its antibacterial effects has not been studied. For this reason, the aim of the present study was to investigate the antibacterial properties of the alcoholic extracts of *C. bungei*.
Compounds extracted from plants have natural antimicrobial activity on a large number of pathogenic bacteria. Most of these compounds are similar in their structure containing active phenolic groups.
In fact, Medicinal herbs contain lots of volatile aromatic compounds. These compounds possess innate antioxidative and antimicrobial properties and play an important role in the plant defense system against microbial diseases and can therefore be used as flavors and preservatives in food industries [9- 11].
In addition, metabolites are usually produced as inactive precursors that are stored in plant tissues and are released in response to environmental stresses.
The precursors in plant tissues include phenolic compounds, flavonols and flavonoids, glycosides, alkaloids and polyacetylene [10-12].
Recently, there has been an increasing interest in these compounds have been due to their inhibitory and antimicrobial effects against pathogenic microorganisms [11]. Identifying the compounds in each extract shows that there is a direct correlation between the compounds in each extract and its antimicrobial properties particularly the major compound.
The phytochemical compound,Phytol was identified as the main component in the leaf extracts of *C. bungei* before (9.52%) and during (3.6%) flowering.
The main effective components in the flowers, stems and roots of *C. bungei* were identified to be linalool (7.8%), aziridine (3.53%), thymol (21.84%), respectively.
Phytol is an alcohol with the molecular formula $C_{20}H_{40}O$ and a molecular weight of 128.705 g/mol. Studies by Pejin *et al*. & kang *et al*. showed that phytol has antimicrobial and anti-inflammatory activities [13, 14].
In our study, phytol was identified as the most abundant compound in the leaf extracts of *C. bungei* and therefore it seems to be the compound responsible for the antimicrobial properties of *C. bungei* leaves. Linalool is an alcoholic compound with the molecular formula $C_{10}H_{18}O$ and a molecular weight of 154.25 g/mol was identified as the most abundant compound in the flower extracts of *C. bungei*. Linalol prevents dental cavity and has antiallergic, antiviral and antibacteriall effects. It prevents cancer and has sputum, sedative and hypnotic effects [15]. The results show that there is a direct correlation between the chemical composition of the flower extracts and their antimicrobial properties.

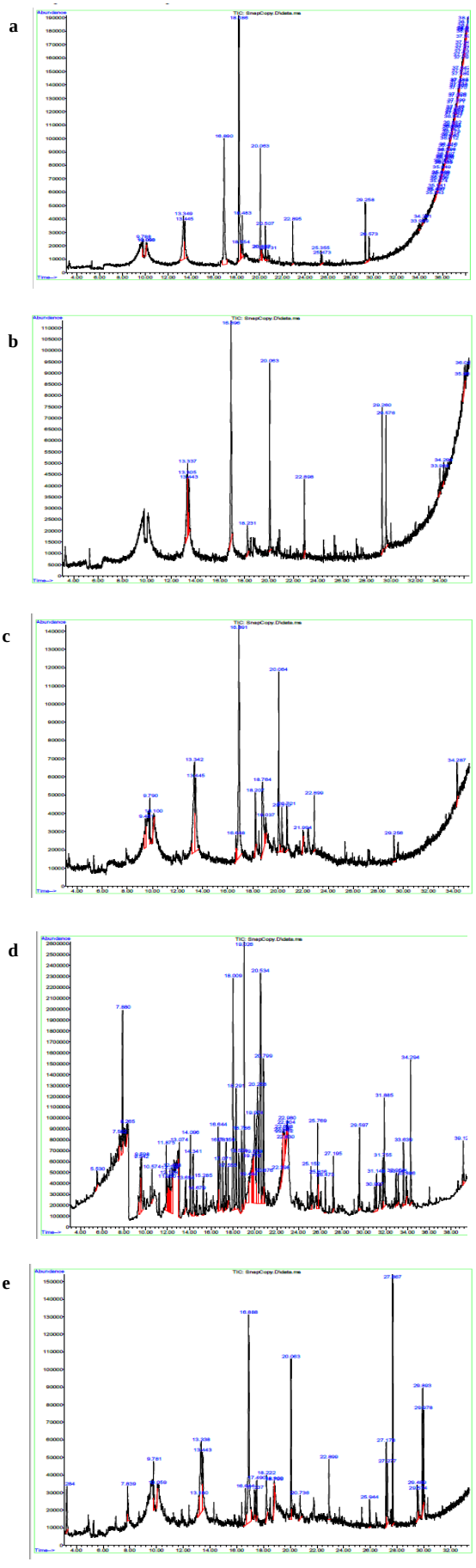


Fig. 6 Chromatogram spectra of *C. bungei* Steud. extract at different growth stages, A: Leaves before flowering, B: Leaves during flowering, C: Flower, D: Stem, E: Root.

Peaks in the chromatogram indicate the number of compounds present in the extract. *Aziridine* is a colorless oily liquid with the molecular formula C_2H_5N and a molecular weight of 43.07 g/mol. It is an organic compound consisting of the three-membered heterocycle (CH_2) 2NH. It is used in the structure of many useful chemical compounds including cationic polymers. Among the chemical compounds identified in the extracts, compounds with functional groups and acircular structure seems to have antibacterial properties [16].

Thymol is a phenolic compound, with the molecular formula $C_{10}H_{14}O$ and a molecular weight of 150.22 g/mol. It has a very strong antimicrobial properties and can destroy outer membrane (OM) of gram-negative bacteria and remove the lipopolysaccharaides and increase the permeability of the plasma membrane [17].

Previous studies showed that thymol has cestocidal anti acne, anti-inflammatory, antioxidant, anti-herpes and anti-rheumatism activities, and can prevent Alzheimer's disease, neuroimflamation, and bronchitis. It can also eliminate bad breath, and prevent dental plaques [18].

The antimicrobial properties of the *C. bungei* roots, therefore, can be due to high amount of thymol in it.

The MBC results of the extracts showed that the alcoholic extracts of flowers, roots, and leaves during flowering at a concentration of 500 mg/ml had a bactericidal effect and at lower concentrations had a bacteriostatic effect. At 500 mg/ml, root extracts showed a bactericidal effect against all bacteria, except *S. aureus*, on which a bacteriostatic effect was observed. Furthermore, leaf extract before flowering – at the concentration of 500 mg/ml - showed a bactericidal effect for *Enterobacter* and *Klebsiella* and a bacteriostatic effect against the other bacteria.

Momoh *et al*., in 2014 studied the efficacy of *Clerodendrum capitatum* for the treatment of tuberculosis. They studied its antibacterial effects against *Klebsiella pneumoniae*, *Corynebacterium ulcerans*, *Salmonella typhi*, *Shigella dysentery*, *S. aureus*, Methicillin resistant *S. aureus* (MRSA), *Proteus mirabilis* and *Streptococcus pneumonia*, and its antifungal effects against *Candida tropicalis*, *C. Cruzei*, *C. albicans*. They showed that growth of all bacteria except *S. aureus* was inhibited at 25.3 mg/ml concentration and *C. albicans* growth was inhibited at 7.5 mg/ml. All bacteria were completely eliminated at 7.5 mg/ml [19].

Unlike *C. capitatum*, there are few studies reporting on the antimicrobial properties of *C. bungei*. Since this plant is native to Gilan province, recognizing its antimicrobial properties is very important. We found out that alcoholic extract of this plant possesses bactericidal effects at high concentration and bacteriostatic effects at lower concentrations. The difference between the MBC obtained in the present study and the one by Momoh *et*

al. (2014) could be attributed to the difference in the effective composition of these two plant species.

In the study by Shan-Shan Liu *et al*. (2009), various compounds of Glycosides and Etanoide from the root (*C. bungei*) were extracted by spectroscopy and examined its cytotoxic effects on proliferation of cervical carcinoma cells in vitro. The results of this study showed that the root biochemical compounds have an inhibitory effect on human erythrocyte proliferation (CCL-2) [5]. In this study, a disc diffusion method was used to extract leaves before flowering; the largest diameter of the inhibition zone was at the concentration of 500 mg/ml for *Pseudomonas* spp. with a diameter of 18 mm. The smallest diameter of the inhibition zone was for *S. aureus* with a diameter of 10 mm. There was no halo for the *E. coli*, *Enterobacter* and *Shigella* in this concentration. At a concentration of 250 mg/ml, the largest diameter of the halos was for *pseudomonas* with a diameter of 18 mm and the smallest was for *S. aureus* with a diameter of 8 mm. At a concentration of 125 mg/ml, except for *S. aureus* with an inhibition zone diameter of 7 mm, the diameter of the halo was 8 mm for the rest of the bacteria. These results indicate that the alcoholic extract of the leaves before flowering was more effective on *Pseudomonas* than the other bacteria studied. These results, to some extent, are consistent with the results of the 2013 study by Borik *et al*. In this research, it was found that the *C. baroianum* extract had an antibacterial effect [20]. Among bacteria *B. subtilis, P. aeruginosa, E. coli, S. aureus*, the most antibacterial effect was on *B. Subtilis, P. aeruginosa, E. coli* and it was found that the agent of this antimicrobial effect is a steroid compound [20]. In the disc diffusion method for the leaf extract during flowering, at concentration of 500 mg/ml, the highest diameter of inhibition zone was for *S. aureus* with a diameter of 10 mm and the lowest diameter of the halo was for *E. coli* with a diameter of 7 mm and for other bacteria, no halo was observed. At a concentration of 250 mg/ml, only *S. aureus* produced an inhibition zone diameter of 8 mm and there was no halo for the other isolates. At a concentration of 125 mg/ml, only *Klebsiella* and *negative coagulase Staphylococcus* produced an inhibition zone with a diameter of 7 mm. These results are not consistent with the results of the study by Venkatanarasimman *et al*. (2012) where they examined the effect of the raw ethanol extract of *C. philippinum Schauer* leaves on *E. coli, S. aureus, Bacillus and Klebsiella*. The plant effectively inhibited the growth of *E. coli, S. aureus, Bacillus* and *Klebsiella*. The maximum diameter of the inhibition zone was 20 mm. Inhibitory concentration (MIC) *C. philippinum* was 100 µg/ml for each of the 4 tested bacteria. The maximum diameter of the inhibition zone was for *E. aerogenes* and the lowest diameter of the inhibition zone was for *Bacillus* and the *P. aeruginosa* inhibition was moderate [21].

Disc diffusion method for the flower extract showed that at a concentration of 250 mg/ml, *E. coli*, had an inhibition zone diameter of about 12 mm. There was no halo for the other bacteria and concentrations. Also, for the stem extract, the highest diameter of the inhibition zone was at the concentration of 500 mg/ml for *E. coli* 20 mm. The smallest diameter of the inhibition zone was for *Klebsiella* (7 mm) and no halo was observed for other bacteria. At the concentration of 250 mg/ml, the largest diameter of the inhibition zone was for *E. coli* (15 mm), and the lowest diameter of the inhibition zone was for *Enterobacter, Pseudomonas* and *S. aureus* (7mm) and there was no halo for other bacteria. At the concentration of 125 mg/ml, the inhibition zone was formed only for the *E. coli* and *Pseudomonas* with a diameter of 13 mm and 7 mm, respectively and no halo was observed for other bacteria.

Disc diffusion method for root extract showed that at the concentration of 500 mg/ml, the highest inhibition zone was for *Pseudomonas* (8mm) and no halo was observed for other bacteria. At the concentration of 250 mg/ml, the highest diameter of the inhibition zone was for *Pseudomonas* (7 mm) there was no halo for other bacteria. At the concentration of 125 mg/ml, the largest diameter of the inhibition zone was for *Enterobacter* (9 mm) and the smallest diameter was for *pseudomonas* and *E. coli* (8 mm) and no halo was observed for other bacteria. Comparison of the diameter of the inhibition zone of the extracts from different parts of the *Clerodendrum* at concentrations of 250, 125, 62.5 and 31.25 (mg/ml) on 7 bacterial isolates showed a significant difference in antimicrobials properties between different parts of the *C. bungei* ($P = 0.006$). In this study, all the extracts of this plant have a bacteriostatic effect. The bactericidal effect of flower extracts, leaves during flowering and bactericidal effect of flower extract and root is greater than that of leaf extract before flowering and stem and thus, it is possible to offer effective ingredients of *C. bungei* for the preparation of herbal medicines.

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References

1. Izadi Z, Modarres Sanavi SAM, Sorooshzadeh A, Sna-Ashari M, Davoodi P. Antimicrobial activity of chamomile (*Matricaria chamomilla* L.) and feverfew (*Tanacetum parthenium* L.). J Armaghane-danesh. 2013;18:31-43.
2. Shahnia M, Khaksar R. Antimicrobial effects and determination of minimum inhibitory concentration (MIC) methods of essential oils against pathogenic bacteria. Iran J Nutr Sci Food Technol. 2013;7:949-955.

3. Oly WT, Islam W, Hassan P, Parween S. Antimicrobial Activity of *Clerodendrum viscosum* (Verbenaceae). Int J Agric Biol. 2011;222-226.
4. Shrivastava N, Patel T. Clerodendrum and Heathcare: An Overview. Med Aroma Plant Sci Biotech, Global Science Books. 2007;142-150.
5. Liu SS, Zhou T, Zhang SW, Xuan LJ. Chemical Constituents from *Clerodendrum bungei* and Their Cytotoxic Activities. Helvetica Chimica Acta. 2009;92:1070-1079.
6. Balaji K, Kilimozhi D. GC-MS Analysis of various extracts of Clerodendrum phlomidis leaf. Int J Pharm Pharmaceut Sci. 2014, 6(1): 226-232.
7. Waliullah TM, Yeasmin AM, Ashraful A, Wahedul IM, Parvez H. Antimicrobial potency screening of *Clerodendrum infortunatum*. Int Res J Pharm. 2014;5:57-61.
8. Awasthi LP, Singh SP. Clerodendrum- A Novel Herb Having Broad Spectrum Antimicrobial Properties. Asi Agri His. 2015;19:33-44.
9. Delaquis PJ, Mazza G. Antimicrobial properties of isothiocyanates in food preservation. J Food Technol. 1995;49:73-84.
10. Lanciotti R, Gianatti A, patrignani F, belletti N, Guerzoni ME, Gardini F. Use of natural aroma compounds to improve shelf life and safety of minimally processed fruits. J Food Sci Technol. 2004;15:201-8.
11. Leitner L. Basic aspect of food preservation by hurdle technol. Int J Food Microbiol. 2000;55:18-186.
12. Kim J, Marshal M, Wei C. Antibacterial activity of some essential oil components against five food borne pathogens. J Agri Food Chem. 1995;43:2839-45.
13. Pejin B, Savic A, Sokovic M, Glamocilija J, Ciric A, Nicolic M, *et al*. Further in vitro evaluation of antiradical and antimicrobial activities of phytol. Nat Prod Res 2014;28:372-6.
14. Kang CH, Jayasooriya R, Choi Y, Moon SK, Kim WJ, Kim GY. β-Ionone attenuates LPS-induced proinflammatory mediators such as NO, PGE2 and TNF-in BV2 microglial cells via suppression of the NF-B and MAPK pathway. Toxicol In Vitro 2013;27:782-7.
15. Dakhili M, Zahrai Salehi T, Torabi Goodarzi M, Khavari A. Evaluation of antimicrobial effects of 4 medicinal plants against salmonella typhymurium and comparison them with common antibiotics in veterinary medicine. J Med Plants. 2006;5:21-24.
16. Ezati Sh, Mirzaie A, Zandi M. Evaluation of Anti-efflux Activity of Anthemis atropatana Extract against Ciprofloxacin Resistant *Staphylococcus aureus* Isolates. J Medicinal Plants. 2018;17:122-195.
17. Burt, S. Essential oils: Their antibacterial properties and potential applications in foods-A review. International J Food Microbiology. 2004;94:223-253.
18. Zargari A. Medicinal Plants. 5th ed. Tehran: Tehran University Press. 1995. p. 77-81
19. Momoh H, Habila JD, Ayo RG, Ndukwe GI. *Clerodendrum capitatum* (Willd) leave extracts inhibits bacteria, fungi and *Mycobacterium bovis*. Sch Acad J Biosci (SAJB). 2015;3:386-391.
20. Borik RM. Isolation and Structural Characterization of a Steroidal Antimicrobial Agent from *Clerodendrum baroianum*. World J Chem. 2013;8:48-54.
21. Venkatanarasimman B, Rajeswari T, Padmapriya B. Antibacterial Potential of Crude Leaf Extract of *Clerodendrum philippinum* Schauer. Int J Pharm Biol Arch. 2012;3:307-310.