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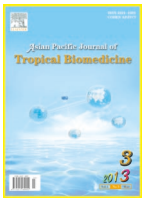

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Activity-guided isolation and identification of anti-staphylococcal components from *Senecio tenuifolius* Burm. F. leaf extracts

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PEER REVIEW

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Comments

This is a good study in which the authors evaluated the antimicrobial properties of *S. tenuifolius*, which are useful in treating skin diseases. The results are interesting and may be used as an effective topical application medicine against drug-resistant *S. aureus* skin infections.
(Details on Page 195)

ABSTRACT

Objective: To investigate activity-guided isolation and identification of anti-*Staphylococcus aures* components from *Senecio tenuifolius* Burm. F. (*S. tenuifolius*). **Methods:** Hexane, chloroform, ethyl acetate, methanol and aqueous extracts of *S. tenuifolius* were prepared by soxilation for antimicrobial activity against one registered *Staphylococcus aureus* (*S. aureus*) (ATCC No: 25923) and two clinical isolates, methicillin resistant and methicillin sensitive *S. aureus*. NCCL standard methods were followed for antibacterial activity. GC-MS was performed to identify the chemical composition of bio active fraction. **Results:** Among all solvent extracts, methanol extract significantly reduced the growth of *S. aureus* (ATCC No: 25923), methicillin resistant and methicillin sensitive *S. aureus* with the best zone of inhibition at 16.23, 14.06 and 15.23 mm and minimum inhibition concentration (MIC) values at 426.16, 683.22 and 512.12 μg/mL, respectively. In order to detect the active component in methanol extract, it was further purified by column chromatography, which yielded four fractions (St1, St2, St3, and St4). Among these four fractions, St3 was effective against the tested strains of *S. aures*, with the best zone of inhibition at 15.09, 13.25 and 14.12 mm and with best MIC values at 88.16, 128.11 and 116.12 μg/mL, respectively. Effective fraction partially purified from *S. tenuifolius* (St3) yielded MIC′s that were at least 20 fold less when compared to crude extract. GC-MS analysis of St3 revealed the presence of 3-[methyl-6,7-dihydro benzofuran-4 (5H)-one], 1,2-benzenedicarboxylic acid, hydroquinone, methyl ester and 3 unknown compounds. **Conclusions:** The study provides scientific evidence for traditional and folklore medicinal use of *S. tenuifolius* in skin infections treatment.

KEYWORDS

Senecio tenuifolius Burm. F., *Staphylococcus aureus*, Chemical composition, GC-MS, Skin infection, Topical application

1. Introduction

During the early period of the 20th century, fewer than 45% of people lived to the age of 65. Until the mid-20th century, infectious diseases were the leading cause of death. Despite Alexander Fleming′s serendipitous discovery in 1928 of the first bactericidal antibiotic, it was not until the early 1940s that penicillin was actually produced and used to treat infectious diseases including infections caused by *Staphylococcus aureus* (*S. aureus*). Just a decade later, a resistant strain of *S. aureus* emerged. It was resistant not only to penicillin, but the new antibiotic arsenal as well as erythromycin, streptomycin, and tetracycline. It was in 1955 when modern medicine was unable to effectively treat the new strain. Faced with this challenge, scientists and health care professionals continued to work collaboratively to control the transmission of the resistant *Staphylococcus* strain and find a cure. By 1960, methicillin was the newest and the most effective weapons against *S. aureus*. In the late 1970s, hospitals in Eastern Australia found the first outbreaks of methicillin-resistant *S. aureus* (MRSA).

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By the 1980s, MRSA had emerged in various places throughout the world[1].

Certain strains of MRSA were found to have the propensity to spread very quickly in hospitals. MRSA infections will transmit from person to person, by direct contact with the skin, clothing, or areas (such as sink, bench, bed, and utensil) that had recent physical contact with a MRSA-infected person[2]. This poses a major threat to public health. MRSA is related to its potential for nosocomial transmission and the limited number of antibiotics are available for its treatment. It has been reported that between the years 1983 and 1994, of 93 new antibacterial agents submitted to analysis by the Food and Drug Administration, six were natural products (teicoplanin, mupirocin, miokamycin, carumonam, isepamicin and RV-11). The screening of plant extracts for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective compounds[3]. Antimicrobial compounds such as benzoin and emetin have been isolated from plants[4]. The systematic screening of antibacterial plant extracts represents continuous efforts to find newer compounds with the potential to act against multi-drug-resistant bacteria[5]. The development of antibiotically resistant strains of microbial pathogens like MRSA, is a growing problem, and it is therefore, extremely important to discover and develop new antimicrobial compounds. One of the measures to minimize the increasing rate of resistance in the long run is to have continuous in-depth investigation for new and effective antimicrobials as alternative agents to substitute the existing ones. Natural resources, especially the plants and microorganisms, are the potent candidates for this purpose[6].

Even though, phytochemicals play a major role as antimicrobial therapeutics, it is important to have awareness of medicinal plants which are poisonous if wrong plant parts or wrong concentrations of its constituents are used. For example, *Chelidonium majus*, which is often prescribed to treat gastric and biliary disorders and as potent antimicrobial agent, can cause cholestatic hepatitis. Some of the poisonous plants such as *Papaver somniferum*, *Datura alba*, *Nerium oleander*, *Strychnos nuxvomica*, *Cleistanthus collinus*, *Cannabis sativa*, *Gloriosa superba*, *Anamirta cocculus*, *Citrulus colocynthis*, *Abrus precatorius*, *Semecarpus anacardium*, *Excoecaria agallocha*, *Digitalis purpurea*, *Aconitum ferrox*, *Croton tiglium* and *Plumbago zeylanica*, which are known to own medicinal properties, are used in the traditional Indian systems of medicine as potent drugs in prescribed doses[7].

Senecio tenuifolius Burm. F. (*S. tenuifolius*) is one of the plants belonging to *Senecio* genus, it is poisonous to livestock, but, the leaves of the plant are used topically as remedy for skin diseases and to reduce swelling and pain according to ethnobotanical information[8,9]. *Senecio* is one of the important genus of the Asteraceae family. The genus *Senecio* has been widely investigated and nearly all species contain pyrrolizidine alkaloids as the most characteristic metabolites, chalcones and flavonoids have also been reported. *Senecio* species were used as food, anti-emetic, anti-inflammatory and also in the treatment of wounds[10]. Hence, the present study aimed to investgate screen and activity guided isolation of active ingredients from *S. tenuifolius* leaves for its anti-staphylococcal activity.

2. Materials and methods

2.1. Source of plant material

S. tenuifolius leaves were collected from Eastern Ghats of Andhra Pradesh, India (Tirumala hills, India) in the month of December 2006 on the basis of ethanobotanical information of traditional Indian herbalists. Identification and authentication were kindly made by Professor Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, India. A classified reference voucher specimen (Voucher No. DOB702) was deposited at Department of Botany, Sri Venkateswara University, Tirupati, India.

2.2. Preparation of the extracts

Collected leaves were washed with distilled water, dried at 37 °C for 72 h, and crushed in a mechanical motor. Powdered sample (100 g) was extracted with 98% hexane (500 mL, w/v), at 69 °C for 10 h using soxhlet extraction apparatus (BOROSIL 3840, India). The leftover powder in thimble was dried and extracted with 99.8% chloroform (450 mL, w/v), at 62 °C for 8 h using soxhlet apparatus. In a similar manner the leftover powder was simultaneously extracted with 99.5% ethylacetate (400 mL, w/v) and 99.9% methanol (400 mL, w/v), at 77 °C and 65 °C for 8 h each, respectively. The whole process was repeated for four times, respective solvent extracts were combined and the solvents were completely evaporated at 65 °C using a rotary vacuum evaporator (BUCHI Rotavapor R-114, Switzerland). Remaining material after solvent extractions, was suspended in 1 L distilled water and boiled for 1 h at 90 °C-95 °C. The supernatant was removed and the extraction was repeated once again. The supernatants thus obtained were combined and filtered through Watmann No. 1 filter paper. The filtrate was concentrated by lyophilization. The extract was designated as aqueous extract[11].

2.3. Preparation of test samples

In the studies of the antimicrobial activity, the soxhlated plant extracts and silica gel G column based fractions were distilled and dissolved in dimethylsulphoxide (DMSO) to obtain the stock concentration of 2 mg/mL and 1 mg/mL, respectively for bioassay. It was established that dilution of DMSO lacked antimicrobial activity against any of the test micro-organisms.

2.4. Test for microorganisms

The soxhlated plant extracts were assayed for antimicrobial activity against one registered bacterial isolate, *S. aureus* (NCIM No: 5021, ATCC No: 25923) which was obtained from the NCIM (NCL, Pune, India). Two clinical isolates, MRSA and methicillin sensitive *S. aureus* (MSSA) were gifted by Dr. P.V.G.K. Sharma, Head, Department of Biotechnology, Sri Venkateswara Institute of Medical Sciences, Tirupati (India).

2.5. Agar well diffusion method

An agar-well diffusion method was employed for determination of antibacterial activities[12]. The extracts

and fractions were dissolved in DMSO. All bacteria were suspended in sterile water and diluted to ~10^6 CFU/mL. The suspension (100 μL) was spread onto the surface of nutrient agar medium (Himedia). Wells (4.6 mm in diameter) were cut from the agar with a sterile borer. Negative controls were prepared using DMSO. Cifroplaxacin, vancomysin and mithicillin (10 μg/well) was used as positive reference standard. The inoculated plates were incubated at 35 °C for 24 h. Antibacterial activity was evaluated by measuring the diameter of inhibition zone of the tested bacteria. All tests were performed in triplicate.

2.6. Determination of minimum inhibition concentration (MIC)

The MICs of the extractions and the fractions were conventionally determined in triplicate for each strain by the micro dilution broth method as described by the National Committee for Clinical Laboratory Standards[13]. Bacterial suspensions were adjusted to the 0.5 Mc Farland standards (approximately 1×10^8 to 2×10^8 CFU/mL). Final inocula were adjusted to the 10^4 CFU/mL. A constant amount of bacteria were added to all tubes and they were incubated at 37 °C for 18-24 h. The MIC was defined as the lowest concentration of extracts and fractions at which there was no visible growth of the organism. MICs of the antibiotic (Cifroplaxacin, vancomysin and mithicillin) were determined in the same way. A positive growth control was included where bacterial suspension was added to a tube filled with nutrient broth without extracts. An uninoculated tube of nutrient broth with extract was also added to serve as negative growth control.

2.7. Column chromatography

Methanol extract from *S. tenuifolius* (45 g of initial concentration, by pooling methanol extracts obtained from 4 different soxhlations) was partially purified. Crude methanol extract was dissolved in 99.9% methanol in the ratio of 1:1 (w/v), adsorbed on silica gel and applied onto the dry packed silica gel column (Merck 60 GF254 70-230 mesh; 500 g; column inner diameter, 90 cm long × 5 cm diameter). The column was eluted by a linear gradient from petroleum ether-ethyl acetate (100:0 to 0:100, v/v; total volume, 4 L) to ethyl acetate-methanol (100:0 to 0:100, v/v; total volume, 6.5 L). All together 25 fractions were obtained. Each fraction (250 mL) was monitored by thin layer chromatography (TLC) on silica gel 60 GF254 TLC aluminium sheets (Merck, layer thickness 0.2 mm) with ethyl acetate:methanol:water (6:3:1; v/v) as the mobile phase. After air drying, spots on the plate were visualized by exposing plate to iodine vapours. Considering the *Rf* values, 25 elutes were pooled into four fractions (designated as St1, St2, St3, and St4). Fractions with similar TLC patterns were pooled. The fractions were then concentrated to complete dryness and stored at 4 °C until use.

2.8. GC-MS analysis

The GC-MS analyses were performed on Agilent 6890 GC system equipped with a 5973 inert mass selective detector and 7863 auto sampler (Agilent Technologies, USA). A CP SIL 8 CB (capillary GC column of 5% phenyl, 95% dimethyl polysiloxane, Varian Chrompack, Middleburg, Netherlands) column of 30.00 mm length, 0.25 mm internal diameter and 0.25 μm film thickness was used. The oven was programmed from an initial temperature 50 °C (hold for 2 min) and to the final temperature 280 °C at a rate of 10 °C/min. The final temperature hold up time was 5 min. Helium at the rate of 1 mL/min was used as the carrier gas in constant flow mode. The inlet and interface temperatures were kept at 280 °C. The electronic impact ionization source was operated at 230 °C and the quadrupole temperature was 150 °C. The MS was scanned from 30 to 600 units for recording full scan spectra. The electronic impact mass spectra obtained from the GC/MS analysis of the sample was usually compared with those of the standard compounds or with the data available in commercial libraries and the library data supplied by the National Institute of Standards and Technology.

3. Results

One hundred gram of the air dried and powdered *S. tenuifolius* leaves were extracted. Subsequently yielded hexane extract (8.9 g), chloroform extract (6.2 g), ethylacetate extract (7.6 g), methanol extract (11.5 g) and the lyophilized filtrate of aqueous extract yields (4.8 g). Among five extracts, four extracts (80%) were observed to possess antibacterial activity against the tested stains of *S. aureus*. Chloroform extract did not produce inhibition zones against either of the strains of *S. aureus*. Aqueous, hexane and ethyl acetate extracts produced moderate anti-staphylococcal activity. All tested strains had not responded for hexane and aqueous extracts at low concentration (250 μg/well; data not presented), while they were sensitive at high concentration of 500 μg/well (Table 1). Methanol extract exhibited the highest antibacterial activity against one reference strain ATCC 25923 and two clinical isolates (MRSA and MSSA) of *S. aureus* with the maximum inhibition zones of 16.23, 14.06 and 15.23 mm, respectively (Table 1). Anti-staphylococcal effects of all the five extracts of *S. tenuifolius* were expressed as MIC and presented in Table 2. Methanol extract was the most active among other extracts, exhibiting very strong activity against tested *S. aureus* strains, with the best MIC values of 426.16, 683.22 and 512.12 μg/mL, respectively. As the methanol extract showed high activity, it was purified or partially purified on silica gel G column as described in experimental section. Four fractions were obtained from column chromatography (designated as St1, St2, St3 and St4) which was further examined for their anti-staphylococcal activities. St3 was the most active fraction with maximum zone of inhibition against *S. aureus* strains (Table 1). St1 and St4 fractions were produced moderate inhibition zones ranged from 10.21 to 12.31 mm. St2 had not shown any inhibiting effect against all tested strains. Partially purified fractions had shown potent activity at 20 fold less concentrations when compared with crude methanol extract. Among four partially purified fractions, St3 expressed strong anti-staphylococcal activity against all tested strains with the best MIC values (Table 2). GC-MS analysis of partially purified potent bioactive fraction (St3) revealed the presence of four known compounds namely,

Table 1
DIZ values of various solvent extracts and methanol fractions of *S. tenuifolius* leaves (mm).

Bacterial strain	Cf	Vc	Ml	Aq	Hx	Cl	EA	Mt	St1	St2	St3	St4
S. aureus ATCC 25923	22.09±1.87	32.22±1.02	30.16±1.00	11.12±1.15	12.23±1.12	–	13.05±1.16	16.23±1.15	12.31±1.02	–	15.09±1.00	12.05±1.10
MRSA	19.89±1.11	31.36±1.12	–	10.06±0.90	10.24±1.23	–	12.24±1.21	14.06±1.12	10.23±1.00	–	13.25±1.00	11.23±1.02
MSSA	20.12±1.12	33.12±1.10	22.14±1.13	10.11± 1.18	11.08±1.14	–	13.05±1.15	15.23±1.09	12.05±1.00	–	14.12±1.04	10.21±1.00

Data are expressed as mean±SD. DIZ: Diameter of inhibition zone, Cf: Ciprofloxacin, Vc: Vancomycin, Ml: Mithicillin, Aq: Aqueous, Hx: Hexane, Cl: Chloroform, EA: Ethyl acetate, Mt: Methanol, Ft: Fractions. The results in the table are average values of triplicate).

Table 2
MIC values of various solvent extracts and methanol fractions of *S. tenuifolius* leaves (μg/mL).

Bacterial strain	Cf	Vc	Ml	Aq	Hx	Cl	EA	Mt	St1	St2	St3	St4
S. aureus ATCC 25923	5.80±1.00	0.78±0.31	1.25±0.08	1706.22±20.16	1365.12±56.02	ND	683.32±30.19	426.16±40.12	215.63±50.21	ND	88.16±22.09	215.20±40.12
MRSA	6.20±1.00	1.50±0.29	⩾100.00	2048.12±30.01	1706.16±34.12	ND	1024.14±20.11	683.22±50.09	482.13±60.21	ND	128.11±18.11	341.21±33.09
MSSA	5.80±1.00	0.78±0.35	1.25±0.09	1024.16±25.12	1365.18±50.11	ND	853.16±30.15	512.12±30.06	341.17±42.09	ND	116.12±28.09	258.11±37.16

Data are expressed as mean±SD. Cf: Ciprofloxacin, Vc: Vancomycin, Ml: Mithicillin, Aq: Aqueous, Hx: Hexane, Cl: Chloroform, EA: Ethyl acetate, Mt: Methanol, Ft: Fractions. The results expressed in the table are average values of triplicate.

3-methyl-6,7-dihydro benzofuran-4(5H)-one, methyl ester Hydroquinone, 1,2-benzenedicarboxylic acid and three unknown compounds (Table 3 and Figure 1).

Table 3
GC-MS analysis of St3 fraction obtained from methanol extract of *S. tenuifolius* leaves by column chromatography.

Retention time (min)	Compound identified
11.94	Hydroquinone
14.20	Methyl ester
14.67	Unknown
15.95	3-methyl-6,7-dihydro benzofuran-4 (5H)-one
16.91	Unknown
19.90	Unknown
24.99	1,2-benzenedicarboxylic acid

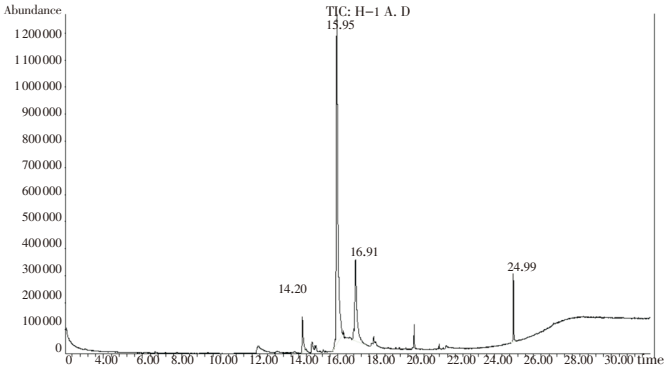

Figure 1. Gas chromatogram of partially purified St3 fraction obtained from methanol extract of *S. tenuifolius* leaves.

4. Discussion

The worldwide distributed genus *Senecio* (family Asteraceae) is a rich source of pyrrolizidine alkaloids. The investigations indicated neurotoxic, mutagenic, carcinogenic, but also antitumor effects of plant's pyrrolizidine alkaloids[14]. On the other hand, many *Senecio* species are used in traditional medicine due to fair amount of therapeutic values, which makes them very interesting.

Agar well diffusion method is a primary *in vitro* method to screen the antimicrobial potential of the drugs/plant extracts. MIC is most commonly used pharmacodynamic parameter for the evaluation of efficacy of anti-infective agent and is a useful predictor of the potency of the drugs-microorganisms interaction. Methanol extracts have shown considerable (MIC) activity against all tested strains. MIC results are in correlating with the results obtained in the agar well diffusion method. With support of these results methanol extract was further subjected to column chromatography to identify the active compounds. The chemical constituents present in St3 were traced out using GC-MS as it is the potent bioactive fraction compared to other fractions. GC-MS analysis revealed that St3 contain a mixer of four known compounds namely, 3-methyl-6, 7-dihydro benzofuran-4(5H)-one, methyl ester, hydroquinone, 1, 2-benzenedicarboxylic acid and three unknown compounds. 3-methyl-6, 7-dihydro benzofuran-4(5H)-one, belongs to dihydro benzofurans which are not cytotoxic and also show anticancer, antimicrobial and antilipidemic activity[15]. 1, 2-benzene dicarboxylic acid, which was reported earlier in *Eurycoma* spp. and also in many plant essential oils with anti-tick and antibacterial activities[16]. Hydroquinone remains the most prescribed bleaching agent worldwide, and is still the gold standard for treatment of hyperpigmentation, although since 2001 its use has been banned in Europe as an ingredient in cosmetics[17]. Methyl ester, a well-known compound which is present commonly in plant oils with antibacterial activity. All the above compounds were identified in St3 may be responsible for the potent anti-Staphylococcal activity. The bioactivity guided purification procedure led to know the potent active compounds in crude methanol extract.

The present findings strongly support the traditional claims of the usage of this plant in treating skin diseases with scientific evidence. Hence it is suggested that, *S. tenuifolius* leaf-extract may be used as an effective topical application medicine against drug-resistant *S. aureus* skin infections. Further investigations are warranted on the isolation and characterization of the unknown active principle(s) for a systematic study which could be one of the prime sources for the development and therapeutical application of new and potent drugs against MRSA infections.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

S. tenuifolius leaves of the plant are used topically as remedy for skin diseases and to reduce swelling and pain according to ethnobotanical information. This work was therefore designated to investigate the antimicrobial activities of extracts and fractions isolated from the leaves of this plant against *S. aureus*.

Research frontiers

Studies are being performed for the activity-guided isolation and identification of anti-*S. aureus* components from *S. tenuifolius*.

Related reports

The present results are in good agreement with the reports of Miert *et al*. and Magano *et al*. focusing on the active compounds as identified in St3 fraction, which may be responsible for the potent anti staphylococcal activity.

Innovations and breakthroughs

MRSA is related to its potential for nosocomial transmission, and the limited number of antibiotics are available for its treatment. Therefore, it is extremely important to discover and develop new antimicrobial compounds. This study led to activity-guided isolation and identification of anti-*S. aureus* components from *S. tenuifolius*, which may be useful for the development of therapeutical application of new and potent drugs against MRSA infections.

Applications

The results of the present study suggest that the methanol extract (St3 fraction) from the leaves of *S. tenuifolius* possesses antimicrobial principles. These results provide promising baseline information for the potential use of the plant in the treatment of skin infections.

Peer review

This is a good study in which the authors evaluated the antimicrobial properties of *S. tenuifolius*, which are useful in treating skin diseases. The results are interesting and may be used as an effective topical application medicine against drug-resistant *S. aureus* skin infections.

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