Compounds from *Vernonia arborea* Buch.-Ham. Inhibit Microbes that Impair Wound Healing

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Authors’ contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

**Aims:** To identify the antimicrobial potency of the leaf fractions of *Vernonia arborea* against selected wound microbes viz., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Stenotrophomonas maltophilia*.

**Background:** Wound healing is often delayed due to the presence of polymicrobial load, that have to be abolished to facilitate the healing process. A major class of antimicrobial phytocompound reported to occur in *Vernonia arborea* species include sesquiterpenes. Reports on the wound healing potency of *V. arborea* in wound models of Wistar rats however did not report antimicrobial activity of the aqueous or methanolic extracts.

**Methodology:** The column fractions of the hexane leaf extract were tested against the selected strains by agar well diffusion assay and the zone of inhibition confirmed with TLC bioautography at specific Rf. The minimum inhibitory concentration (MIC) of the bioactive fractions was identified using resazurin microtiter assay (REMA) and the minimum bactericidal concentration (MBC) was determined. HPTLC quantification was also performed.

**Results:** Out of the 30 pooled fractions, six showed antimicrobial potency against all the five tested wound microbes. The minimum inhibitory concentrations of these fractions were determined.
ranging from 15.62 µg/mL to 500 µg/mL for the different microbes. Quantitative High-Performance Thin Layer Chromatography (HPTLC) revealed two compounds (a and b) in the bioactive fraction with yields of 633 mg (63%) and 97 mg (9.7%) per gram of the extract.

**Conclusion:** The findings suggest the potential use of the bioactive compound in chronic infectious wound management therapy.

**Keywords:** Vernonia arborea; Wound microbes; Minimum inhibitory concentration; HPTLC.

1. INTRODUCTION

*Vernonia arborea*, the tree species of the family Asteraceae is found in Western Ghats of India and Sri Lanka [1]. Apart from the traditional decoctions used for wound healing, the methanol and aqueous leaf extracts of this species showed wound healing activity in excision, incision and dead space wound models of Wistar rats [1-5].

Wounds require a complex repertoire of cellular events to heal. The major reason for impairment in wound healing in most cases has been wound infection caused by a poly-microbial community [6]. Some of these opportunistic strains maybe resistant to regular antibiotics while the others impact the wound microenvironment by producing digestive enzymes like proteases and hyaluronidase, contributing to the increasing surface area and depth of the wound [7].

In the ordered series of events, the early onset and resolution of wound inflammation is a kick start for the actual healing process, apparently delayed by the microbial load at the wound site. Notwithstanding a wide range of the microbes in the polymicrobial community, the most commonly found microbes in clinical cases were chosen for this study, viz., *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 27736) and *Stenotrophomonas maltophilia* (ATCC 13637) [8].

Among the various classes of phytocompounds, sesquiterpenes showed promising antimicrobial potency and happens to be a major phytocompound reported to occur in *V. arborea* species [1,2]. Sesquiterpenes from other plant species like, Cnicin, Cynaropicrin, Ferusinol, Secodesma showed activity against *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae* respectively [9,10].

Guaianolide and Germacraneolide class of sesquiterpene lactones were found active against *S. aureus* [11]. Isabelin, a sesquiterpene lactone was reported to be active against *S. maltophilia* [12].

Reports on the wound healing potency of *V. arborea* in wound models of Wistar rats, prompted us to investigate the antimicrobial activity of hexane leaf extracts [1,3,5]. Zaluzanin D, a sesquiterpene from the hexane leaf extract of *V. arborea* was reported to possess anti-fungal activity [10,12]. Considering the polymicrobial community colonising a wound and impairing the healing process, novel compounds active against a wide range of wound microbes are vital in wound management [7-10,13,14].

In this study we report fractionation of *V. arborea* leaf extracts, the HPTLC profiles and the bioactivity of selected fractions against the five wound microbes using agar well plate assays, the minimum inhibitory and bactericidal concentrations.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Authentication

The *Vernonia arborea* leaves were collected from Anaimalai Hills, Pollachi District (10°22′N 77°07.5′E) in the month of October. The collected material was identified and authenticated and a voucher specimen maintained in the department (PARC/2012/1392).

2.2 Extraction and Column Fractionation of the Crude Extract

The dried, ground leaf material (1 kg) was extracted by exhaustive percolation for three days in *n*-hexane with intermittent stirring for every 18 hours at room temperature. The extract was concentrated in rotary vacuum evaporator (IKA Werke ® HB4 Basic, Germany) and yielded 29.68 g of the crude hexane leaf extract (HLE). Five grams of the HLE was chromatographed over a silica gel column in an eluotropic series
with hexane and ethylacetate [9]. The pooled elutions were concentrated on a rotary vacuum evaporator and stored in a cool dry place in a glass container for further analysis [1,2,15-18].

2.3 Antimicrobial Activity of the Column Fractions

The column fractions were tested against five wound microbes to screen for their antimicrobial activity using agar well diffusion assay. The wound microbes tested include Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Stenotrophomonas maltophilia [19,20].

Thin layers of (1% Muller Hinton) agar seeded with the test strains (0.5 MacFarland Standard concentrations) were added along with the indicator, tetrazolium chloride (0.1%) [18]. The column fractions were added to the agar wells at 1000 μg concentration in triplicates. Ampicillin at the inhibitory concentrations for the respective bacteria was used as positive control. Ampicillin has been studied to inhibit the growth of the test strains by mechanisms including induction of cell wall damage by action on autolysin inhibitor, inhibition of cell wall penicillin-binding proteins and β-lactamase inhibition [19-22]. After incubation for 18 h at 37 °C, the mean diameter of inhibition zones as (mm) ± SD were determined. Reproducibility of these assays was tested at least five times. The fractions that had antimicrobial activity against all of the five bacterial strains were selected for further analysis.

2.3.1 TLC bioautography

TLC of the bioactive fractions were developed and bioautography was performed by overlaying agar containing the test organism on the developed chromatogram. The zone of inhibitions at the specific RI were observed.

2.3.2 MIC microbroth dilution method

The minimum inhibitory concentration (MIC) of the selected bioactive fractions were determined using resazurin microtiter assay (REMA) plate method individually against all the five bacterial strains as per the standard reference method (CLSI 2000). The selected bioactive fractions were serially diluted from 1mg/mL concentration with 100 μL of uninoculated broth in the microtiter plate. Ten microlitres of the standard inoculum (1:100 dilution of the McFarland standard) was added and incubated overnight. After overnight incubation, 30 μL of 0.01% resazurin was added to all the wells and observed for color change after one hour of incubation. Uninoculated broth control, inoculated broth control and with ampicillin drug (at inhibitory concentration for the respective strain) were controls used for comparison. The MIC was identified to be the least concentration of the fraction that showed absence of growth (i.e., inhibition) indicated by no change in color of the dye. Growth was seen as change from purple to pink color due to reduction of the indicator dye [15,16]. The assay was independently performed twice for reproducibility and duplicates for each concentration were maintained.

2.3.3 Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) of the fractions were determined by plating the sample from the REMA plates after determining the MIC through visual observation. A loop full of the suspension from the wells at which MIC was noted and with no apparent color change was streaked onto Muller Hinton agar plates and incubated overnight. The concentration of each fraction inhibiting 99.9% of bacterial growth (yielding no bacterial colonies) on the solid medium was taken as MBC.

2.4 HPTLC Quantification

Quantification of the F10 fraction using the photo-densitometric HPTLC method with a stock solution of the column purified compound (5 mg/mL) dissolved in chloroform was done. A CAMAG Automatic TLC Sampler 4 (ATS4) was used to spot (2 μl, 4 μl, 6 μl and 8 μl) and developed using hexane: ethylacetate (95:5, v/v) and derivatized with methanol: sulphuric acid (90:10, v/v) reagent and chromatograms scanned at 254 nm. The hexane crude leaf extract (5 mg/mL) was (4 μl and 6 μl) spotted on the HPTLC plates precoated with Si-gel Si60F254 (E. Merck). The calibration curve for linearity was obtained by plotting peak area vs. concentration of compound (ng/spot). The data analysed by the linear least square regression method was used to estimate the percentage of the compound (F10) in the HLE of V. arborea sample. Limit of detection and limit of quantification were estimated statistically for three determinations of the sample [19,23-27].

3. RESULTS AND DISCUSSION

Wound microbes contribute to major impairment in healing process, altering the wound
microenvironment by prolonging inflammation, inducing excessive exudate formation, digesting wound tissue with extracellular enzymes, exacerbating devascularization caused by the wound, impeding re-epithelialization [28,29]. Among the listed mechanisms, prolonged inflammation is the primary stumbling block that expedites the repertoire of the other challenging events. Chronic inflammation due to polymicrobial wound infection, is characterised by increased expression of proinflammatory mediators, altering the infiltration of inflammatory cells like neutrophils and macrophages, secretion of tissue degrading enzymes like protease and hyaluronidase. The early resolution of inflammation is an indispensable factor to progress with the actual healing phase, including reepithelialisation and tissue remodelling [30,31].

Hence, clinical wound management predominantly entails destruction of the microbial load. Though wound management strategies including mechanical debridement and regular dressing of the wound microenvironment attempts to clear the necrotized, avascular tissue with exudates and bacterial load, wound palliation in immunocompromised patients warrants the need for a topical exogenous drug that can keep a check on the wound microbes and accelerate the healing process as well. Drugs which are claimed to possess wound healing activity fail to do so either due to the lack of inherent antimicrobial property or due to surging resistance to antibiotic agents among the wound microbes [32-35].

3.1 Antimicrobial Activity of the Column Fractions

The 790 elutions obtained were grouped based on their TLC characteristics. They were pooled into 30 fractions and tested for antimicrobial activity in agar well assays.

3.1.1 Agar well diffusion assay

The 30 fractions tested (at 1mg/mL) against the wound microbes, showed varying inhibition zones as compared to the standard ampicillin drug at inhibitory concentrations for the respective microbe. Six of them showed activity against all five or four bacteria with over 10 mm diameter of zones [Table 1, Fig. 1].

![Fig. 1. Zones of inhibition seen in agar well bioassay of the bacteria tested with 5 fractions of V. arborea leaf extracts and ampicillin. Fractions 10 and 30 inhibited all the tested bacteria](image-url)
Table 1. Antibiosis of the six selected fractions tested at 1mg/ml concentration recorded in the agar well plate assay against wound microbes

| Selected column fractions | Zone of inhibition (mm) of selected bacterial strains |
|---------------------------|-----------------------------------------------|
|                           | S. aureus | E. coli | S. maltophilia | P. aeruginosa | K. pneumoniae |
| Ampicillin tested at 50μg or 75μg | 16 | 22 | 19 | 13 | 11 |
| F10 | 16 | 16 | 16 | 12.7 | 10.8 |
| F25 | 0 | 0 | 17.2 | 0 | 9.7 |
| F26 | 9 | 0 | 16.4 | 9.5 | 8.2 |
| F28 | 11 | 0 | 17 | 10.6 | 0 |
| F29 | 0 | 16 | 18 | 12 | 10.2 |
| F30 | 13 | 14.6 | 15 | 12 | 10.3 |

Triplicates for each fraction and organisms were tested and the values are mean readings. Over 34 fractions were assayed for antibacterial activity against the five bacteria in agar well plate assays. Six of the pooled fractions chosen for agar well plate assay were active against two or more bacteria with zones equal to that obtained with ampicillin.

Among them, fractions F10 and F30 inhibited all the five bacterial species with zones of inhibition > 10 mm. F25 inhibited two of the test strains while F26 inhibited all bacteria except E. coli with moderate inhibition zones. F29 fraction showed two compounds, while one did not inhibit any of the bacteria, the other showed inhibition against four of the bacteria. We therefore selected F10 and F30 fractions which showed promising ability to inhibit all the tested bacterial species. A TLC bioautography overlay assay was also used to identify the Rf of these two fractions that were active against all five microbes [Fig. 2].

![Fig. 2. Bioautography of fraction 10 and 30 overlayed with five wound microbes showing zones of inhibition at Rf 0.05 and 0.85 respectively](image-url)
3.1.2 Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentrations of the six fractions were determined using resazurin dye reduction method and recorded as shown in Table 2.

As shown in Fig. 3, F10 showed an MIC ranging from 31.5 µg/mL against S. aureus and K. pneumoniae to 125 µg µg/ mL against P. aeruginosa. Fraction 30 was also effective with MICs ranging from 15.62 µg/ mL against S. maltophilia to 125 µg/mL against P. aeruginosa. Other fractions showed higher MICs against the five bacteria tested. Among the bacteria, the most resistant was P. aeruginosa. The MBC also supports the same conclusions. Fraction 10 showed MBC at 31.5 µg/mL against S. aureus and K. pneumoniae. The other fractions showed a slightly higher MBC.

Leaf extracts from Vernonia cinerea have been reported to show approximately 10, 13, 12, 12 mm zone of inhibition in agar well diffusion assay and MICs ranging from 2-6, 3-6, 3-5-6, 3-12 mg/mL against S. aureus, E. coli, K. pneumoniae and P. aeruginosa, respectively [36,37]. The compound vernolide from Vernonia amygdalina showed a maximum activity of 19 mm inhibition zone against S. aureus in agar well diffusion assay [38-40]. Hexane leaf extract from Vernonia colorata showed 13 mm inhibition zone and 3.12 mg/mL MIC against S. aureus [41]. The extract inhibited P. aeruginosa at 25 mg/mL. Hexane leaf extracts of Vernonia tenoreana showed 11, 12.3, 16.3, 18.3 mm zone of inhibition and 15, 20, 10, 20 mg/mL MIC against S. aureus, E. coli, K. pneumoniae, P. aeruginosa, respectively. In this study the results obtained from Fraction 10 are better than most of the other species studied earlier [42-45].

Several phytocompounds from the Genus Vernonia have been demonstrated to be antimicrobial in earlier studies. Vernolide from Vernonia galamensis was found to be potent against S. aureus, E. coli, B. subtilis, K. pneumoniae and Proteus mirabilis, with the maximum inhibition zone being 17 mm against S. aureus. This is comparable to the action of the phytocompound isolated from Vernonia arborea (16 mm) against S. aureus in the present study. Chondrillasterol isolated from acetone leaf extract of Vernonia adoensis is reported to be very effective against P. aeruginosa, with an MIC of 16 mg/mL, while this study reports a several fold lower MIC of 125 µg/mL. The compound Vernolide from the species V. galamensis was reported to be effective against Shigella boydii and Salmonella typhi with maximum inhibition zones of about 25-28 mm; studies against wound microbes are not reported though. There are no distinct reports on antimicrobial activity reported from the species of Vernonia against the burn wound microbe, S. maltophilia (16 mm inhibition and 62.2 µg/ml MIC) considered in this study. The crude extracts of the material from the tree Vernonia have also been reported to be active against a number of pathogens, the most predominant being the chloroform stem extract of Vernonia schimperi showing 38 mm zone of inhibition against E. coli [46-48].

Six selected samples of the 30 column fractions of the leaf extract showed activity at their minimum inhibitory and bactericidal concentrations as comparable with the standard antibiotic, ampicillin [49,50]. A few of these samples were found to be mixture of two phytocompounds thus leaving behind a possibility of synergistic role in inhibiting or killing the bacteria.

3.2 Quantification of the Partially Purified Bioactive Fraction in HLE by HPTLC Analysis

The partially purified column fraction, F10 which showed good activity (in terms of MIC and MBC) was selected as the standard for HPTLC analysis and the quantity of the same in the HLE was deduced. The densitogram in Fig. 4 showed two prominent peaks in the putative standard and the HLE with the linear regression correlation shown for the two peaks (a and b) [38]. From the regression equation, the compounds a and b were quantified to be 633mg/g (63%) and 97mg/g (9.7%) of HLE respectively [Table 3] [26-27,51].

The quantification of the bioactive compounds in HLE revealed good yield from the leaf material. Further structural elucidation would open way to QSAR studies and understanding the class of phytoconstituent(s) that could be a lead compound to develop an effective drug. The fraction with potent anti-wound microbial activity could be an effective constituent of medications used in topical management of infectious wounds and is the future objective of this study.
Table 2. Minimum inhibitory concentration of the column fractions tested against wound microbes

| Wound microbe                  | MIC (µg/mL) of fractions |
|--------------------------------|--------------------------|
|                                | F10  | F25  | F26  | F28  | F29  | F30  |
| Staphylococcus aureus          | 31.5 | 250  | 250  | 31.5 | 31.5 | 31.5 |
| Escherichia coli               | 62.2 | 250  | 250  | 125  | 125  | 62.2 |
| Stenotrophomonas maltophilia   | 62.2 | 250  | 250  | 125  | 125  | 15.62|
| Pseudomonas aeruginosa         | 125  | 500  | 500  | 125  | 250  | 125  |
| Klebsiella pneumoniae          | 31.5 | 125  | 125  | 125  | 250  | 31.5 |

Fig. 3. Resazurin microtiter assay plates showing MICs of the fractions 10 and 30 tested against the wound microbes. a, *E. coli* (62.2 µg/mL); b, *S. aureus* (31.5 µg/mL); c, *S. maltophilia* (62.2 and 15.6 µg/mL); d, *P. aeruginosa* (125 µg/mL) and e, *K. pneumoniae* (31.2 µg/mL)

Fig. 4. HPTLC densitogram of the standard (F10, Left) at various concentration ranges and the hexane leaf extract (HLE) scanned under optimum chromatographic conditions at 254 nm. Right, calibration plots of concentration (ng/spot) vs peak area for compound a and b
Table 3. Statistical parameters from the calibration curve for two compounds in F10 and the interpolated amount of bioactive compounds a and b in hexane leaf extract (HLE) using data from HPTLC densitograms

| Calibration parameters          | Compound a       | Compound b       |
|--------------------------------|------------------|------------------|
| Number of standard, n (F10)    | 4                | 4                |
| Linearity range                | 1000-4000 ng/spot| 1000-4000 ng/spot|
| Peak area range                | 3000-8000        | 2000-8000        |
| Mean peak area                 | 5509.75          | 5384.25          |
| Regression equation            | Y=1.3405x + 2158.5| Y=1.6767x + 1192.5|
| Correlation coefficient        | 0.9777           | 0.9738           |
| LOD                            | 1.92µg           | 1.51µg           |
| LOQ                            | 5.84µg           | 4.58µg           |
| Concentration of HLE/spot      | 20µg/µl          | 20µg/µl          |
| Amount/g HLE                   | 633mg            | 97mg             |
| % recovery                     | 63%              | 9.7%             |

4. CONCLUSION

The hexane leaf extracts were fractionated with at least two fractions being antimicrobial against the five wound microbes tested in this study. Fractions 10 showed MICs of 31.5-125 µg/mL while fraction 30 showed a low MIC of 15.62 µg/mL against *S. maltophilia*. Wound healing by methanol and aqueous leaf extracts of this species in wound models of Wistar rats were reported. The antibiosis against the polymicrobial community impairing wound healing was however not reported and this study demonstrated this in the hexane leaf extracts for the first time.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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