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Original Research Article

Evaluation of anti-leishmanial and antibacterial activity of Waldheimia tomentosa (Asteraceae), and chemical profiling of the most bioactive fraction

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Abstract

Purpose: *To evaluate the anti-leishmanial and antibacterial activities of a relatively unexplored whole plant of Waldheimia tomentosa (Asteraceae) and the chemical profiling of its most bioactive fraction.*
Methods: *The whole plant material was extracted with methanol - water (9 : 1) and fractionated into n-hexane (C_6H_{14} or n-Hex), dichloromethane (CH_2Cl_2 or DCM), ethyl acetate ($C_4H_8O_2$ or EtOAc) fractions and aqueous residue. The fractions were screened for leishmanicidal activity against promastigotes and intracellular amastigotes of L. donovani, while antibacterial activity was evaluated against four multi-drug resistant (MDR) clinical isolates by bioassay guided fractionation. Scanning Electron Microscopy (SEM) performed on S. aureus at the minimum inhibitory concentration (MIC). Chemical profiling of the most bioactive fraction was performed using gas chromatography-mass spectrometry (GC-MS).*
Results: *The most significant leishmanicidal activity was exhibited by n-Hex fraction against promastigotes (DD8 strain) with half maximal inhibitory concentration (IC_{50} of 89.85 ± 0.84 µg/mL) and intracellular amastigotes (IC_{50} of 48.3 ± 0.40 µg/mL). The same fraction also exhibited maximum potency against S. aureus and E.coli at MIC of between 62.5 and 125 µg/mL. The fraction comprised mainly fatty acids and alkyl ketones. SEM examination performed on S. aureus at MIC revealed swelling and multiple blisters on cell surface compared to untreated control.*
Conclusion: *The profound antibacterial activity of Waldheimia tomentosa justifies the use of the plant in traditional medicine for stomach ache and food preservation.*

Keywords: *Waldheimia tomentosa, Antibacterial activity, Leishmanicidal activity, Stomach ache, Food preservation*

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INTRODUCTION

The trans-Himalayan belt of Ladakh and Kashmir valley are rich in ethnobotanical wealth. The traditional medicine of this region uses a number of plant extracts or concoctions in combination with salts or minerals for numerous ailments like common infections, memory loss, osteoporosis, age related disorders, AIDS and cancer [1]. Due to extreme climatic condition, the plants produce a number of unique metabolites which help in their sustenance and various other ailments. Barring few scattered examples, the claim of local medical practitioner has been validated by modern scientific parameters. Isolated living environment and poor access to healthcare is a threat for the management of highly prevalent communicable diseases for e.g., tuberculosis

(TB), visceral leishmaniasis (VL) and malaria in this region. Considering the importance, a relatively unexploredplant, *Waldheimia tomentosa,* commonly used in traditional medicine was selected for the present investigation.

Waldheimia tomentosa, known as white leaf ground daisy belonging to the family Asteraceae is a perennial herb growing on the stony slopes of the Himalayas, at altitudes of 3600-5000 m. The plant is used for common infections, acidity, arthritis and rheumatism [2] in traditional medicine. Report of any pharmacological study was not available on *W. tomentosa.* Few secondary metabolites possessing antioxidant activity were reported from another species of Asteraceae family, *Waldheimia glabra* (Decne.) Regel [3]. A number of plants have been reported to have leishmanicidal activity against various forms of leishmanial parasites [4]. Visceral leishmaniasis; a vector-borne disease caused by *L. donovani,* is the second most dreaded parasitic disease after malaria, causing considerable morbidity and mortality [5]. The life cycle of *Leishmania* involves two forms, promastigote and amastigote; one which evolves and thrives extracellularly in flagellar form in the gut of sandfly vector and another which develops intracellularly in the mammalian host respectively [6]. Most of the existing drugs for the treatment of kala-azar provoke a number of side effects and require long-term treatment [7]. Hence, there is an urgent need to explore new drug candidates. It is established fact as scientific evidences also suggests that several plants are rich in bioactive chemical components and several drugs that are analogues of plant origin substances are available and being used in modern day medicine [8]. Therefore, for the development of alternative drug line to treat leishmaniasis and bacterial infections, *W. tomentosa* was considered for further investigation. This is the first report of investigation of the plant for antibacterial and antileishmanial activity along with chemical profiling of the most active fraction.

EXPERIMENTAL

Chemicals and reagents

Solvents used for extraction and fractionation were of analytical grade from Merck, India. All media was procured from HiMedia chemicals, India. Gas Chromatography and Mass Spectrometry analysis was performed on GCMS-Q2010 Ultra, Shimadzu gas chromatograph coupled with a mass selective detector. The separation was achieved using SPB-5 fused-silica capillary column (30 m x 0.25 mm x 0.25 µm), Supelco, Sigma-Aldrich. SEM was performed on Zeiss Evo 40 electron microscope with magnifications ranging from 10K to 25K

Plant Material and bacterial strains

W. tomentosa was procured from the natural habitat of Khardung La region of Ladakh (4500-5300 meters), in the flowering stage and was authenticated by Dr. S. Kitchlu, from Indian Institute of Integrative medicine, Jammu (India). A voucher specimen, bearing vocher number AUUP/AIB/2013/01 is kept at the herbarium of Amity Institute of Biotechnology, Amity University, Uttar Pradesh. MDR bacterial isolates, viz, *S. aureus* (2413), *E. coli* (2461), *Acinetobacter* sp (2457) and *Serratia* sp (2442), were obtained from Dr Kumardeep Dutta Choudhary, Department of Medical Oncology, Rajiv Gandhi Cancer Research Institute, Delhi, India with their respective antibiotic resistance profiles (Table 1).

Preparation of plant extract

Air dried whole plant material of *W. tomentosa* (0.92 kg) was crushed and extracted with organic solvents overnight. Next day, the mixture was sonicated twice in an ultrasonic bath at 35 ^{o}C for 30 min, twice and filtered. The filtrate was collected and the residue was extracted with minimum volume of water. The combined organic extracts were concentrated under reduced pressure, below 50 ^{0}C while, the aqueous extract was concentrated in a lyophilizer. The concentrated organic fraction was resuspended in 75 mL of water, and was fractionated with the solvents in increasing order of polarity viz, *n*-Hex, DCM and EtOAc successively as described previously by Mishra *et al* [9]. The organic and aqueous (Aq) fractions were concentrated appropriately and dry weight of each fraction was recorded. Thereafter, the fractions along with aqueous extract were screened for anti-leishmanial and antibacterial activity.

Phytochemical tests

The presence of alkaloids, flavonoids, steroids, tannins, reducing sugar, cardiac glycosides, terpenoids, anthraquinones and phlobatanins were examined by the methods described by Rajesh *et al* and Sawant *et al* [10,11].

Alkaloids

Methanolic extract with 1 mg/mL concentration was taken in the test tube and 1 mL HCL was added to the extract, thereafter is was gently heated and cooled for 10 mins and filtered.

Table 1: Antibiotic resistance profiles of MDR clinical isolates

Antibiotic	*S. aureus* (2413)	*Serratia sp.* (2442)	*Acinetobacter sp.* (2457)	*E. coli* (2461)
Amikacin	S	S	R	S
Ampicillin	-	R	-	R
Ciprofloxacin	S	R	R	R
Ceftriaxone	S	R	-	R
Chloramphenicol	-	R	-	R
Gentamicin	S	R	R	S
Imepenem	S	S	R	S
Levofloxacin	S	R	-	R
Meropenem	S	S	R	S
Nalidixic acid	-	-	-	R
Nitrofurantoin	-	-	-	S
Norfloxacin	-	-	-	R
Ofloxacin	S	R	-	R
Piperacillin	S	S	R	R
Vancomycin	S	-	-	-
Tobramycin	-	R	R	R

R: Resistant; S: Sensitive

To the filtrate 2 - 3 drops of Dragendorff's reagent was added. The development of a creamy precipitate was indicative of the presence of alkaloids.

Flavonoids

Alkaline reagent test- methanolic extract was treated with 10 % NaOH solution, the change in the color to intense yellow indicates presence of flavonoid.

Steroids

The methanol extract (1 mg/mL) was dissolved in 10 mL of $CHCl_3$ and conc. H_2SO_4 (5 mL) was added from the sides of test tube. As the upper layer turns to brownish red color and H_2SO_4 layer showed greenish yellow fluorescence, indicates the presence of steroids.

Tannin

The methanol extract (1 mg/mL) was treated with 1 mL $FeCl_3$ and formation of green color indicated the presence of condensed tannins.

Reducing sugars

To the methanolic extract (1 mg/mL), 4 mL of Fehling's solution A and B mixture (1:1) was added and was boiled in water bath for five minutes. Presence of free reducing sugars was indicated by brick-red precipitate.

Cardiac glycosides (Keller-Killani's test)

To the methanol extract, 2 mL glacial acetic acid containing one or two drops of $FeCl_3$ was added. A brown colored ring confirms the presence of cardiac glycosides.

Triterpenoids

Acetic anhydride (2 mL) was added to the methanolic extracts (1 mg/mL) and cooled well in ice. H_2SO_4 was added from the sides of test tubes. If color changes from violet to blue or green, presence of terpenes is indicated.

Anthraquinones

The methanolic extract was hydrolyzed with dilute H_2SO_4, 2 mL of benzene and NH_3 (1:1) was added, formation of rose pink color suggests the presence of Anthraquinone.

Phlobatannins

When the methanol extract was boiled with 1 % aqueous HCl, deposition of a red precipitate confirms the presence of phlobatannins.

Bacteria and culture media

Four clinical isolates including *Staphylococcus aureus* (2413)*, Escherichia coli* (2461), *Acinetobacter sp*. (2457) and *Serratia sp.* (2442) were grown on 5 % Muller Hilton broth (MHB). Following initial incubation (37 °C, overnight in shaker incubator), secondary bacterial cultures were suspended in 15 mL of MHB and optical density readings were adjusted and compared to a 0.5 McFarland standard. For the MIC determination bacterial suspension of 5×10^6 colony-forming units (CFU) mL^{-1} was employed [12].

Agar well diffusion assay

Antibacterial activity of plant fractions was determined by agar well diffusion method as described by Rojas *et al* [13]. To the solidified Muller Hilton agar plates, 0.1 mL each of bacterial suspensions (1×10^6 CFU/mL) were added and spread well with sterile glass spreader. Subsequently, wells of 6 mm size were punched aseptically with cork borer into the agar set plates and filled with 30 µL of the plant extracts (the concentration of the extract employed was 30 µg/mL). The extracts were prepared in 2 % DMSO and water expect for the aqueous fraction which was dissolved in autoclaved distilled water. Standard antibiotic disc of tetracycline (30 µg) was used as positive control and autoclaved distilled water was used as negative control. The plates were incubated at 37 ± 2 ºC for 24 h or overnight. The assay was performed in triplicate and the antibacterial activity was expressed as mean diameter of inhibition zones (mm) with standard deviation produced by the tested fractions.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC was determined by broth dilution method described by Weigand *et al* [14]. The bacterial cultures were diluted in Mueller-Hilton broth and density adjusted to 0.5 McFarland turbidity. bacterial suspension (0.5 mL) containing 5×10^6 CFU/mL was added to 4.5 mL of susceptibility test broth containing diluted extracts solution which was already prepared by two-fold serial dilution from the stock solution of extract starting from 1024 to 15.62 µg/mL, in autoclaved tubes. Wells were reserved in each plate as control of sterility (where no inoculum was added), inoculum viability (where no sample solution was added) and DMSO inhibitory effect control. The plates were then incubated for 24 h at 37 °C. After 24 h of incubation, the absorbance was read at 570 nm in ELISA reader. MIC of antibiotic tetracycline was used as standard reference drug, determined in parallel experiment for comparison. The lowest concentration of the sample that prevented visible growth was considered as MIC of the extracts.

MBC assay was performed as described by Celiktas *et al* [15], with slight modifications. The wells in which no growth or complete absence of bacterial growth were identified, aliquots (10 µL) of each well were transferred to Muller Hilton agar plates and incubated at 37 ºC for 24 h. MHA plates with complete absence of growth of bacteria was examined as the minimum bactericidal concentration.

Leishmanicidal assay

Pan sensitive strain of *L. donovani* (DD8) was obtained from Department of Laboratory Medicine (AIIMS), New Delhi, India. The culture was routinely maintained at 24 °C in M-199® (Gibco, USA) medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) (Invitrogen, USA) and 10 % heat inactivated fetal calf serum (FCS; GIBCO®, USA).

Log phase promastigotes were added in 96-well microtiter plate with different concentrations of extracts (250 - 15.6 µg/mL) and incubated at 24 °C for 48 h. Thereafter, 100 µL of MTT (5 mg/ mL) solution was added to each plate and incubated for 4 h at 24 °C. Finally, 100 µL of DMSO was added in each well to dissolve the formazan crystal produced, followed by 18 h of incubation. The absorbance was measured at 570 nm and DMSO (0.5 %) was considered as untreated control while, amphotericin B and miltefosine (1 µg/mL) were used as reference standard drugs. Each experiment was performed in triplicates [16].

To produce intracellular amastigotes, J774G8 (5×10^5 cells mL^{-1}) macrophage cells were plated onto 13 mm coverslips in 24-well plates for 1 h at 37 °C in a CO_2 incubator. Non adherent cells were removed and the cells were further incubated overnight. The cells which remained adhered to the plate were infected with *L. donovani* promastigotes at a parasite: macrophage ratio of 10: 1 and further incubated for 1 h. Unbound promastigotes were removed by extensive washing with PBS (pH 7.2). The infected macrophages (intracellular amastigote form) were incubated with different concentrations of samples (as for promastigotes) and the mean percentage of viable amastigotes was calculated in comparison to control and the results were expressed as concentration inhibiting the parasitic growth. The leishmanicidal effect of each sample was expressed as half maximal inhibitory concentration IC_{50} values.

Cell cytotoxicity assay

Cell cytotoxicity assay was performed by MTT method as described by Kakad and Dhembare [17]. In brief, from chick embryo, fibroblast cells were obtained and cultured in DMEM medium supplemented with Fetal Bovine Serum (FBS) and tetracycline. The cells suspension (2 mL) was treated with sample solution at MIC and twice of MIC concentration. The 96-well plate

was incubated aseptically in CO_2 incubator for 24 h at 37 ^oC. After incubation, cells were disaggregated using trypsin (0.25 %) and cell viability (%) was calculated.

GC-MS analysis

The most potential bioactive, non-polar *n*-Hex fraction of *W. tomentosa*, was accurately weighed to 5 mg and was dissolved in acetonitrile. The sample was centrifuged at 3000 rpm for 15 min. The supernatant was concentrated to dryness and the residue was reconstituted with methanol as required. An aliquot of 1.0 µL was injected for the GC-MS experiment. The GC-MS analysis was repeated three times on GCMS-Q2010 Ultra, Shimadzu gas chromatograph coupled with a mass selective detector. The injector and interface were operated at 260 and 270 ^oC, respectively. The oven temperature was raised from 80 to 280 ^oC at a heating rate of 5 ^oC for 3 min and then isothermally held for 17 min. Helium, at a flow rate of 1.0 mL/min, was used as a carrier gas. Sample solution (1 µL) in methanol (1:100) was injected in pulsed split mode (first 3 min at 1.5 mL/min and the rest time period at 1.0 mL/min; split ratio1:10). Mass selective detector operated at the ionization energy of 70 eV, in 40 - 650 amu range with a scanning speed of 0.33 s. Retention Indices (RI) were determined in relation to a homologous series of *n*-alkanes (C_7 - C_{33}) under the same conditions. GC (FID) analysis was carried out under the same experimental conditions using the same column as described for GC-MS the percentage composition was computed from the GC peak areas without the use of correction factors. Peak identification was accomplished by comparison of their mass spectra with those stored on GC - MS databases (NIST 11 and Wiley 8).

Preparation of cells for SEM

Scanning Electron Microscopy (SEM) analysis was performed on *S. aureus* treated with MIC of *W. tomentosa n-Hex fraction*. Bacterial *cells* were cultured to reach mid exponential growth phase in nutrient broth as described by Tang *et al* [18]. The cells were collected by centrifugation and washed thrice with sodium phosphate buffer. The samples were then fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, at 4 ^oC overnight, and post fixed in 1 % osmium tetroxide in the phosphate buffer for 1 h at room temperature. This was followed by three washings in phosphate buffer for 10 min and subsequently dehydration by a series of ethanol concentrations (20, 50, 70, 90 and 95 %), for 15 min each. The samples were finally subjected to 100 % ethanol and CO_2 to achieve the critical point drying. An aliquot of 20 µL of bacterial pellets were applied on poly-L-lysine slide, and subjected to gold coating and observed under ZeissEvo 40 scanning electron microscope.

Statistical analysis

All experiments were carried out in triplicate and statistical analysis was by Graph Pad Prism, version 4.00 for Windows (GraphPad Software, San Diego California USA). Data are presented as mean ± SD, and $p < 0.05$ for leishmanicidal assay and $p < 0.001$ for antibacterial assay, were set as statistically significant.

RESULTS

The *in-vitro* leishmanicidal activity was checked against promastigotes and intracellular amastigotes of *L. donovani*. The result of the analysis is represented in Table 2.

The antibacterial screening was conducted by agar well diffusion assay using bioassay guided fractions against Gram-positive *S. aureus* and three Gram-negative strains viz., *Serratia sp, Acinetobacter sp.* and *E. coli* MDR clinical isolates. The result of the analysis is represented in Table 3.

Table 2: *In vitro* leishmanicidal activity against promastigotes and intracellular amastigotes of *L. donovani* with fractions of *W. tomentosa*

Extract	IC_{50} (mean±SD, µg/mL) promastigotes	IC_{50} (mean±SD, µg/mL) amastigotes
n-Hex	89.85±0.84	48.3±0.40
DCM	500	250
EtOAc	500	250
Aq	> 1000	> 1000
Control 1 (amphotericin B)	0.055±0.5	0.25±0.48
Control 2 (miltefosine)	8.11±0.36	4.37±0.51

Table 3: Antibacterial activity of *W. tomentosa* plant fractions against four MDR clinical isolates

MDR bacterial isolates	*n*-Hex	DCM	EtOAc	Aqueous	Positive control (tetracycline)
E. coli (2461)	10.6±0.9	18.3±0.8	11.1±0.3	10.3±0.4	21.79±0.58
Acinetobacter sp.(2457)	20.7±0.5	16.2±0.2	12.1±0.6	9.2±0.3	25.33±0.50
S. aureus (2413)	24.1±0.3	18.5±0.2	18.7±0.7	8.0±0.2	25.76±0.58
Serratia sp.(2442)	15.2±0.5	16.1±0.3	17.1±0.6	9.7±0.1	22.33±0.57

Antibacterial activity expressed as diameter of zone of inhibition in mm including 6 mm as diameter of the well. Values represented as mean ± SD of three replicates. All compounds were tested at concentration of 1mg / mL; 30 µg Tetracycline discs were used as positive control

The antibacterial activity was also evaluated by determining MIC values against the selected strains. The MIC value of *S. aureus* and *E. coli* lied in the range of 62.5 - 125 µg/mL (Table 4). Cell cytotoxicity of the active fraction was evaluated at MIC and twice MIC concentration against chick embryo culture based fibroblast cells and the result was well within the permissible limit.

To know the occurrence of different classes of phytochemicals in the fractions, qualitative analysis for alkaloids, flavonoids, steroids, reducing sugar, cardiac glycosides, terpenoids, anthraquinones, tannins, phlobatanins and saponins had been conducted and being presented in Table 5.

In order to gain an insight into the chemical constituents of *n*-Hex fraction of *W. tomentosa*, GC-MS analysis was performed. The constituents were identified by comparing their retention indices (RI) with those stored on GC - MS database. The RI of each compound was determined in relation to a homologous series of *n*-alkanes (C_7 - C_{33}) under the same operating conditions. The GC - MS profile of *W. tomentosa* *n*-Hex fraction showed the presence of twenty compounds as revealed in Table 6.

Table 4: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the most potent *n*-Hex fraction of *W. tomentosa*

Test organism	MIC[a]	MIC[a]	MBC	MBC
	n-Hex	Tetracycline	*n*-Hex	Tetracycline
E. coli (2461)	125	0.5	250	>0.25
S. aureus (2413)	62.5	0.8	62.5	0.5
Acinetobacter sp (2457)	250	2	250	>1
Serratia sp (2442)	250	1.2	500	>2

[a] *Results are presented as MIC_{90} values determined by microdilution method and expressed in µg/mL. Experiments were carried out in triplicate and results are expressed as mean of three replicate experiments. All crude extracts or fractions were dissolved in 0.2 % DMSO and distilled water*

Table 5: Phytochemical profile of soluble fractions of methanol extract of *W. tomentosa*

Phytochemical class	*n*-hex	DCM	EtOAc	Aqueous residue
Alkaloids	++	+	++	-
Flavonoids	++	++	++	++
Steroid	+	+	++	-
Tannins	+	+	+	+
Reducing sugars	-	-	-	++
Cardiac Glycosides	+	++	++	+
Triterpenoids	+	++	_	+
Anthraquinones	-	-	-	-
Phlobatanins	++	++++	+++	+

Flavonoids, steroids, alkaloids and tannins were detected by NaOH - HCl test, Salkowski's reaction, Dragendorff's reaction and ferric chloride test respectively. Additional tests were carried out to check the presence of reducing sugar, cardiac glycosides, phlobatannins, anthraquinones, saponins and terpenoids (10 - 11)

Table 6: GC-MS analysis of *n*-Hex fraction of *W. tomentosa*

Peak	RT (min)	Area (%)	RI[b]	Name of compound[a]	Class of compound	Activities reported
1	23.076	1.84	1670	Bisabolol oxide b (furan oxide)	Sesquiterpene alcohol	Anti-inflammatory, antioxidant, apoptosis inducer
2	28.63	6.3	1934	Heptadecanoic acid, methyl ester	Fatty acid methyl ester	Antibacterial,antifungal, antioxidant
3	28.686	1.82	1937	2,3-diisopropylquinoxaline	Quinoxaline derivative	Not reported
4	29.399	14.4	1973	Tridecanoic acid	Saturated fatty acid	Antioxidant and perfumery
5	31.891	10.99	2105	Linolelaidic acid, methyl ester	Fatty acid methyl ester	Inhibit production of uric acid, urine acidifier
6	32.004	6.54	2111	Linoleic acid, methyl ester	Fatty acid methyl ester	Antibacterial, antifungal
7	32.544	37.72	2142	1-Phenyl-2-pentanone	Aryl ketone	Insecticidal
8	32.664	3.42	2148	Cyclodecene	Acyclic alkene	Not reported
9	33.115	0.51	2174	2-Nitro-2-methyl-1-propanol	Aliphatic alcohol	Agonist of the ER-alpha signalling pathway
10	35.355	5.37	2303	2-methyl-3-buten-1,2-diol	Aliphatic diol	Not reported
11	35.429	1.75	2307	1-(2-hydroxyethoxy)-pentadecane	Aliphatic hydrocarbon	Not reported
12	35.942	0.51	2338	Hexanoic acid, methyl ester	Fatty acid methyl ester	Flavouring agent, exhibit beta-oxidant function, found in melon, raspberry, wine grapes etc
13	38.886	1.4	2508	Pentadecane	Alkane hydrocarbon	Volatile oil component/ antibacterial
14	38.966	0.5	2512	2,4,4-trimethyl-2-penten-1-ol	Aliphatic alcohol	Not reported
15	39.524	0.25	2538	Acetic acid, methyl ester	Fatty acid methyl ester	VOC emission from branches of plants
16	43.42	3.41	2198	Docosane	Acyclic hydrocarbons	Antibacterial
17	43.597	0.51	2722	Acetic acid, trichloro-, propyl ester	Fatty acid	Not reported
18	44.028	0.56	2746	2-Methylpentanoic acid	Fatty acid	Food-flavor ingredient, fragrances, soaps etc.
19	45.729	0.52	2849	Acetonyl decyl ether	Ether	Not reported
20	46.728	1.68	2911	Hexadecane	Alkane hydrocarbon	Volatile oil component/ antibacterial

Components listed in order of elution; [a] compounds identified through NIST11 /Wiley8 Mass Spec library; [b] Retention index on SPB-5 column

GC-MS data revealed that four chemical classes including fatty acid and fatty acid esters (40.06 %), aryl ketone (37.72 %), hydrocarbon (11.66 %) and aliphatic alcohol (6.34 %) constituted almost 95.8 % of the total constituents (Figure 1).

The SEM result showed substantial morphological changes in the treated cells compared to the untreated negative control (Figure 2).

DISCUSSION

Based on the results, the *n*-Hex fraction of *W. tomentosa* demonstrated substantial leishmanicidal activity against promastigotes and intracellular amastigotes, while the other fractions displayed much lower potential.

It is well documented that IC_{50} < 100 µg/mL is

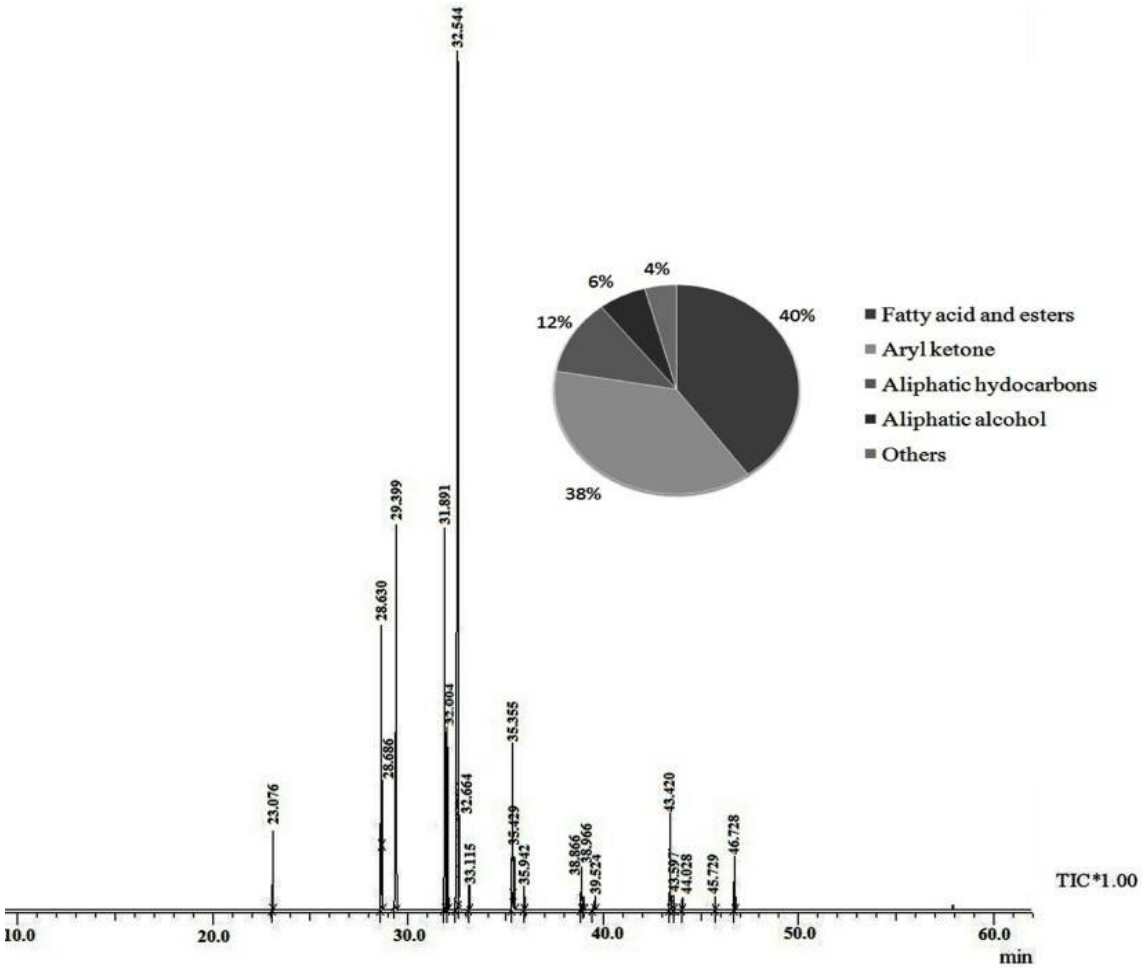

Figure 1: GC-MS spectra of *n*-Hex fraction. The compounds are classified in four major chemotypes and miscellaneous represented as pie-chart

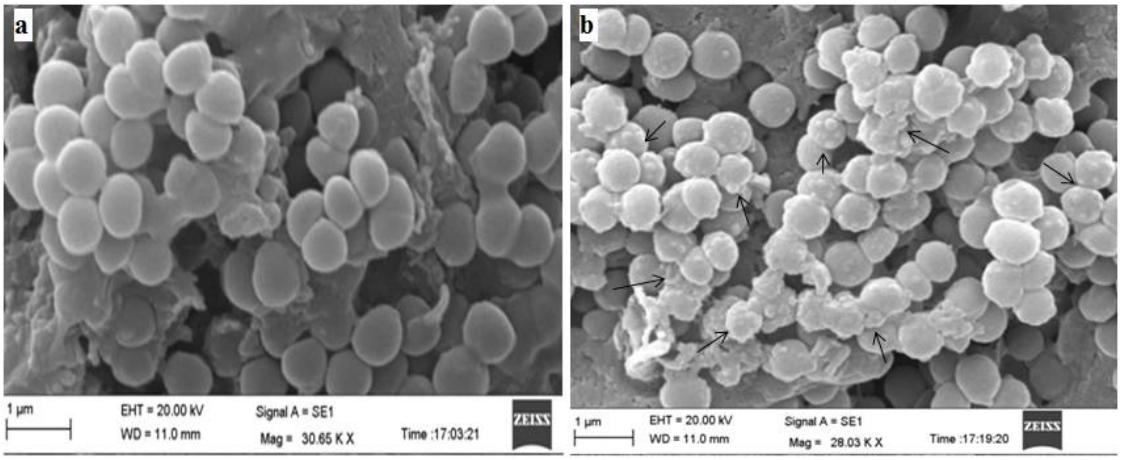

Figure 2: Scanning electron micrograph of *S. aureus* treated with *W. tomentosa n*-Hex fraction. Cells were treated as described in Experimental section. The untreated *S. aureus* cells (2a) can be seen round and intact. The treated cells, i.e. *S. aureus* with *W. tomentosa n*-Hex fraction (2b) can be seen with blebs and distortions at 30000 x magnification

considered as potent while, 100 - 625 µg/mL represent moderate activity [19]. The antibacterial activity evaluated against Gram-positive and three Gram-negative clinical isolates identified *n*-Hex fraction of *W. tomentosa* possessing maximum potency against *S. aureus* with maximum zone of inhibition (ZI) of 24.1 ± 0.3 mm, followed by *Acinetobacter sp.* while, the DCM and EtOAc fractions were moderately active against *S. aureus, Serratia sp.* and *E.coli*.

Also, it is well established that ZI in the range of 11 - 15 mm is considered as high, 16 - 20 mm is considered as very high and more than 21 mm is considered as significant [20]. Minimum inhibitory concentration against the selected strains was determined for *n*-Hex fraction, the values against *S. aureus* and *E. coli* lied in the range of 62.5 - 125 µg/mL.

It is worthy to mention that though, a number of plant extracts exhibit promising biological activity but majority of them suffers from high toxicity on normal cells and could not be considered for drug development [21]. However, our study identified *n*-Hex fraction possessing both leishmanicidal and antibacterial activity without any cell cytotoxicity.

The result of the qualitative test provides a broad idea about phytochemical classes and their relative proportion in a particular fraction. GC - MS analysis data revealed that four chemical classes including fatty acid and fatty acid esters (40.06 %), aryl ketone (37.72 %), hydrocarbon (11.66 %) and aliphatic alcohol (6.34 %) constituted almost 95.8 % of the total constituents. Literature search revealed that six compounds were reported to have antibacterial activity, four compounds were known for their fragrance and seven compounds had not been reported for any biological activity. The source of quinoxaline derivative in GC - MS spectrum is not known. However, it is well known fact that quinoxaline derivatives are used as herbicides in soil and also it is produced by chemical degradation of certain chemicals in soil and soil fungal cultures [22]. Though, 2-nitro-2-methyl-1-propanol, had been reported in a number of GC - MS spectrum [23], but it is difficult to justify the occurrence of the compound (0.51 %) in plant extract. The use of fatty acids is safe and finds wide applications in the field of medicine, agriculture and food preservation due to its broad spectrum and non-specific mode of action.

Better performance demonstrated by non-polar fractions might involve the lipophilic constituents, which causes disturbance to the lipid portion of the plasma membrane, leading to a loss of permeability and leakage of intracellular materials [24], this might be the case as the Scanning Electron Microscopy (SEM) showed morphological changes induced by the extract and revealed that *n*-Hex fraction of *W. tomentosa* induces deleterious morphological changes in the bacterial cell membrane of *S. aureus* indicating membrane damage in bacterial cells.

CONCLUSION

The investigation conducted by bioassay guided fractionation for leishmanicidal and anti-bacterial activity identified *n*-Hex fraction of *W. tomentosa* as the most active fraction. The GC - MS profile was distinctive in nature which could be used further to differentiate *Waldheimia tomentosa* from other *Waldheimia* species. The occurrence of leishmanicidal and antibacterial activity in the same fraction could be beneficial for commercialization of the fraction in herbal medicine and food preservation.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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