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In vitro antimicrobial activity of essential oils and their acetylenic constituents

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The emergence of new infections and increase of bacterial drug resistance has prompted interest for the development of new antibacterial agents from natural sources. This study is an attempt to assess the therapeutic potential of plant constituents as new antimicrobial drugs. The essential oils from six Asteraceae species belonging to the genus *Erigeron*, *Aster*, and *Senecio* were evaluated for their antibacterial activity against six bacteria namely *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Aeromonas hydrophilla*, *Klebsiella pneumonia*, and *Streptomyces candidus* using agar well diffusion method. The results revealed (*Z*)-lachnophyllum (<68 %) and 2(*E*), 8(*Z*)-matricaria esters (<62 %) as the major acetylenic constituents in four *Erigeron* species. *E. mucronatus* showed the highest activity against Gram-positive bacteria *S. candidus* (8.3 mm, MIC 5 µL/mL) and *B. subtilis* (11.0 mm, MIC 10 µL/mL). (*Z*)-Lachnophyllum and 2(*E*), 8(*Z*)-matricaria esters showed maximum activity against *S. candidus* (MICs 5 µL/mL). The results showed that the oils containing acetylenic constituents have potential as natural agents for treatment of infections caused by these bacteria.

Keywords: Asteraceae, Antibacterial activity, Essential oils, (*Z*)-lachnophyllum ester, 2(*E*), 8(*Z*)-matricaria ester.

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Introduction

The indiscriminate use of synthetic antimicrobial drugs has resulted in the emergence of wide range of drug-resistant bacteria, fungi, and viruses. Since bacteria are able to adapt rapidly to the presence of antimicrobial molecules, the resistance increases with the antimicrobial misuse[1]. To overcome the increasing resistance of pathogenic microbes, more effective, alternative biodegradable antimicrobial agents with novel modes of action and safer biomolecules need to be developed.

Aromatic and medicinal plants are known to produce bioactive molecules that can inhibit pathogens and have little toxicity to host cells. These have the potential for being developed as new antimicrobial drugs. Recent *in vitro, in vivo*, and clinical studies confirm that large number of essential oils exert antimicrobial activity against different Gram-positive and Gram-negative bacteria and fungi[2,3].

Asteraceae includes the most diverse and widespread plant families in terms of ethnomedicine as antispasmodic, antiseptic, antiasthmatic, antitumor, cytotoxic, and antimicrobial activities among others[4-6]. There is no specific report on the antibacterial activity of essential oils from the aerial parts of *Aster albescens, Erigeron annuus, E. karwinskianus, E. mucronatus, E. multiradiatus*, and *Senecio nudicaulis*. Thus, we present the results of antimicrobial activity of essential oils and major acetylenic constituents from these Asteraceae species.

Materials and Methods

Plant materials

Aerial parts of *Aster albescens* (Acc.No. 113968), *Erigeron annuus* (Acc. No. 113641), *E. karwinskianus* (Acc. No. 113642), *E. mucronatus* (Acc. No. 113639), *E. multiradiatus* (Acc. No. 113962) and *Senecio nudicaulis* (Acc. No. 113640) were collected at the flowering stage from Himalayan region of Uttarakhand during April-July 2012 at altitude range of 1900-3200 m. The plant specimens were identified from Botanical Survey of India, Dehradun and voucher specimens were deposited in the Phytochemistry Laboratory, Chemistry Department, Kumaun University, Nainital and Botanical Survey of India, Dehradun.

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Chemicals and reagents

All chemicals and reagents used were of analytical grade. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were obtained from Hi-Media, India.

Extraction of the essential oil

Fresh aerial parts (~3 kg) of each species were subjected to steam distillation. The distillate obtained by steam distillation of the fresh plant material was treated with n-hexane for the extraction of organic constituents. The distillate was further shaken with dichloromethane to ensure complete extraction of constituents. The n-hexane and dichloromethane extracts were combined and dried over anhydrous Na_2SO_4. Solvent was distilled off in a rotary vacuum evaporator (Perfit-RV 1240, Buchi type) to get residual oil which was stored at ~4 ºC.

GC and GC-MS analysis

The oil samples were analyzed using a gas chromatograph (GC) (Nucon 5765, New Delhi, India) equipped with Rtx-5 non-polar fused silica capillary column (30 m × 0.32 mm, film thickness: 0.25 μm). The oven temperature (60-210 °C) was programmed at 3 °C/min and retained at 210 °C till analysis was complete. The injector and detector temperatures were 210 °C each and the injection volume 0.5 μL, using a 10 % solution of the oil in *n*-hexane. The GC–MS was conducted on a Thermo Quest Trace GC 2000 (ThermoQuest/ Finnigan) interfaced with a Finnigan MAT Polaris Q ion trap mass spectrometer using similar operating parameters as in GC with injection volume of 0.10 μL and the split ratio was 1:40. The MS were taken at 70 eV with a mass range of 40-450 amu. Identification of components of the essential oils was done with published data[7] and NIST and WILEY mass spectral library data.

Isolation and characterization of acetylenic compounds

The essential oil (1 mL) was subjected to silica gel column chromatography (230-400 mesh, Merck, 20 g) with hexane: diethyl ether (99:1-85:15) as eluent and fractions were collected and screened by TLC and GC to produce compound 1 (80 mg, >98 % purity) and compound 2 (70 mg, >96 % purity). These compounds were identified by using Mass, ^{1}H-NMR, and ^{13}C-NMR spectral data[8,9].

Bacterial strains

The *in vitro* antibacterial activity was evaluated against a panel of pathogenic and clinically isolated 6 bacterial strains, including four Gram-negative bacteria {*Pseudomonas aeruginosa* (MTCC No. 424), *Escherichia coli* (MTCC No. 443), *Aeromonas hydrophila* subsp. *hydrophila* (MTCC No. 646), *Klebsiella pneumoniae* (MTCC No. 3384)} and two Gram-positive bacterium {*Bacillus subtilis* (MTCC No. 441), *Streptomyces candidus* subsp. *azaticus* (MTCC No. 703)}. The test strains were provided by the Department of Biotechnology, Bhimtal, Kumaun University, which were procured from the Institute of Microbial Technology, Chandigarh. The cultures of bacteria were maintained on agar slants at 4 °C throughout and used as stock cultures.

Antibacterial activity evaluation

Antimicrobial activity evaluation of the oils and isolated compounds was done by the agar well diffusion method[10]. The samples were dissolved in dimethyl sulphoxide (DMSO) to prepare desired concentrations. Inoculums of the microbial strains (1×10^6 CFU/mL) were plated using sterile swabs into petri dishes (90 mm) with 20 mL of Mueller-Hinton agar, where 3 mm wells were cut and filled with 30 μL/mL of sample. Standard antibiotic gentamycin was used as positive control and DMSO as negative control. The petri dishes were pre-incubated for 3 h at room temperature, allowing the complete diffusion of the samples and then incubated at 37±1 ^{o}C for 24 h[11]. The zones of inhibition were observed and measured in mm.

Antibacterial activity evaluation by agar dilution method

The evaluation of MICs was done by using the agar dilution method with slight modifications described by the National Committee for Clinical Laboratory Standards[12]. Equal volume of each microbial strain culture containing approximately 1×10^6 CFU/mL, were applied onto MHB supplemented with the essential oils at concentration ranging from 5-100 μL/mL in tubes. These cultures were then incubated at 37 ^{o}C for 24 h.

Statistical analysis

The principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed using XLSTAT statistical computer software package, version 14 for evaluating correlation between antibacterial activity and essential oils.

Results and Discussion

Major chemical constituents of the oils are presented in the Table 1 and Fig.1. The essential oil of

Table 1—Chemical constituents of Asteraceae species

Species	Biochemical markers
E. mucronatus	(*Z*)-lachnophyllum ester (21.2%) (1), 2(*E*), 8(*Z*)-matricaria ester (62.1%) (2), caryophyllene oxide (3.8%) (3)
E. karwinskianus	(*Z*)-lachnophyllum ester (4.7%) (1), 2(*E*), 8(*Z*)-matricaria ester (25.4%) (2), caryophyllene oxide (5.5%) (3), germacrene D (13.1%) (4)
E. annuus	(*Z*)-lachnophyllum ester (68.1%) (1), germacrene D (10.4%) (4)
E. multiradiatus	(*Z*)-lachnophyllum ester (10.3%) (1), 2(*E*), 8(*Z*)-matricaria ester (50.7%) (2), spathulenol (13.4%) (5), kaurene (8.0%) (6)
A. albescens	germacrene D (8.4%) (4), β-phellandrene (13.8%) (7), δ-selinene (13.3%) (8), β-eudesmol (12.3%) (9)
S. nudicaulis	β-caryophyllene (22.3%) (10), α-humulene (30.2%) (11), germacrene D (26.3%) (4), linoleic acid (9.7%) (12)

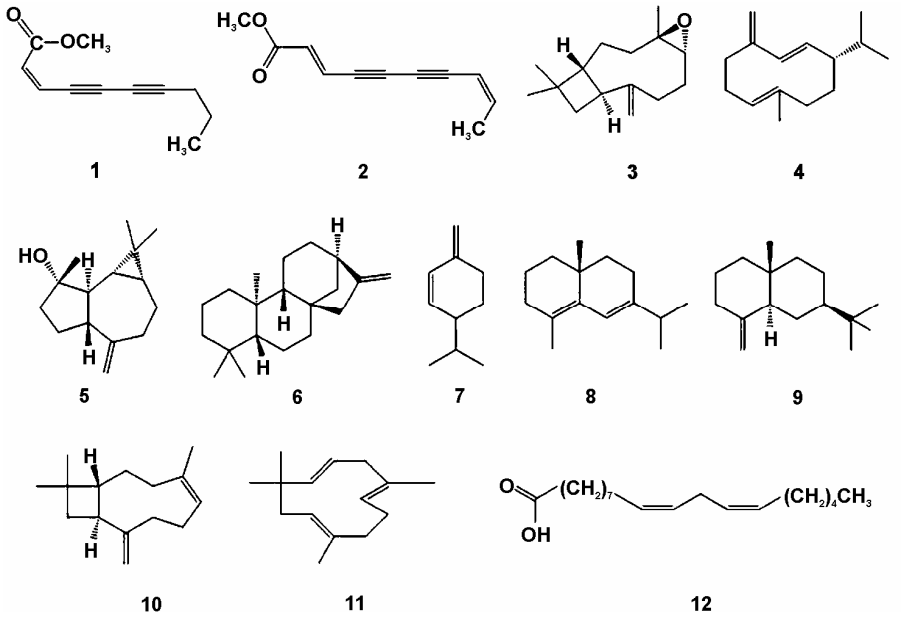

Fig. 1—Chemical constituents of Asteraceae species.

E. multiradiatus showed presence of acetylenic compounds (*Z*)-lachnophyllum ester (10.3 %), 2(*E*), 8(*Z*)-matricaria ester (50.7 %), kaurene (8.0 %), and spathulenol (13.4 %). β-Phellandrene (13.8 %), δ-selinene (13.3 %), and β-eudesmol (12.3 %) were the main components of the *A. albescence* oil. *S. nudicaulis* oil was dominated by the presence of sesquiterpene hydrocarbons such as α-humulene (30.2 %), germacrene D (26.3 %), and β-caryophyllene (22.3 %). Essential oils from three *Erigeron* species namely *E. mucronatus, E. karwinskianus,* and *E. annuus* were characterized by high content of acetylenic compounds[9].

The antibacterial activity data of the oils and their acetylenic constituents are given in Table 2 and 3. *E. mucronatus* showed higher activity against *S. candidus* (8.3 mm, MIC 5 µL/mL) and *B. subtilis* (11.0 mm, MIC 10 µL/mL). Oil was also active against *P. aeruginosa* (MIC 35 µL/mL), *E. coli* (MIC 30 µL/mL), *A. hyrdophilla* (MIC 40 µL/mL), and *K. pneumoniae* (MIC 45 µL/mL). The essential oil of *E. karwinskianus* showed MIC value within the range of 5-50 µL/mL and was more active against *S. candidus*, *B. subtilis*, and *E. coli.* All strains were inhibited by *E. annuus* oil, which showed higher activity against Gram-positive bacteria (MICs 10-25 µL/mL) than Gram-negative bacteria (MIC 25-50 µL/mL). *E. multiradiatus* was very active against *S. candidus* (6.0 mm, MIC 5 µL/mL).

Quantitative antibacterial activity evaluation of the acetylenic isolates (*Z*)-lachnophyllum ester and 2(*E*), 8(*Z*)-matricaria ester recorded identical MIC values of 5 and 10 µL/mL against *S. candidus* and *B. subtilis*, respectively. 2(*E*), 8(*Z*)-Matricaria ester showed lower activity against *P. aeruginosa* (MIC 65 µL/mL) and *K. pneumoniae* (MIC 70 µL/mL), while (*Z*)-lachnophyllum ester showed low activity against *A. hyrdophilla* and *E. coli* (MIC 60 µL/mL each) (Table 3).

Table 2—Antibacterial activity of the Asteraceae species and acetylenic compounds in agar well diffusion test.

Samples	Diameter of Inhibition Zone (mean ± SD) mm[a]					
	Bacterial strains					
	P.a.	B.s.	E.c.	A.h.	K.p.	S.c.
Essential oils						
E. mucronatus	10.7±0.6	11.0±0.5	7.0±0.3	12.3±0.6	7.6±0.6	8.3±0.8
E. karwinskianus	10.6±0.6	8.0±1.0	8.3±1.1	10.5±0.5	6.3±0.6	5.5±0.5
E. annuus	10.5±0.5	9.7±1.1	10.2±0.8	8.0±0.5	9.5±0.5	8.7±0.6
E. multiradiatus	7.2±0.3	6.7±0.6	7.7±1.1	6.3±0.6	7.3±0.6	6.0±1.0
A. albescens	6.7±0.6	6.8±0.2	7.7±1.1	8.5±0.5	7.3±0.6	7.7±1.1
S. nudicaulis	8.8±0.3	10.2±0.8	6.0±1.0	10.3±0.6	8.3±0.8	6.7±0.6
Acetylenic compounds						
AC-1	6.0±1.0	8.2±0.8	9.7±1.1	7.7±1.1	7.5±0.9	6.2±0.8
AC-2	10.2±1.0	8.6±0.6	9.1±0.6	8.1±0.5	5.2±0.5	5.9±0.8
Reference antibiotic						
Gentamycin	20.3±0.2	21.3±0.1	27.7±0.1	21.0±0.3	25.3±0.1	24.0±0.1

[a]Inhibition zone diameter includes well diameter; P.a.= *Pseudomonas aeruginosa*, B.s.= *Bacillus subtilis*, E.c.= *Escherichia coli*, A.h.= *Aeromonas hydrophilla*, K.p.= *Klebsiella pneumoniae*, S.c.= *Streptomyces candidus* subspecies *azaticus;* AC-1= 2(*E*), 8(*Z*)-matricaria ester, AC-2= *(Z)*-lachnophyllum ester; SD=Standard deviation.

Table 3—Minimum inhibitory concentration (µL/mL) of Asteraceae species and acetylenic compounds against bacterial strains.

Samples	Minimum inhibitory concentration (µL/mL)					
	Bacterial strains					
	P.a.	B.s.	E.c.	A.h.	K.p.	S.c.
Essential oil						
E. mucronatus	35	10	30	40	45	5
E. karwinskianus	40	25	30	45	50	5
E. annuus	30	30	25	50	40	10
E. multiradiatus	40	30	45	40	40	5
A. albescens	40	20	30	10	30	10
S. nudicaulis	30	45	30	25	60	30
Acetylenic compounds						
AC-1	65	10	45	55	70	5
AC-2	45	10	60	60	55	5
Reference antibiotic						
Gentamycin*	5	5	5	5	5	5

P.a.= *Pseudomonas aeruginosa*, B.s.= *Bacillus subtilis*, E.c.= *Escherichia coli*, A.h.= *Aeromonas hydrophilla*, K.p.= *Klebsiella pneumoniae*, S.c.= *Streptomyces candidus* subspecies *azaticus*; AC-1= 2(*E*), 8(*Z*)-matricaria ester, AC-2= (*Z*)-lachnophyllum ester; *MIC of antibiotic in µg/mL

A. albescens essential oil was more active against *S. scandidus* and *A. hyrdophilla* (MIC 10 µL/mL, each). It showed activity against *B. subtilis* (MIC 20 µL/mL), *K. pneumoniae* (MIC 30 µL/mL), *E. coli* (MIC 30 µL/mL), and *P. aeruginosa* (MIC 40 µL/mL), whereas *S. nudicaulis* oil was much less active except against *A. hyrdophilla* (10.3 mm; MIC 25 µL/mL).

The MIC values were subjected to PCA and HCA (Fig. 2 and 3). The statistical analysis of the antibacterial activity of the oils showed significant difference among the oils and the tested bacterial strains (p <0.05). The PCA horizontal axis explained 68.4 % of the total variance, while the vertical axis a further 14.2 % (Fig. 2).

Group I represented by the Gram-negative bacteria, *P. aeruginosa*, *E. coli*, and *A. hyrdophilla* were the most resistant to the majority of *Erigeron* essential oils and acetylenic compounds. This group is divided into two subgroups Ia and IIb. Subgroup Ia was limited to *A. hyrdophilla*, which was characterized by the resistance to *E. annuus*, (*Z*)-lachnophyllum ester and 2(*E*), 8(*Z*)-matricaria ester (MICs 45-60 µL/mL). Subgroup IIb represented by *P. aeruginosa* and *E. coli*, showed particularly less susceptibility to the *E. multiradiatus* oil and both acetylenic compounds

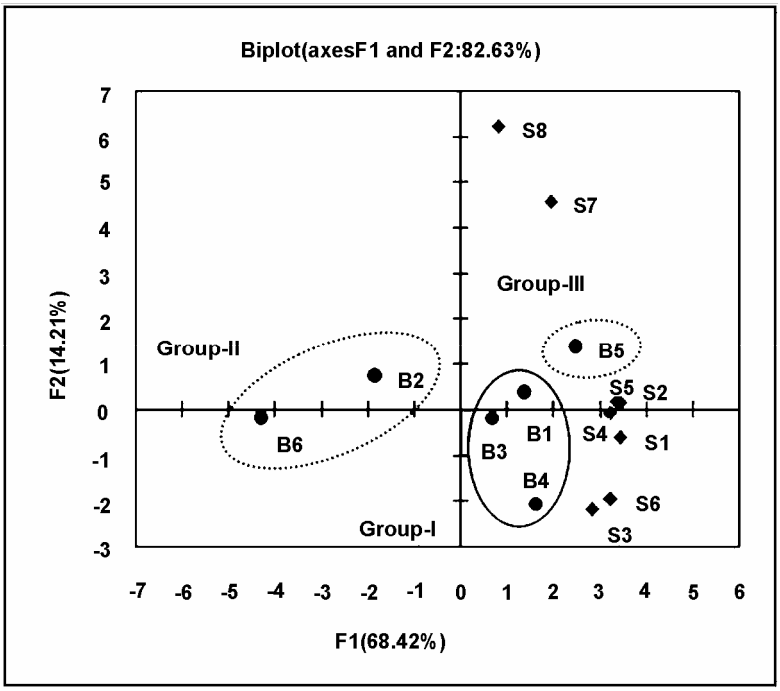

Fig. 2—Principal component analysis of the antimicrobial activity of essential oils of Asteraceae species and acetylenic compounds against six bacteria. (S1= *E. mucronatus*; S2= *E. karwinskianus*; S3= *E. annuus*; S4= *E. multiradiatus*; S5= 2(*E*), 8(*Z*)-matricaria ester; S6= *(Z)*-lachnophyllum ester; S7= *A. albescens*; S8= *S. nudicaulis;* B1= *P. aeruginosa*; B2= *B. subtilis;* B3= *E. coli*; B4= *A. hydrophila*; B5= *K. pneumoniae*; B6= *S. candidus*)

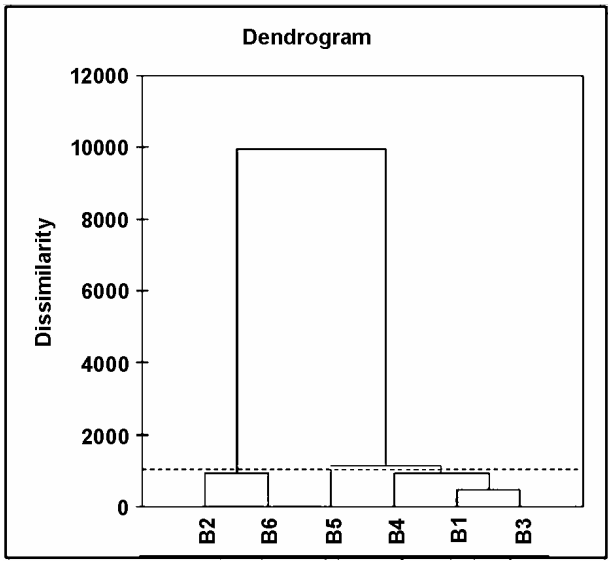

Fig. 3—Hierarchical cluster analysis based on the Euclidean distance between groups of the antibacterial activity of essential oils Asteraceae species and acetylenic compounds. (B1= *P. aeruginosa*; B2= *B. subtilis;* B3= *E. coli*; B4= *A. hydrophila*; B5= *K. pneumoniae*; B6= *S. candidus*)

(MICs 40-65 µL/mL), in which *P. aeruginosa* showed less susceptibility for 2(*E*), 8(*Z*)-matricaria ester (MIC 65 µL/mL) and *E. coli* for (*Z*)-lachnophyllum ester (MIC 60 µL/mL).

Group II was limited to the Gram-positive bacteria, *B. subtilis* and *S. candidus*. These strains showed more susceptibility to all the essential oils and especially for acetylenic compounds (MICs 5-10 µL/mL). Group III**,** consisting of *K. pneumoniae* showed high resistance for all the essential oils and both the acetylenic compounds with MIC values varying from 30-70 µL/mL.

Overall, result revealed that Gram-negative bacteria were less inhibited than Gram-positive bacteria. The greater resistance of Gram-negative bacteria to essential oils, as observed in this study might be owing to the great complexity of the double membrane-containing cell envelope of these bacterial in contrast to the single membrane structures of Gram-positive bacteria. The outer membrane acts as a barrier to the biomolecules[2,3].

(*Z*)-Lachnophyllum ester and 2(*E*), 8(*Z*)-matricaria ester, which were the principle components of the essential oils from *Erigeron* species, had lower activity than the essential oils containing them. The efficiency might be explained by the synergistic or additive effects of several constituents of the oil rather than arising from a single compound alone. Different bioactive compounds in a mixture can interact to provide a combined effect[13]. Synergic effect between

major and minor components occurs and these acetylenic compounds together could be assumed as major contributors of the total antimicrobial activity of *Erigeron* essential oils.

Conclusion

Results show broad spectrum antibacterial effect of the essential oils from *Erigeron*, *Aster*, and *Senecio* species against bacterial strains. *E. mucronatus* oil showed highest activity against Gram-positive bacteria *S. scandidus* (8.3 mm, MIC 5 µL/mL) and *B. subtilis* (11.0 mm, MIC 10 µL/mL), while (*Z*)-lachnophyllum ester and 2(*E*), 8(*Z*)-matricaria ester showed maximum activity against *S. candidus* (MICs 5 µL/mL) comparable with the reference antibiotic gentamycin. This study, records the potential of acetylenic ester containing oils as natural agents for fight against bacterial infections.

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