Gautam et al. Clinical Phytoscience (2016) 2:4
DOI 10.1186/s40816-016-0018-3

Clinical Phytoscience
a SpringerOpen Journal

ORIGINAL CONTRIBUTION Open Access

A new derivative of ionone from aerial parts of *Viola odorata* Linn. and its antibacterial role against respiratory pathogens

Shiv Shanker Gautam[1*], Navneet Bithel[1], Sanjay Kumar[1], Deepak Painuly[2] and Jashbir Singh[2]

Abstract

Background: *Viola odorata*, widely distributed in Eastern and Western Himalaya region is extensively used in treatment of various respiratory ailment, calculous affections and nervous disorders. It is a rich source of alkaloids, terpenes, flavonoids, glycosides, tannins as well as viola-quercitin and salicyclic acid known as natural aspirin. The present study was aimed to isolate bioactive compounds from the aerial parts of *V. odorata*.

Methods: The isolated compound from aerial parts of *V. odorata* was identified grounded on observed spectral data including ultraviolet–visible (UV–vis), Fourier-transform infrared, gas chromatography-mass spectroscopy, ^{1}H and ^{13}C-nuclear magnetic resonance. The antibacterial efficacy of isolated compound was determined by disc diffusion method against five bacterial strains namely, *Haemophilus influenzae* (MTCC 3826), *Pseudomonas aeruginosa* (MTCC 2474), *Staphylococcus aureus* (MTCC 1144), *Streptococcus pyogenes* (MTCC 422) and *Streptococcus pneumoniae* (MTCC 655) and Minimum inhibitory concentrations (MICs) by a serial dilution method by using 96 well microtiter plates.

Results: Based on spectral data analysis, one new structurally related ionone-like compound known as 3-(2′,4′,6′,6′-tetramethylcyclohexa-1′,4′-dienyl)acrylic acid (**1**) has been isolated from aerial parts of *V. odorata*, with significant bioactivity. The antibacterial efficacy of compound **1** ranged between 7.3 ± 0.28 to 9.3 ± 0.28 mm against selected respiratory bacteria at 6 mg/disc concentration. The MICs were recorded at 32–128 μg/ml. *Streptococcus pyogenes* was fairly resistant and *Haemophilus influenzae* more sensitive than others.

Conclusions: By this study, it can be concluded that compound **1** has significant bioactive properties against tested microorganisms. It validates its use as a new potential source of natural drug for curing the respiratory diseases caused by selected microorganisms.

Keywords: Antibacterial activity, Ionone-like compound, 3-(2′,4′,6′,6′-tetramethylcyclohexa-1′,4′-dienyl)acrylic acid, *Viola odorata*

* Correspondence: gautam12shiv@gmail.com
[1]Department of Botany and Microbiology, Gurukul Kangri University, Haridwar 249404Uttarakhand, India
Full list of author information is available at the end of the article

Background

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide a limitless opportunities for novel drugs discovery due to outstanding availability of chemical diversity. *Viola odorata* Linn. (Violaceae), commonly known as Sweet Violet, English Violet or Common Violet and Gulbanafsa in Hindi, is a medicinal herb natively from Mediterranean countries and Asia Minor. The genus *Viola* contains about 500 species worldwide. Currently, numerous species and varieties of violet is known and used for the extraction of their natural occurring phytoconstituents. *V. odorata* has excessive medicinal values. Its aerial parts were reported as antibacterial, antioxidant, anti-inflammatory [1–3], and in treatment of bronchitis, tonsillitis, respiratory catarrh, cough, asthma, calculous affections and nervous disorders [4, 5]. Recent studies have reported the presence of antibacterial cyclic proteins known as cyclotides, the positively charged bracelet cyclotide cycloviolacin O_2 (cyO_2) in *V. odorata*. It has been found efficient with killing properties against gram negative bacteria including *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium LT2 [6, 7].

The ionones, dihydroionones, ionols and epoxy ionones are some few representative group of compounds explored in violet flowers [8–10]. They are responsible for well natural fragrance with pleasant woody-floral, violet-ionone like scent characteristics [11]. Ionones and their derivatives are a member of terpene, the naturally occurring unsaturated hydrocarbons whose carbon skeletons are composed of isoprene C5 units. The isomers and its derivatives differ due to presence of double bond at 1 or 2 position, and shifting of methyl group in cyclohexane ring. They occur mainly in plant containing beta-carotene. Based on the close structurally related derivatives of ionone, they could be expected to exhibit similar biological activities.

In present study, qualitative chemical screening of aerial parts of *V. odorata* has led to isolation of compound **1** named as 3-(2′,4′,6′,6′-tetramethylcyclohexa-1′,4′-dienyl)acrylic acid, a new derivative of ionone, along with its antibacterial efficacy.

Methods

Chemicals and reagents

All the chemicals and solvents, silica gel for column chromatography of mesh 60–120, silica gel-G for TLC, and silica gel 60 F_{254} (0.25 mm layer thickness precoated on aluminium back), and iodine resublimed were commercial and purchased from Merck, Aldrich and Fluka and were used as received. All the other solvents and chemical used were of HPLC or analytical grade.

Plant material and isolation procedure

The aerial parts of *V. odorata* were collected in April, 2012 from Haridwar and authenticated at Botanical Survey of India, Dehradun (BSD No. 113405). Powdered material (1.2 Kg) was extracted successively with petroleum ether (PET), acetone (ACE) and methanol (MeOH) by using Soxhlet apparatus. The yield of MeOH extract was 1.22 % w/w (14.6 g). The MeOH extract was fractionated by column chromatography (CC) on a silica gel (600 × 30 mm, 60–120 mesh size), and sequentially eluted with chloroform ($CHCl_3$)/ACE with a gradual increment of the $CHCl_3$ content (v/v 100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, each 1000 ml), resulting in 11 lots (A-K) with each 20 fractions (A1-A20). Fraction G6-G20 and H1-H15 showed similar TLC pattern and purified by preparative TLC (prep-TLC) and finally at $CHCl_3$/MeOH/H_2O mixture (v/v, 75:17:8). A yellowish powdered compound **1** (21 mg) was obtained.

Instruments

UV–vis absorption spectra was recorded in the wavelength range 190–800 nm with Shimadzu UV-1800 series Pharmac-spec UV–vis spectrophotometer (Japan). The signals were acquired four times and the mean signals were taken as the best value of the UV spectra. Before every measurement the blank spectrum was also recorded, and automatically subtracted from the sample spectrum by the instrument software. The IR spectra were recorded in the wave number range 400–4000 cm^{-1} on Varian 3100 FT-IR spectrophotometer (Perkin Elmer Spectrum Version 10.03.05). The GC-MS spectra were recorded on Varian Saturn (mass spec model). ^1H and ^{13}C NMR spectra were recorded on JEOL AL 300 FT-NMR spectrometer. Chemical shifts are given as δ value with reference to tetramethylsilane (TMS) as the internal standard.

Spectroscopic data of compound 1

Yellow powdered compound; UV λ_{max} (MeOH) nm: 246.00, 202.00, 222.50; IR (KBr) ν_{max}: 3429.50, 2919.91, 2851.39, 1563.04, 1436.62, 1384.56, 1167.41, 1093.92, 880.64 cm^{-1}; GC-MS: m/z 207 $[M + H]^+$ calcd: for $C_{13}H_{19}O_2$ (207.281); ^1H NMR (300 MHz, DMSO-d_6): δ 7.613 (1H, d, J = 8.14 Hz, H-3), 6.532 (1H, d, J = 8.17 Hz, H-2), 5.406 (1H, s, H-5′), 2.436 (2H, s, H_a-3′, H_b-3′), 1.558 (3H, s, H_a-9′, H_b-9′, H_c-9′), 1.164 (3H, s, H_a-10′, H_b-10′, H_c-10′), 0.757 (6H, s, H_a-7′, H_b-7′, H_c-7′, H_a-8′, H_b-8′, H_c-8′), 3.330 (m, DMSO-d_6); ^{13}C NMR (75.45 MHz, DMSO-d_6): δ 171.645 (C-1), 158.716 (C-3), 154.310 (C-5′), 130.834 (C-1′), 122.652 (C-2), 113.833 (C-4′), 107.423 (C-2′), 34.477 (C-3′), 34.405 (C-6′), 31.269 (C-7′), 28.969 (C-8′), 21.722 (C-9′), 21.431 (C-10′), 39.529 (DMSO-d_6).

Antibacterial assay

Microorganisms used

The bacterial strains causing respiratory tract infections namely, *Haemophilus influenzae* (MTCC 3826), *Pseudomonas aeruginosa* (MTCC 2474), *Staphylococcus aureus* (MTCC 1144), *Streptococcus pyogenes* (MTCC 422) and *Streptococcus pneumoniae* (MTCC 655) were procured from Institute of Microbial Technology (IMTECH), Chandigarh. Bacterial strains were maintained at 4 °C on nutrient agar medium.

Antibacterial testing

The in vitro antibacterial activity was tested by disc diffusion method [12]. This method depends upon the diffusion of the tested material to such an extent that growth of the added microorganism is prevented entirely in a zone around the disc containing a solution of tested material. Muller Hinton Agar (MHA) was used for antibacterial screening. 6 mg/disc concentration of compound was impregnated on the discs. These discs were placed on the surface of the agar plates already inoculated with selected bacterial organisms. An additional control disc without any sample but impregnated with an equivalent amount of solvent (DMSO) was used in the assay. Efficacy of compound **1** against bacteria was compared with a broad-spectrum antibiotic erythromycin as positive control. Plates were incubated at 37 °C for 24 h in BOD incubator. The antibacterial activity was interpreted from size of diameter of zone of inhibition measured in millimetre (mm).

Determination of Minimum Inhibitory Concentrations (MICs)

The broth microdilution method employed for the determination of minimum inhibitory concentrations (MICs) of compound **1** as according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) by a serial dilution method by using 96 well microtiter plates. Bacterial strains were cultured at 37 °C in MHA medium. After 18 h incubation, bacterial suspensions were prepared in Mueller Hinton Broth (MHB) and their turbidity was standardized to 0.5 McFarland. Optical density of test strains were confirmed on spectrophotometer (Shimadzu UV-1800 series Pharmac-spec UV–vis spectrophotometer, Japan) at A_{600}. The bacterial density was 5×10^5. By using multipipettor, 100 μl of broth medium dispensed into all wells of microtitre plate. Total 11 dilutions (1024–1.0 μg/ml) of compound **1** were prepared by two-fold serial dilution method and 100 μl of each concentration was placed into each well and mixed properly. Column 12 was used as negative control. 5 μl of bacterial cultures were dispensed into wells in column 11 to 1. The plates were incubated at 37 °C. After 18–22 h, the plates were observed and bacterial cultures streaked on culture plates to check their purity.

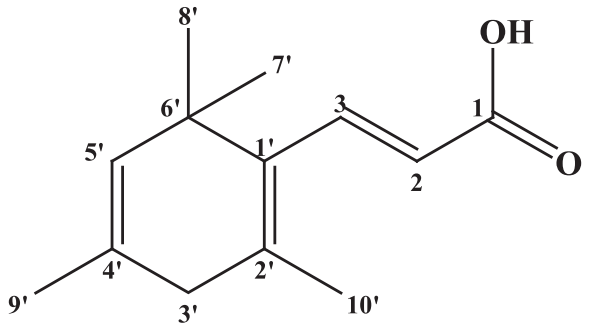

Fig. 1 Chemical structure of compound **1** i.e. 3-(2′,4′,6′,6′-tetramethylcyclohexa-1′,4′-dienyl)acrylic acid

Results and discussion

Chemistry

Analysis of spectroscopic data revealed that compound **1** is 3-(2′,4′,6′,6′-tetramethylcyclohexa-1′,4′-dienyl)acrylic acid (Fig. 1). Its molecular formula was assigned to be $C_{13}H_{18}O_2$ [Elemental composition: (in %) C, 75.69; H, 8.80; O, 15.51] according to ^{1}H NMR, ^{13}C NMR and a quasi-molecular ion molecular ion peak in GC-MS in at *m/z* 207 ($[M + H]^+$ calculated for $C_{13}H_{19}O_2$, 207.281).

The UV–vis spectrum of compound **1** [see Additional file 1] indicated the absorption maxima characteristic for $\pi \rightarrow \pi^*$ transitions for CH = CH of aromatic ring (246.00 nm). FT-IR spectrum of compound **1** [see Additional file 2] revealed the absorption band of O–H stretching (3429.50 cm^{-1}), C-H stretching (2919.91, 2851.39 cm^{-1}), C = O stretching (1563.04 cm^{-1}), C-H deformation of alkenes (1436.62, 1384.56 cm^{-1}), C-O stretching of alcohol (1167.41 cm^{-1}), O-H deformation of alcohol (1093.92 cm^{-1}), and out plane deformation of CH in alkene (880.64 cm^{-1}).

The mass spectrum for compound **1** obtained by GC-MS [see Additional files 3 & 4] revealed its molecular formula could be assigned as [M] = [$C_{13}H_{18}O_2$] with a

Table 1 Zone of inhibition of compound **1** and reference drug (erythromycin) against respiratory tract pathogens

S. no.	Microorganisms	Diameters of inhibition zone (mm) ± SEM[a]		
		Compound **1**	DMSO	Erythromycin
1.	*H. influenzae*	9.3 ± 0.28	-	9.6 ± 0.23
2.	*P. aeruginosa*	7.6 ± 0.28	-	11.0 ± 0.40
3.	*S. aureus*	7.6 ± 0.57	-	11.3 ± 0.23
4.	*S. pneumoniae*	8.0 ± 0.50	-	8.3 ± 0.23
5.	*S. pyogenes*	7.3 ± 0.28	-	13.3 ± 0.47

[a]zone of inhibition in millimeter (mm) in triplicate expressed as means and standard error of means (SEM); Disc diameter: 5 mm; Compound **1** concentration was 6 mg/disc

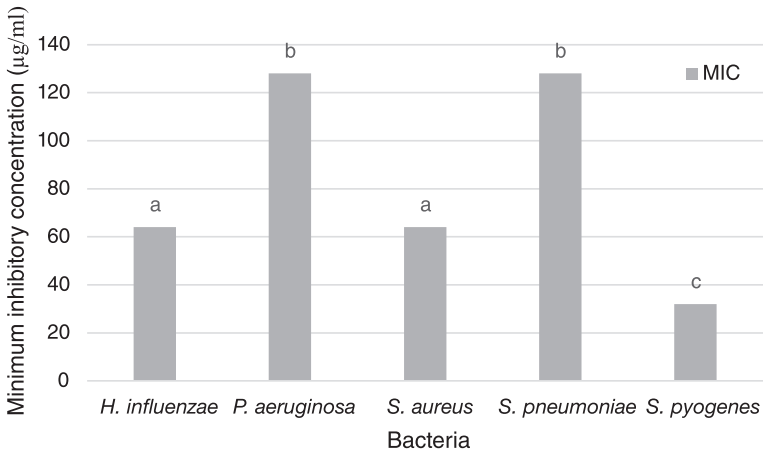

Fig. 2 Minimum inhibitory concentrations (MICs) of compound **1**. The inhibition is noted at a) 64 µg/ml against *H. influenzae* and *S. aureus*, b) 128 µg/ml against *P. aeruginosa* and *S. pneumoniae*, and c) 32 µg/ml against *S. pyogenes*

quasi-molecular ion molecular ion peak at *m/z* 207 $[M + H]^+ = [C_{13}H_{19}O_2]$. Loss of a neutral ethane molecule from quasi-molecular ion generates first daughter ion with *m/z* 177 which corresponds to $[M + H - C_2H_6] = [C_{11}H_{13}O_2]$; further fragmentation of first daughter ion and elimination of a neutral C = O molecule generates a stable ion which corresponds to base peak at *m/z* 149 $[M + H - C_2H_6 - CO] = [C_{10}H_{13}O]$. Successive fragmentation and loss of CO_2, C_2H_2 and two CH_4 neutral molecules from quasi-molecular ion leads to generate a molecule with *m/z* 105 corresponding to $[M + H - CO_2 - C_2H_2 - 2CH_4] = [C_8H_{10}]$.

The ^{1}H NMR spectrum of compound **1** [see Additional file 5] exhibited that it contain two ethylenic protons indicated by two doublets each for 1H at δ_H 7.613 (1H, d, $J = 8.14$ Hz) and 6.532 (1H, d, $J = 8.17$ Hz), their spin coupling pattern indicates presence of one *cis*-disubstituted ethylene moiety in the molecule. It also represented two singlet one for a methine proton which appear at δ_H 5.406 (1H, s) and one for two methylene protons at δ_H 2.436 (2H, s). The compound also illustrated three characteristic singlet for twelve methyl protons at δ_H 1.558 (3H, s), 1.164 (3H, s) and 0.757 (6H, s).

The ^{13}C NMR spectrum of compound **1** [see Additional file 6] marked the presence of thirteen signals indicating the presence of thirteen carbon atoms in the molecule. The highest downfield shift at δ_H 171.645 indicates the presence of one carbonyl group (in form of carboxylic acid). It represented six signals between δ_H 158.716 and 107.423 characteristic for six olefinic carbon atoms. The spectrum also showed two signals each for a secondary carbon at δ_H 34.477 and a quaternary carbon at δ_H 34.405. The compound also demonstrated the presence of four methyl carbon which signals at δ_H 31.269, 28.969, 21.722 and 21.431.

Antibacterial activity

The purified compound **1** was tested for antibacterial activity against selected respiratory tract pathogens at 6 mg/ml concentration and showed better inhibitory activities (Table 1). Compound **1** was found most efficient against *H. influenzae* (9.3 ± 0.28 mm) and *S. pneumoniae* (8.0 ± 0.50 mm) respectively. While erythromycin showed inhibition ranged between 8.3 ± 0.23 to 13.3 ± 0.47 mm which slightly higher values at similar concentration.

The MICs for compound **1** were ranged from 32 to 128 µg/ml (Fig. 2). The inhibition was observed similar at 64 µg/ml against *H. influenzae* and *S. aureus*, 128 µg/ml against *P. aeruginosa* and *S. pneumoniae*, and 32 µg/ml against *S. pyogenes*.

Conclusions

In conclusion, present paper has shown the isolation and structure elucidation of one new derivative of ionone like compound named as 3-(2′,4′,6′,6′-tetramethylcyclohexa-1′,4′-dienyl)acrylic acid (compound **1**) from methanol fraction of *V. odorata* with antibacterial activity. In regards to antibacterial activity, compound **1** was significantly active against tested respiratory tract pathogens including *H. influenzae*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae* and *S. pyogenes* respectively.

Additional files

Additional file 1: UV–VIS spectrum of compound 1. (DOC 109 kb)
Additional file 2: IR spectrum of compound 1. (DOC 256 kb)
Additional file 3: GC-MS spectrum of compound 1. (DOC 134 kb)
Additional file 4: Mass spectrum of compound 1. (DOC 38 kb)
Additional file 5: ^{1}H-NMR spectrum of compound 1. (DOC 97 kb)
Additional file 6: ^{13}C-NMR spectrum of compound 1. (DOC 139 kb)

Competing interests
Shiv Shanker Gautam, Navneet, Sanjay Kumar, Deepak Painuly and Jashbir Singh declare that they have no competing interests.

Authors' contributions
SSG participated on designing the study, carried out the research, collection and analysing data and drafted the manuscript. N and SK participated in planning and designing of study, supervised in laboratory data collection and analysis. DP and JS participated in designing, data analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgments
Authors are grateful to the Head, Department of Botany & Microbiology, Gurukul Kangri University, Haridwar for providing necessary facilities to enable this research and Dr. Dmitry Kosyakov, Northern (Arctic) Federal University, Arkhangelsk, Russia for help and suggestions in spectral analysis. This investigation was supported by grant from UGC, New Delhi, India.

Author details
[1]Department of Botany and Microbiology, Gurukul Kangri University, Haridwar 249404Uttarakhand, India. [2]Department of Chemistry, Gurukul Kangri University, Haridwar 249404Uttarakhand, India.

Received: 16 July 2015 Accepted: 23 January 2016
Published online: 12 February 2016

References

1. Arora DS, Kaur GT. Antibacterial activity of some medicinal plants. J Nat Med. 2007;61:313–17.
2. Akhbari M, Batooli H, Kashi FJ. Composition of essential oil and biological activity of extracts of *Viola odorata* L. from central Iran. Nat Prod Res. 2012;26(9):802–09.
3. Koochek MH, Pipelzadeh MH, Mardani H. The effectiveness of *Viola odorata* in the prevention and treatment of formalin-induced lung damage in the rat. J Herbs Spices Med Plants. 2003;10(2):95–103.
4. Vishal A, Parveen K, Pooja S, Kannappan N, Kumar S. Diuretic, laxative and toxicity studies of *Viola odorata* aerial parts. Pharmacol Online. 2009;1:739–48.
5. Pullaiah T. Encyclopedia of world medicinal plants, vol. IV. New Delhi: Regency Publications; 2006. p. 2048.
6. Ireland DC, Colgrave ML, Craik DJ. A noval suite of cyclotides from *Viola odorata*: sequence variation and the implications for structure, function and stability. Biochem J. 2006;400:1–12.
7. Pranting M, Loov C, Burman R, Goransson U, Andersson DI. The cyclotide cycloviolacin O_2 from *Viola odorata* has potent bactericidal activity against gram-negative bacteria. J Antimicrob Chemother. 2010;65:1964–71.
8. Cu JQ, Perineau F, Gaset A. Volatile components of Violet leaves. Phytochemistry. 1992;31(2):571–73.
9. Legnani L, Luparia M, Zanoni G, Toma L, Vidari G. A full conformational characterization of natural ionones and irones, as well as 13-alkyl-substituted α-ionones. Eur J Org Chem. 2008;28:4755–62.
10. Anca T, Philippe V, Ilioara O, Mircea T. Composition of essential oils of *Viola tricolor* and *V. arvensis* from Romania. Chem Nat Comp. 2009;45(1):91–2.
11. Brenna E, Fuganti C, Serra S. Enantioselective perception of chiral odorants. Tetrahedron Asymmetry. 2003;14:1–42.
12. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolke RH. Manual of clinical microbiology. 4th ed. Washington: ASM; 1999. p. 1527.