Chemical and antimicrobial studies on the essential oil from 
*Salvia santolinifolia* Boiss.

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**Abstract:** In view of the reputation of genus *Salvia* in folklore medicine and its abundance in our region, the chemical composition and antimicrobial activity of essential oil from *S. santolinifolia* Boiss. was analyzed. Chemical analysis, using gas chromatography and gas chromatography mass spectrometry, retention indices and C-13 nuclear magnetic resonance spectroscopy has resulted in identification of 116 constituents, comprising about 97% of the total constituents. Out of these 116, 78 constituents are hitherto unreported from this source. The species belongs to *α*-pinene chemotype. In antibacterial assay, gram negative gastropathogens (*Shigella boydii*, *S. flexneri*, *S. dysenteriae*, *Vibrio cholerae*); causative agent of urinary tract infection (*Proteus mirabilis* and *P. vulgaris*) and pneumonia (*Klebsiella pneumoniae*) were found sensitive to this essential oil while *Corynebacteria species* and *Staphylococcus epidermidis* were significantly inhibited in antibacterial assay against gram positive bacteria. Clinical and Laboratory Standards Institute protocol was used for determining antimicrobial activity. Thus the essential oil from this species can be utilized as potential chemotherapeutic agent.

**Keywords:** *Salvia santolinifolia* Boiss., essential oil, C-13 NMR, GC-MS, antimicrobial activity.

**INTRODUCTION**

*Salvia* is a member of the family Lamiaceae (Sefidkon and Khajavi, 1999). Latin word “salvare” is the source of the word *Salvia*, which means to restore to health or to secure health. It is with reference to the medicinal properties shown by some of the species (Kamatou et al., 2008a and the references cited there in). *Salvia* acquired a great reputation in folk medicine (Gulluce et al., 2006). A number of species of this genus are frequently used for the cure of heart diseases, amenorrhea, sleeplessness and dysmenorrhea (Mehmood et al., 2006).

*Salvia santolinifolia* Boiss. is suffruticose and a much branched herb. It is an abundant plant found in many parts of Pakistan, growing in sandy plains, rocky slopes, valleys, shale slopes, road sides and cultivated fields (Ali and Nasir, 1990). *S. santolinifolia* leaves and seeds are used in diarrhoea and haemorrhoids conditions due to its demulcent property. In this herb a high quantity of mucilage is present which is combined with demulcent property. In this herb a high quantity of mucilage is present which is combined with demulcent property. In this herb a high quantity of mucilage is present which is combined with demulcent property. In this herb a high quantity of mucilage is present which is combined with demulcent property. In this herb a high quantity of mucilage is present which is combined with demulcent property. In this herb a high quantity of mucilage is present which is combined with demulcent property.

A total of 62 components have already been identified in the essential oil of *S. santolinifolia* by three different research groups from Iran (Sefidkon and Khajavi, 1999; Javidnia et al., 2008; Sonboli et al., 2006). To our best of knowledge it is the first study on essential oil from Pakistani *S. santolinifolia* and current study has resulted in identification of 116 constituents.

It was reported that some members of the species of the genus *Salvia*, including *Salvia triloba* and *Salvia officinalis* possessed marked antibacterial and antifungal activity (Delamare et al., 2007). The antimicrobial activity of essential oil from *Salvia santolinifolia* is determined in the current study to search its use as a natural therapeutic substance. One of the major problems in antimicrobial chemotherapy is the increasing rate of resistance to antibiotics and chemotherapeutics, which is primarily responsible for the inefficiency of antimicrobial treatment (Schelz et al., 2006). The overuse of antibiotics and consequent antibiotic selective pressure is considered to be the most critical and obvious reason in contributing to the emergence of various potentially pathogenic resistant microorganisms (Mimica-Dukic et al., 2003). Potentiation of the pure and crude essential oil preparations from species of genus, *Salvia officinalis* was also carried out successfully in combination with aminoglycosides for synergistic action in certain infectious diseases (Horiiuchi et al., 2007). Thus, there remains always a strong need to explore new agents for combating infectious diseases caused by resistant and multi-resistant bacteria species. The aim of the investigation was to determine the chemical constituents and the antimicrobial activity of essential oil and utilizing its potential as a therapeutic agent.

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MATERIALS AND METHODS

Plant material
The wild-growing plants of *S. santolinifolia* were collected during the flowering period (June-July) from surroundings of Umer Basha Institute of Information Technology at University of Karachi, Karachi and were authenticated by Jan Alam, Senior Taxonomist, Department of Botany, University of Karachi, Karachi. A voucher specimen was deposited in the Herbarium of the Department of Botany, University of Karachi, with general herbarium No. 78351.

Extraction of essential oil
1.5 kg of the chopped aerial parts (flowers, stem and leaves) of the plant was ground into coarse powder and was subjected to hydro-distillation for 4 hours (British Pharmacopeia, 2005). The essential oil was collected in ether, dried over anhydrous sodium sulfate, concentrated under N2 stream and stored at 4°C in a dark amber vial before chemical and antimicrobial analyses. The yield was 0.16%.

Analyses of essential oil

GC-FID analysis
The dried essential oil from *S. santolinifolia*, diluted with Et2O, was subjected to GC-FID thermal gradient analysis, on an Agilent model 6280 gas chromatograph, fitted with an HP-5e (30 m × 0.25 mm, 0.25 µm film thickness) capillary column. The initial temperature of the column was kept at 50°C for 5 min and was heated to 300°C at a rate of 4°C/min and then kept at final temperature for 20 min. The temperature of the detector and injector were kept at 250°C and 280°C respectively. Helium, as carrier gas, was used at flow rate of 1 ml/min. 1 µl of sample was injected with a split ratio of 1:20.

GC-MS analysis
The GC-MS analysis was performed on similar conditions and parameters as described for GC-FID, using Perkin-Elmer Clarus 500 gas chromatograph equipped with an HP-5MS® (30 m × 0.25 mm, 0.25 µm film thickness) capillary column. The MS was operated at standard conditions 70 eV, and 250°C. The processed mass spectra of components from essential oil of *S. santolinifolia* were identified by comparison with the electronic MS library (NIST, 2005) and by comparing the calculated retention indices (RI) with literature values (table 1).

C-13 NMR Analyses
C-13 (BB and DEPT) NMR spectra were recorded in CDCl3 on Bruker Avance 400B spectrometer operating at 100 MHz. The chemical shifts were in ppm (δC) with

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Table 1 continued

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Class of compounds

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Continued....
Muhammad Nadir et al.

Table 1 continued

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<td>Unidentified</td>
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<td>2.86</td>
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Fig. 1: GC-MS (TIC) chromatograms of the essential oil from *S. santolinifolia* Boiss.

Microorganism cultures

Microorganisms were obtained from the microbial collection of Department of Microbiology, University of Karachi, Karachi. The 13 gram positive strains studied were *Staphylococcus aureus*, Methicillin Resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Streptococcus pneumoniae*, *Corynebacterium hofmannii*, *Corynebacterium xerosis*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus thuringiensis* and *Micrococcus luteus*; and 19 gram negative bacteria included *Shigella dysenteriae*, *Shigella boydii*, *Shigella flexneri*, *Salmonella typhi*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *Vibrio cholerae*, *Proteus vulgaris*, *Proteus mirabilis*, *Escherichia coli*, *Escherichia coli* MD40, *Escherichia coli* FPL5014, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. Purity was checked by gram staining and cultures were identified using conventional biochemical tests and rapid tests using Quick Test Strip (QTS 10). All cultures were grown in nutrient broth under continuous shaking in a water bath for 24 hours set to 37°C. 500 µl of cultural suspension was transferred into vials and overlaid with 30% glycerol. Vials were frozen until required.

Antimicrobial screening

The antimicrobial susceptibility and minimum inhibitory concentration (MIC) of the essential oil of *S.
Chemical and antimicrobial studies on the essential oil

**santolinifolia** against 13 gram positive and 19 gram negative clinical isolates (the test microorganisms) were determined by agar well dilution method and microbroth dilution method respectively applying the procedures as recommended by the Clinical and Laboratory Standards Institute (CLSI) and National Committee for Clinical Laboratory Standards (NCCLS) (Wayne, 1998).

Fig. 2: Structures of monoterpenes and monoterpenoids.
Antimicrobial assay
The dilutions of essential oil were made in 10% aq. DMSO with Tween 80 (0.5% v/v for easy diffusion) and sterilized by filtration through membrane filter (0.45 µm). All the bacterial cultures were revived in Mueller-Hilton broth for 2 hours at 37°C in shaking water bath. The tubes were matched with 0.5 McFarland index to obtain 10^8 cells. Microbial lawn was prepared on Mueller-Hilton agar plates. Wells were punched into the agar using sterile borer and 50 µl of Salvia crude oil was poured in all the wells. Sterile laboratory parafilm were used to seal the plates to avoid any change in concentration of the test samples due to evaporation. After incubation at 37°C for 24 hours, susceptibility was evaluated on the basis of size and measurement of zone of inhibition.
Determination of minimum inhibitory concentration (MIC)
Minimum inhibitory concentration (MIC) of susceptible essential oil was determined by micro broth dilution method using 96-well microtitre plate (Aboaba et al., 2006). Stock solution of 1 mg/ml of essential oil of S. santolinifolia was dissolved in 10% aq. DMSO with Tween 80 (0.5% v/v for easy diffusion) and sterilized by filtration through membrane filter (0.45 µm). Two fold serial dilutions of oil was made in 100 µl broth and subsequently 10 µl of 2 hours fresh culture matched with 0.5 McFarland index was added to each well. One well served as antibiotic control while other served as culture control. Plates were incubated for 24 hours at 37°C. The MIC was read as the well showing no visible growth.

RESULTS
The essential oil was subjected to GC-FID, GC-MS (fig. 1) and C-13 NMR analysis using similar protocols as reported in earlier communications (Siddiqui et al., 2004; 2005; 2009; Gilani et al., 2009). More than 97% of the constituents of the essential oil were identified and quantified by the area normalization method using their FID responses. Altogether 116 constituents were identified. Their structures are given in fig. 2 and 3. Identification were done via electronic mass spectral survey (NIST, 2005), confirmed and authenticated by Kovats retention indices (Kovats, 1958) and C-13 NMR data published in literature. The C-13 NMR data of the identified constituents were search in the literature (Ahmad and Rehman, 1992; Bohlmann et al., 1975; Kubeczka and Formacek, 2002; SDBS, 2008). The signals of pure constituents were then traced in the C-13 NMR of the essential oil. Constituents present in higher concentration showed prominent signals in the C-13 NMR of the essential oil, thus supported the identification.

The details on contribution and classification of constituents are mentioned at the end of table 1. The oil was found to contain more than 91% of terpenes and just less than 6% of other constituents. Almost 46 and 37% components were those belonging to mono- and sesquiterpenes respectively. The amount of oxygenated monoterpenes was higher than oxygenated sesquiterpenes (22.92 and 17.25% respectively). The total contribution of diterpenes and oxygenated diterpenes was about 8%.

Antimicrobial assay
The results of antimicrobial study indicated that the essential oil of S. santolinifolia possessed significant antimicrobial activity against a number of gram positive and gram negative potential human pathogens. The results of the antimicrobial studies are presented graphically (fig. 4). Other tested bacterial cultures, mentioned in materials and methods, were found resistant.

DISCUSSION
Owing to the medicinal properties, the genus Salvia has been explored well for the chemistry of essential oil (Sajjadi and Shahpiri, 2004; Tepe et al., 2005; Karaman et al., 2007; and related references cited at the foot note of table 1). Thus wildly growing Pakistani S. santolinifolia was selected for chemical and antimicrobial investigation, as essential oil from this origin has not been studied yet.

Chemical Analysis
Chemical constituents, identified primarily via electronic mass spectral survey (NIST, 2005), were further confirmed by the Kovats retention indices (Kovats, 1958) and C-13 NMR data published in literature. The C-13 NMR data of the identified constituents were search in the literature (Ahmad and Rehman, 1992; Bohlmann et al., 1975; Kubeczka and Formacek, 2002; SDBS, 2008). The signals of pure constituents were then traced in the C-13 NMR of the essential oil. Constituents present in higher concentration showed prominent signals in the C-13 NMR of the essential oil, thus supported the identification.

Identified compounds and their importance
α-Pinene with 13.86% is the major contributor. It is used as a fragrance material in industrial products such as insecticides and antiseptics (Bamoniri et al., 2009) and is also an effective insect repellent (Wang et al., 2009). β-
Caryophyllene (3.81%) and its oxide (4.38%) were other major constituents. Caryophyllene oxide is well recognized as stabilizer in foodstuff, drugs and cosmetics and has also shown growth inhibiting activity against dermatophytes (Yang et al., 1999). β-Caryophyllene, is a commonly distributed sesquiterpene in plants, having allelopathic potential (Wang et al., 2009) and nematicidal activity (Park et al., 2007). (E,E)- and (Z,E)-Farnesol, present in the oil in a concentration of 3.83 and 3.70% respectively, are used as fragrance (Eriksson et al., 2003) and have also shown growth inhibitory effects on Staphylococcus aureus, a normal skin flora, thus, are used in skin care products (Katsuyama et al., 2005). L-Borneol, identified in 3.70%, strongly inhibits the microsomal CYP2B6 activity (Kim et al., 2008). Limonene, also present in concentration of 3.70%, is abundantly distributed in high quantity in volatile oils and can easily be biotransformed into compounds, such as carvone, perillyl alcohol and α-terpineol, which in turn are more valuable. It is found in citrus extracts and is used as a flavoring and odor agent (Cadwallader et al., 1989; 1992; Filho et al., 2003; Trytek and Fiedurek, 2005). It is also used as a part in various synthetic essential oils (Bamoniri Filho, 2010). Germacrene D has antimicrobial and insecticidal properties (Bamoniri et al., 2010). Some other compounds, enlisted in identifications, having different beneficial activities are as follows; myrtenyl acetate reported to have DPPH scavenging activity (Mimica-Dukic et al., 2010). Eugenol, the major constituent of clove essential oil, possesses significant antifungal and antibacterial activity (Ali et al., 2005; He et al., 2007; Mimica-Dukic et al., 2010). Methyl eugenol has shown antifungal activity (Ahmad et al., 2010). Myrtanol has fragrance and flavoring properties (Bell et al., 2003). cis-Verbenol has anti-ischemic, anti-oxidative, anti-inflammatory and antifungal activities (Choi et al., 2010). Germacrene D has antimicrobial and insecticidal properties (Bamoniri et al., 2009). Terpenine-4-ol has strong antimicrobial, anti-inflammatory, antifungal, bacteriostatic and bactericidal activities (Mondello et al., 2006; Loughlin et al., 2008). Decanal, present in citrus fruits, is responsible for specific odor of buckwheat (Janes et al., 2008). Elemene has shown antitumor activity (Yang et al., 1997).

Current study has revealed 22 compounds which are not reported from essential oil from any species of Salvia. Few of these are noteworthy. These included oleic acid, which is a potent inhibitor of cholesterol and fatty acid synthesis (Natalli et al., 2007); Thujopsene, which showed antignawing activity in mice (Ahn et al., 1995); Neocembrene A, a pheromone in termites and may induce various behavioral effects on the insect and can trigger various biological activities (Sillam-Dusses et al., 2009).

**Earlier reports on S. santolinifolia essential oil**

Earlier communications on S. santolinifolia has reported up to 62 components (Sefidkon and Khajavi, 1999; Sonboli et al., 2006; Javidnia et al., 2008), of these 39 were present in the studied oil. The major constituents of the two previously studied oils were α-pinene, β-pinene and limonene (Sefidkon and Khajavi, 1999; Sonboli et al., 2006) and that of third one are α-pinene, borneol, camphene, geranylinalool and carvophyllene (Javidnia et al., 2008) while in present study the chief components are α-pinene, caryophyllene oxide, caryophyllene, (E,E) and (Z,E)-farnesol, L-borneol and limonene. A comparison for all chemical and antimicrobial studies (vide infra) is presented in table 2. The chemical composition of the Salvia santolinifolia reported from Iran and currently studied sample was found varying. This may be due to the phytogeographical distinction but all four studied essential oils from Salvia santolinifolia are α-pinene chemotype. However, the current study is further reporting 78 new constituents from S. santolinifolia, of these 56 constituents were found reported in the essential oil of different species of genus Salvia while 22 constituents composing 12.17% of the total oil were hitherto unreported from any species of this genus (vide table 1).

**Chemotypes of genus Salvia**

The literature survey showed that the chemical composition of essential oil from the genus Salvia demonstrated a vast variation. Majority of species of Salvia were found rich in α-pinene and β-pinene while another set of species have β-caryophyllene and its oxide as major components (Sefidkon and Khajavi, 1999; Sajjadi and Shahpiri, 2004; Tepe et al., 2005; Kivrak et al., 2009). These trends were also observed in current studies. Some other species of Salvia contain 1,8-cineole and thymol as major component as much in concentration as 60 and 69% respectively (Ozer et al., 2007; Anackov et al., 2009; Liang et al., 2009).

**Antimicrobial results against gram-positive bacterial species**

Among gram positive candidates, the best activity was observed in case of non-pathogenic Corynebacteria species; C. hofmannii (16 mm; MIC 3000 µg/ml) and C. xerosis (20 mm; MIC 2500 µg/ml). It is expected that the oil would also show similar activity against the
pathogenic member of the same genus, *C. diptheriae* and could be helpful in treating a severe upper respiratory tract infection of diphtheria. Moderate activity was also observed against *Staphylococcus epidermidis* (18 mm; MIC 5000 µg/ml), a normal skin flora but a potential biofilm producing organism in surgical implants, prosthetics, and catheters and in other devices (fig. 4). Previously studied essential oil from *S. santolinifolia* showed activity against *Staphylococcus aureus* and *Bacillus subtilis* (Sonboli et al., 2006; Javidnia et al., 2008), however, in current study these organisms are resistant.

**Antimicrobial results against gram-negative bacterial species**
Antibacterial results of this oil were remarkable against gram negative gastro-pathogens, potential causes of cholera, diarrhea and dysentery in Pakistan. These included *Shigella dysenteriae* (22 mm; MIC 1500 µg/ml), *Shigella boydii* (20 mm; MIC 1000 µg/ml), *Shigella flexneri* (18 mm; MIC 2500 µg/ml), *Vibrio cholerae* (25 mm; MIC 1000 µg/ml) and *Escherichia coli* (16 mm; MIC 2000 µg/ml); while no activity was found against the rest of the tested gastrophathogens like *Helicobacter pylori*, *Campylobacter jejuni*, *Salmonella typhi*, *Salmonella paratyphi A* and *Salmonella paratyphi B*. Good antibacterial activity was also found against potential urinary tract infection causing agents like *Proteus mirabilis* (24 mm; MIC 2000 µg/ml) and *Proteus vulgaris* (26 mm; MIC 1000 µg/ml) and also against an important causative agent of pulmonary infection and pneumonia, *Klebsiella pneumoniae* (22 mm; MIC 300 µg/ml). Significant activity was noted against *Acinetobacter baumannii* (20 mm; MIC 2500 µg/ml), which is a potential opportunistic pathogen causing serious infection in immuno-compromised individuals (fig. 4). Antimicrobial investigation and results of the essential oil from *S. santolinifolia* suggest the possible use in the treatment of infections and as a potential candidate to increase the efficacy of chemotherapeutics.

**CONCLUSION**
The essential oil from *S. santolinifolia* is rich in mono- and sesquiterpenes. The ratio of oxygenated and non-oxygenated terpenes is almost equal. These detailed chemical identifications will be helpful in the chemotaxonomic studies. It also possesses antimicrobial potential against a number of potential human pathogens. It is a preliminary screening and to our knowledge is not conducted for this species of Pakistan origin and thus study can be extended in various directions.

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Chemical and antimicrobial studies on the essential oil


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