Medicinal plant: Assessment of the chemical composition and in vitro antibacterial activities of the *Viola odorata Linnoil'sagainstBacillussubtilis* (ATCC No. 21332) in west of Iran.

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Abstract: Considering the drug resistance and the side effects of chemical antibacterial drugs, the research approach is increasingly going toward using natural resources. Certainly, using herbal plants is the oldest way of mankind to treat bacterial diseases. As we know, there is no documented proof on antibacterial properties of Viola odorataLinn (VOL) oil in west of Iran. The aim of the study was to assessment the chemical composition and in vitro antibacterial activities of the oil from VOL against Bacillussubtilis (BS (ATCC No. 21332))in west of Iran (In Kermanshah). Gaschromatography mass spectrometry was run tospecify their chemical composition. As a screen test to detect antibacterial effects of the oil, agar disk and agar well diffusion methods were employed. Macrobroth tube test was performed to determinate MIC. The results indicated that the most substance found in VOL was Pentane 2, 3, 4-Trimethyl (41.85%). Also, The MIC and MBC values were 0.031 g/ml for the oil intested bacterium. The results demonstrate that oil of VOL has its own chemical composition, which may be correlated with its antibacterial properties. It can be used as antibacterial supplement in the developing countries towards the development of recent therapeutic agent.

Keywords: Viola odorata Linn, oil, Antibacterial activities, Chemical composition.

Introduction

Infection is the invasion of an organism's body tissues by disease-causing agents such as virus, bacterium, and fungus. Infections disease due to bacterial species also stay a serious clinical problem.Antibiotics provide the primary basis for the treatment of microbial (bacterial and fungal) infections. But overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms [1-4]. The spread of drug resistant pathogens is one of the most crucial threats to successful therapy of microbial diseases. Down the ages plants have evoked interest as sources of innate products. The uses of plants in remedy of animal and human diseases have long been established. They have been screened for their potential uses as alternative remedies for the treatment of different infectious diseases [5-10]. Most plants have been shown to possess antimicrobial agents active against microorganisms in vitro. Some medicinal plants used in traditional Iranian medicine are efficacious in treating several ailments caused by bacterial and oxidative stress [11-13]. Aromatic oils are used in many

industries including food preservation, pharmacy and medicine [14-16]. They are expected to form recent sources of antimicrobial drugs especially against bacteria [17]. The antibacterial properties of aromatic oils has been divided into a good, medium or bad [18, 19]. These oils can also produce some defense products (some secondary metabolites) against various natural enemies [20, 21].

VOL is a species of the genus *Viola*, native to Europe and Asia, but has also been introduced to North America and Australia[22]. VOL is one of the edible plants which have generated a lot of interest throughout human history as a medicinal panacea. In herbal medicine, VOL has been used for a variety of respiratory ailments, insomnia, skin, and infectious disorders [23, 24].The study about antimicrobial effects of aqueous extract has been reported against certain opportunistic/secondary invaders and pathogenic bacteria of respiratory tract region [25-27]. Hence, exploring the scientific justifications behind its traditional use could support to find the probability to develop further modern biotechnological applications. The aim of this study was to screen the in vitro antibacterial activity of the plant oil againstBS.

Materialsand Methods

Source of microorganisms

Bacterium specie namely BS (ATCC No. 21332) was procured from Iranian Research Organization for Science and Technology as lyophilized. Bacterium strain was activated on Tryptic Soy broth, constant at 37°C for 18 h. Then 60 µl of the broth was transferred to Nutrient agar and incubated at 37°C for another 24 h; cell concentration was then adjusted to obtain final concentration of 10^8cfu/ml using Muller Hinton broth.

Culture media

Mueller-Hinton Agar (Müller-Hinton agar is a microbiological growth medium that is commonly used for antibiotic susceptibility testing) was prepared according to the manufacturer's instruction, autoclaved and dispensed at 20 ml per plate in 12 x 12cm Petri dishes. Set plates were incubated overnight to ensure sterility before use.

Plant sample collection

In this empirical-experimental study, medicine plant collected from Kermanshah.The sample was cleaned from any strange, plants, dust, or any other contaminants.

Oil extraction of VOLwith Hot Liquid Fats

At one time widely used for extracting the oil from flowers other than tuberose and jasmine, extraction with hot liquid fats is now seldom employed commercially. It is cumbersome and the products obtained do not represent the true perfume of the flowers. VOLis immersed in a specially prepared fat. The mixture is heated to about 80°C for about half an hour and then allowed to cool for an hour. It is finally reheated and then strained or filtered to remove VOL. The proportion by weight of flowers to fat is about 1 to 4. New charges

of VOLare introduced until the total weight of VOLimmersed and macerated is about twice the weight of the fat solvent used. The perfume-saturated fat is sold andmade oil from it in high temperature.

Gas chromatography mass spectrometry (GC/MS)

To analyze oil ofVOL by GC-MS, fused silica DB-5 column with 0.25 μm thickness film was used. The oven temperature was kept at 500°C for 5 minutes and then programmed from 50-2800°C for 40 minutes. Helium flow rate was maintained at 2 ml/min, with the split ratio of 1:3. Sample injection of 1μl and ionization voltage of MS-analysis was run by EI technique at 70ev. The volatile oil constituents were identified by matching their MS and retention index data with those of the standards spectra and by matching their fragmentation pattern in Mass Spectra [28]. NIST standard reference database (AMDIA version 2.70) was used to interpret the mass spectral data.

Evaluation of antimicrobial activities

Agar well and agar disk diffusion methods were used as screen tests to evaluate antibacterial properties of the oil from VOLbased on standard protocol. The solution of the plantwere yielded in 1g/ml from which six fold serial dilutions (v/v) were prepared. 60 μl of each dilution was poured on each disk and well in order. After a period of 24 hours incubation, the diameters of growth inhibition zones around the disks and wells were measured. DMSO was used as negative control whereas cephalexin was used as positive control in case of bacterium. Minimum inhibitory concentration (MIC) means the lowest concentration of the probable antimicrobial agent which prevents growing of bacteria (regardless of killing the bacteria or stopping the growth of them). The lowest dilution which no gross microbial growth has been seen indicates MIC. Minimum bactericidal concentration (MBC) means the lowest concentration of the agent which causes death to test bacteria. The last can be revealed by pouring 60 μl of MIC tube and six dilutions before contents on agar plate. In this case, after incubation period, the lowest concentration which makes no growth indicates MBC. For determination of MIC value, macrobroth dilution method was applied. Interpretation of the results was done due to national accepted letter [29].

Statistical Analysis

Antibacterial effect was determined by One way variance analysis (ANOVA), using the SPSS 18 software package. Data were considered statistically significant at $p \leq 0.01$.

Results

Chemical composition

GC-MS analysis in VOL revealed the presence of Pentane 2, 3,4- Trimethyl, N- Hexadecanoic acid, 10-Undecyn-1-l and Pentadecanoic acid.

Agar disk diffusion test

About VOL, the widest zone was seen in 0.125 g/ml concentration (The value of growth inhibition zone was 10 mm in this dilution). No inhibition zone was observed due to DMSO.Growth inhibition zones due to different dilutions are listed in table 1.

Dilution(g/ml)	**Inhibition zone (mm) in disk diffusion**
Microorganism	***B. Subtilis***
Positive control	22
1/8 (0.125)	10
1/16 (0.062)	9
1/32 (0.031)	8
1/64 (0.015)	0
1/128 (0.007)	0
1/256 (0.003)	0
Negative control	0

Table 1. The diameters of growth inhibition zones in agar disk diffusion test in different dilutions of the oil from VOL.

Agar well diffusion test

In regard to VOL, the widest zone was seen in 0.125 g/ml concentration (The value of growth inhibition zone was 10 mm in this dilution). There was no inhibition zone ofBS due to 0.031, 0.015, 0.007,and 0.003 g/ml concentrations. Growth inhibition zones due to different dilutions are listed in table 2. No inhibition zone was observed due to DMSO.

Dilution(g/ml)	**Inhibition zone (mm) in well diffusion**
Microorganism	***B. Subtilis***
1/8 (0.125)	10
1/16 (0.062)	8
1/32 (0.031)	0
1/64 (0.015)	0

1/128 (0.007)	0
1/256 (0.003)	0
Negative control	0

Table 2. The diameters of growth inhibition zones in agar well diffusion test in different dilutions of the oil from VOL.

MIC andMBC ascertaining

In oil ofVOL, MIC and MBC were 0.031 g/ml for bacterium (table 3).

The oil of VOL.	
Microorganism	***B. Subtilis***
MIC(g/ml)	1/32 (0.031)
MBC(g/ml)	1/32 (0.031)

Table 3: MIC and MBC of the oil of VOL.

Discussion

The development of resistance in bacteria is one of the mechanisms of natural adaptation to the presence of an antimicrobial agent that prevents susceptible organisms and selects the resistant ones.The problem of antibiotic resistance, which has limited the use of cheap and old antibiotics, has necessitated the need for a continued search for novel antimicrobial compounds. Cefalexin or cephalexin, is an antibiotic that can treat a variety of bacterial infections. It destroys gram-positive and some gram-negative bacteria by interrupting the growth of the bacterial cell wall. But, this antibiotic like other antibiotics have several side effects. Current side effects of cephalexin include stomach upset, diarrhea and allergic[30].Plants as a source of medicinal compounds have continued to play a dominant role in the maintenance of human health since ancient times. Interest in plants with antimicrobial effects has revived as a result of common problems associated with the use of antibiotics [31-34]. Also, because of their safety and low cost as well as their effect on a large range of microbesin traditional medicine uses plants. Medicinal plants may have the ability to treat bacterial resistance to several types of antibiotics. The type and level of antibacterial effect exhibited by any plant material depends on many factors, including the plant part, soil conditions, drying method, storage conditions, and post-harvest processing [35]. Oils of medicinal plants stand out as veritable sources of potential resistance modifying agents. Oils are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial activities [36, 37].

VOL belongs to family *Violaceae*. It is commonly known as Sweet Violet, English Violet, Common Violet or Garden Violet and Gulbanafsa in Hindi. VOL is a native of Mediterranean countries and Asia Minor. From old ages, it has been grown in gardens and now it has spread to most of Europe. It is used either alone orin mixture with other herbs for catarrhal and pulmonarytroubles and for calculous affections[23]. VOL possess various important biological effects including uterotonic, insecticidal, anti-HIV, antimicrobial, antineurotensive, cytotoxic and haemolytic activity [24].

Concerning the method of oil and preventing fromusing high temperature to decrease the rate of destruction of efficient herbal compound.GC-MS analysis in oil of VOLindicated the presence of Pentane 2, 3, 4-Trimethyl (41.85%), N-Hexadecanoic acid (29.35%), 10-Undecyn-1-ol (16.79%) and Pentadecanoic acid (9.14%). Pentane 2, 3, 4-Trimethyl is a branched alkane. It is one of the isomers of octane. The results of GC/MS demonstrated that oil of VOL was rich of Pentane 2, 3, 4-Trimethyl, which may be correlated with its antibacterial activities.But, the other compounds of VOL have antibacterial properties. In studies showed N- Hexadecanoic acid, pentadecanoic acid and 10-Undecyn-1-ol have considerable antibacterial effects [38, 39].

As the tables shows, the oil of VOL have prevented the growth of BS. By increasing the concentrations of the oil of VOL, the inhibition zone increased ($p \leq 0.01$). The results determined that in tested bacterium, there was a notable difference ($p \leq 0.01$) in terms of sensitivity to the oil. Also, the results demonstrated that VOLwith concentration about 0.031 g/ml has inhibited BS and destroyed it. Thus, the research suggests the antibacterial effectsof the medical plant on Gram-positive pathogenic bacterium. There are correspondences between this result and the similar studies.In a study documented the antibacterial activities of aqueous extract of VOL(aerial parts) against BS [40].In other study reported that aqueous extract of VOL(flowers) showed strong antibacterial action against BS[25].

Conclusion

VOL is an aromatic medicinal plant with antibacterial effects toward BS (ATCC No. 21332). The results indicated that oil of VOL with concentration about 0.031 g/ml has prevented BS and destroyed it. The research represents the antibacterial activities of the medical plant on BS. We believe that the article providesupport to the antibacterial properties of the oil. The results demonstrate the fact that the oil of the VOL can be useful as medicinal or preservatives composition.Additional *in vivo* studies and clinical trials would be needed to justify. Also, further evaluation is necessary on potential of it as an antibacterial agent in topical or oral applications.

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Authors' Contribution

The core idea of this work came from Mohammad Mahdi Zangeneh and AkramZangeneh, also the experiments, evaluation and Statistical Analysis of antimicrobial activities done byFaribaNajafi, Reza Tahvilian, Mohammad Mahdi Zangeneh, AkramZangeneh, RohalahMoradi.

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