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Research Article

Antimicrobial Properties of Plants of Chungtia Village Used Customarily to Treat Skin Related Ailments: From Antimicrobial Screening to Isolation of Active Compounds

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Abstract

Ethno pharmacological relevance: Chungtia villagers of Nagaland, India, have a strong reliance on plants as medicines. Previous studies have shown that 31 Chungtia medicinal plants (and parts therein) used customarily for skin related treatments possess antimicrobial properties against skin pathogens, strongly supporting the use of these plants by the Chungtia villagers. Five plants, namely *Albizia lucidior*, *Begonia picta*, *Cassia floribunda*, *Holboellia latifolia* and *Maesa indica* have no previous studies on their antimicrobial properties, while *Prunus persica* has only antimicrobial activity reported on its fruit, with no reports on its roots, which are used by the Chungtia villagers. The aim of the study was to investigate these six plants for their antimicrobial properties against dermatologically relevant pathogens and undertake phytochemical analysis of the most active species, *Prunus persica*.

Materials and Methods: 70% aqueous ethanoic extracts were prepared of leaves of *B. picta, C. floribunda, H. latifolia* and *M. indica* and roots of *A. lucidior* and *P. persica*. The crude extract of *P. persica* was successively partitioned between water and n-hexane, dichloromethane (DCM) and ethyl acetate (EtOAc). TLC bioautography guided fractionation using column chromatography of the *n*-hexane and EtOAc partition afforded five compounds. The structures of the compounds were elucidated by 1D and 2D NMR spectroscopic techniques and comparison with published data. The crude extract and partitioned fractions of *P. persica* as well as the isolated compounds were screened against antibiotic sensitive and resistant strains of *Staphylococcus aureus* and *Escherichia coli* as well as antibiotic sensitive *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Salmonella typhimurium* and the fungus *C. albicans* using the MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) microtitre dilution and disc diffusion assay methods.

Results: All of the plant aqueous ethanolic extracts exhibited antibacterial activity against at least two of the tested microorganism. The most noteworthy activity was shown by *P. persica* root extract against antibiotic sensitive as well as resistant (methicillin resistant [MRSA] and multi drug resistant [MDRSA]) strains of *S. aureus* (0.156mg/mL for all tested strains). None of the plants were active against the fungus *C. albicans*. This is the first report of antibacterial activity for all of these plants except for *P. persica* of which the fruit has been previously reported to be anti bacterially active. The partitioned fractions of *P.persica* showed antibacterial activity, with the highest antimicrobial activity for the EtOAc fraction with MIC 312µg/mL (susceptible as well as resistant strains of *S.aureus*), followed by *n*-hexane MIC 625µg/mL (susceptible *S. aureus*) and MIC 312µg/mL (MRSA and MDRSA). Bioassay guided fractionation of the EtOAc partition resulted in isolation of ent-epiafzelechin-(2α→O→7, 4α→8)-(-)-ent-afzelechin (1), afzelechin (2), Caffeic acid phenylethyl ester (3) and Gallic acid (4). GC-MS analysis of the *n*-hexane extract showed the presence of the antibacterial compounds palmitic acid, linoleic acid and oleic acid. Bioassay guided fractionation of the *n*-hexane partition resulted in isolation of three ant bacterially active compounds: α-cyanobenzyl benzoate (4), MIC 0.78µg/mL for susceptible and resistant *S. aureus* strains, MIC 312µg/mL for susceptible and resistant *E. coli* strains and MIC 625µg/mL for *P. aeruginosa*), β-sitosterol (5) MIC 2500µg/mL for susceptible and resistant *S. aureus* strains and antibiotic susceptible *E. coli*, MIC 625µg/mL for antibiotic susceptible *S. typhimurium* and MIC 1250µg/mL for antibiotic susceptible *P. aeruginosa*) and stigmast-4- en-3-one (6) MIC 156µg/mL for

susceptible and resistant *S. aureus* strains, MIC 312µg/mL for antibiotic susceptible *E. coli*, MIC 625µg/mL for antibiotic susceptible *S. typhimurium* and MIC 1250µg/mL for antibiotic susceptible *P. aeruginosa*). This is the first report for antibacterial activity for α-cyanobenzyl benzoate and stigmast-4-en-3-one.

Conclusion: Aqueous ethanolic extracts of six Chungtia medicinal plants used for skin related ailments were found to have antibacterial properties against dermatologically relevant bacteria, supporting their customary uses by Chungtia villagers. Good antibacterial activity of the EtOAc and *n*-hexane partitioned fractions from the *P. persica* root extract and the isolated compounds ent-epiafzelechin-(2α→O→7, 4α→8)-(-)-*ent*-afzelechin, α-cyanobenzyl benzoate, β-sitosterol and stigmast-4-en-3-one as well as antibacterially active compounds identified from the GC- MS studies further supports the customary use of roots of this species in treating skin related ailments.

Abbreviations: PAR: Participatory Action Research; CBD: Convention of Biological Diversity; TLC: Thin Layer Chromatography; GC: Gas Chromatography; SEC: Size Exclusion Chromatography; CSMT: Chungtia Senso Mokokchung Town; BSI: Botanical Survey of India; MIC: Minimum Inhibitory Concentration; EI: Electron Impact.

Introduction

The presence of pathogenic bacteria and fungi can cause skin infections and exacerbate the healing of and seriousness of sores and wounds [1-6]. Skin disease and infections cause a significant global disease burden, and with the escalating occurrence of multidrug resistant microorganisms, there is heightened concern that the rates of skin infections will only worsen [7-15]. Much research effort is therefore being focussed on identifying new antimicrobial compounds, including those isolated from nature [16-25]. Since the introduction of conventional antibiotics in the 1950's, there has been little use of plant derivatives as antimicrobials. However, interest in using phytochemicals (products from secondary plant metabolism) for the treatment of microbial infections has increased from the late 1990's following the poor efficacy of conventional antibiotics, due in part to their often excessive and inappropriate use in mammalian infections [26-40]. The Chungtia villagers of Nagaland, North East India, have developed a wealth of knowledge on medicinal flora over many generations [41-49]. In a recent ethno botanical study [50-57], we documented 37 medicinal plants used by Chungtia villagers for the treatment of skin related ailments consistent with a microbial aetiology. In this study, we provide a literature review of these plants with a focus on the antimicrobial properties of relevance to their applications. Six of these plants were then analysed for the first time using antimicrobial screening methods against dermatological relevant microbes. The antimicrobial properties of fractions and compounds isolated from the roots of the most active species, *Prunus persica,* are also reported here.

Materials and Methods

Ethics

This research was approved by the Human Research Ethics Committee at Macquarie University (Ref: HE22JUN2007-R05316 and Ref: 5200700334). It was governed by collaborative research agreements that followed the principles of the Convention of Biological Diversity (CBD) along with the stepwise participatory Action Research (PAR) methodology of UNESCO [58] and was conducted under the framework of best ethical practice, working in partnership with Indigenous people (NHMRC 2003).

General

Preparative TLC (PTLC) was carried out using Uniplate preparative TLC plates (Sigma- Aldrich, Australia). Analytical normal phase thin layer chromatography (TLC) was performed on fluorescent Merck silica gel F^{254} plates (Germany). The TLC plates were visualised using UV light (254nm and 365nm) and vanillin-sulphuric acid spray reagent. UV-visible spectra were recorded on a CARY 1 Bio spectrophotometer (Varian, USA). IR spectra were recorded on a NICOLET iS10 (Thermo Scientific, USA). Optical rotations were measured using a P-1010 polarimeter (JASCO, Japan). Analytical gas chromatography (GC) was carried out on a Shimadzu GC-17A gas chromatograph with an FID detector. Normal phase column chromatography was performed using silica gel 60 (0.040-0.063mm, Merck, Germany). Size exclusion chromatography (SEC) was carried out using Sephadex LH-20 (18-111µm, GE Healthcare Biosciences AB, Sweden). All chemical solvents used for extraction and chromatographic separations were of analytical HPLC grade from Merck, Germany. Organic solvents were evaporated using a Buchi rotary evaporator (Switzerland). ^{1}H, ^{13}C, HSQC, COSY, HMBC and NOESY NMR spectra were recorded on Bruker Avance AMX 400 and Bruker DRX600K 600 MHz NMR Spectrometers (Germany) using standard pulse sequences. The ^{1}H and ^{13}C chemical shifts were referenced relative to the residual chloroform ($^{1}H\delta$ 7.24 and $^{13}C\delta$ 77.2), acetone (δH 2.04 and δc 205.8 and 30.6) and methanol (δH 3.31 and δc 49.0) solvent peaks. A Shimadzu 2010 LC-MS system was used for electro spray ionisation mass spectrometry (ESI-MS) analyses. A Shimadzu GC-17 system

was used for electron impact mass spectrometry (EI-MS) analyses. High resolution mass spectrometry (HR-MS) was determined using a Bruker Apex 3 instrument and circular dichroism was measured on Jasco Circular Dichroism Spectropolarimeter. Freeze drying was conducted with a CHRIST alpha 1-4 LD plus (Labconco, USA) freeze drier.

Plant material

Albizia lucidior (roots), *Begonia picta* (leaves), *Cassia floribunda* (leaves), *Holboellia latifolia* (leaves), *Maesa indica* (leaves) and *Prunus persica* (roots) were collected from Chungtia village, Nagaland, India, by the villagers with the assistance of Mr Anungba Jamir, a Chungtia Senso Mokokchung Town (CSMT) representative. The collections were done between the months of June and August 2007 and 2009. All plant materials were thoroughly inspected by Mr Anungba Jamir for precise identification of the species and dried in the shade for 10 to 20 days. Voucher specimens for each plant were deposited at the Botanical Survey of India (BSI), Shillong Branch, India. For further processing, *A. lucidior*, *B. picta*, *C. floribunda*, *M. indica*, *H. latifolia* were transported to Chennai, India to Dr Velmurugan, while *P. persica* was transported to Dr Udaya Sankar of Central Food Technological Research Institute (CFTRI), located in Mysore, India. Upon receipt by Dr Velmurugan or Dr Sankar, the plant materials were separated from foreign particles, washed with clean water, dried in the shade for 24 hours then dried in a vacuum drier at 75 to 85°C. After 48 hours of drying under vacuum, the plants were kept in the shade for three days and rechecked for foreign particles. The plant materials were then chopped and passed through a micro pulveriser for grinding. The process was repeated until 130-200 mesh size was obtained. The powders were then sieved and dried again under vacuum to ensure that they did not contain any moisture [59-70]. The plant materials were allowed to cool in the shade and packed into plastic bags, which in turn were packed in plastic containers and sealed. The sealed containers were couriered to Macquarie University, Australia; under the import permit IP12012991 from the Department of Agriculture, Fisheries and Forestry (DAFF).

Antimicrobial activity assays

Culture preparation: The use of all microbial strains was approved by the Macquarie

University Biosafety Committee (approval references 05/14 LAB, TEM170512BHA) [71-74]. All cultures were provided by Dr John Merlino (Department of Microbiology, Concord Hospital, Sydney). These included a susceptible strain of *Staphylococcus aureus* (ATCC 29213), community acquired methicillin resistant (MRSA) *Staphylococcus aureus* (ATCC BAA 1026), wild multidrug resistant (MDRSA) clinical isolate *Staphylococcus aureus*, β lactamase negative – sensitive to common antibiotics *Escherichia coli* (ATCC 25922), β lactamase positive, resistant to antibiotics *Escherichia coli* (ATCC 35218), sensitive to common antibiotics *Pseudomonas aeruginosa* (ATCC 27853), clinical isolates *Streptococcus pyogenes* and *Salmonella typhimurium* as well as a clinical isolate *Candida albicans.* Stock cultures of the bacterial strains were maintained in Mueller-Hinton II (MH II) broth containing 10% *v/v* glycerol. The stock culture of the fungus was maintained in Sabouraud Dextrose broth (SAB) containing 10% *v/v* glycerol. Fresh subcultures were made by inoculating the bacterial cultures in MH II broth with the exception of *S. pyogenes* which was inoculated in Todd Hewitt (TH) broth and the fungus in SAB broth, followed by an overnight incubation at 37 °C (bacteria) and 30 °C (fungus). For all antimicrobial assays, the bacteria were grown overnight in MH II broth and *C. albicans* in SAB broth. After overnight incubation the optical density at 600nm (OD600) was measured and the density was adjusted to 0.08 with fresh MH II, TH or SAB broths, as appropriate [75,76]. The CFU/mL of used bacteria was 1.5×10^6 cfu/mL. Vancomycin and kanamycin (Amresco, USA) were used as positive controls for *S. aureus* strains (susceptible *S. aureus*, MRSA, MDRSA) and gentamycin was used for β-, β+ *E. coli*, *P. aeruginosa* and *S. typhimurium* and *S. pyogenes*. Fluconazole was used as a positive control for *C. albicans*.

Aqueous ethanoic extracts preparation: Nagaland Plant materials were prepared in

Nagaland as described in the 2.3 section. Tested plant materials were suspended in 70% aqueous ethanol, shaken overnight at room temperature and vacuum filtered to collect the filtrates. The materials were re-extracted as above, two more times, and the combined filtrates were rotary evaporated (Büchi) at 37 °C. The water residues were freeze dried overnight [77-80].

Minimum inhibitory concentration (MIC): The MTT microdilution assay was used to

quantify the minimum inhibitory concentration (MIC) values of the plant extracts. The plant samples (10mg/mL) or the antibiotic (1mg/mL) were dissolved in 200µL DMSO and diluted with MH II broth to a final volume of 1mL for all bacterial strains, with the exception of *S. pyogenes* for which TH broth was used. SAB broth was used for *C. albicans*. Using a 96 well microtitre plate, 100µL of suitable broth was dispensed into wells 1-11 (from left to right) for each row, 100µL of the samples or antibiotic was added to well 1 (in different rows for each sample) and mixed thoroughly, after which 100µL was taken out and dispensed to the next well (i.e. well 2) [81-92]. This process of two-fold serial dilution was carried out until well 10, and skipping well 11, with the final volume dispensed into well 12. 100µL each of the microbial inoculum was dispensed into wells 1 to 11 leaving well 12. Since well 11 was free of the test sample or the antibiotic, this acted as a positive control for the growth of the inoculum and well 12 being free of inoculum

served as the sterile control of the assay. 2% DMSO/H_2O was also included as a negative control. The plate was incubated at 37 °C for 18 to 20 hours. 20µL of MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazoliium bromide) solution in methanol (5mg/mL) was added to each well followed by incubation for 30 minutes. The MIC values were determined as the lowest concentration of the test sample or antibiotic that showed no visible colour change from yellow to blue [93-99].

Disc diffusion method: For the disc diffusion assay freeze dried samples (10mg) were

dissolved in 200µL DMSO, and then diluted to a final volume of 1mL in distilled water. Discs were loaded with the samples and dried with a hair dryer to afford a sample concentration of 2mg per disc. MH II agar medium was prepared for all bacterial strains (except *S. pyogenes*) as per the manufacturer's protocol and autoclaved at 121 °C for 20 minutes. Potato dextrose agar was used for *C. albicans* and horse blood agar for *S. pyogenes*. The molten media were poured into petri plates (10 to 15mL) and allowed to solidify. Using a sterile cotton swab, the diluted culture of the microorganism was swabbed evenly on the entire surface of the appropriate agar plate to provide a lawn of microbes [100-111]. What man discs (6mm) impregnated with the samples were then placed on the inoculated plate and pressed down gently. The plates were incubated at 37 °C for 18 hours for bacteria and at 30 °C for 24 hours for *C. albicans*. The diameter of the zones of inhibition was measured with a ruler (including the 6mm disc diameter) [112-119]. Negative control discs were prepared with 20% DMSO/H2O. Positive control discs were impregnated with appropriate antibiotics (2µg per disc). Vancomycin and kanamycin were used as positive controls for *S. aureus* strains (susceptible *S. aureus*, MRSA, MDRSA) and gentamycin was used for β-, β+ *E. coli*, *P. aeruginosa*, *S. typhimurium* and *S. pyogenes*. Fluconazole was used as a positive control for *C. albicans* [120].

TLC Bio autography analysis: For the TLC bio autography, the method described by [121-

125] with modifications was used. In brief, developed TLC plates were placed face up on sterile petri dishes and molten, warm, inoculated agar was rapidly distributed over each of them. After solidification of the medium, the TLC plates were incubated overnight and stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) dye. An inoculum of test bacteria (susceptible strain of *S. aureus*) was prepared in the broth by overnight incubation. After incubation, optical density of the culture was measured and adjusted to 0.16 by diluting with the broth (1:50), then an equal volume of molten agar was added, resulting in a final dilution of 0.08 optical densities. Approximately 10mL of the inoculum was rapidly distributed over the TLC plates (10×10cm). After solidification of the medium, the over layed TLC plate was incubated overnight at 37 °C. The bio autogram was then gently covered with a methanolic solution (2.5mg/mL) of MTT with a sterile micropipette, and then incubated for 5 minutes at 37°C for the visualisation of results [126-130].

Solvent-solvent fractionation of P. persica

The freeze dried crude aqueous ethanolic extract of *P. persica* roots (25g) was re suspended in water (900mL) and successively partitioned with *n*-hexane (300mL), DCM (300mL) and EtOAc (300mL). The process was repeated three times [131-137]. Rotary evaporation of the combined extracts and freeze drying afforded four partitions, i.e. *n*-hexane (2g, green solid), DCM (6g, light red solid), EtOAc (8g, dark red solid) and water (5g, blackish red solid).

Phytochemical analysis

The *n*-hexane, DCM, EtOAc and water partitioned fractions (10mg/mL dissolved in methanol, 20µL) were applied on TLC plates in duplicate and developed with petroleum ether:diethyl ether (7:3) for the *n*-hexane and DCM partitions and chloroform: methanol: water eluent systems (7:3:0.4 and 7:3:0.2) for the EtOAc and water partitions, respectively [138-140]. Chemical constituents were detected by visualisation under visible and ultraviolet light (365 and 254nm) and by spraying with vanillin-sulfuric reagent and heating of the plates at 100 °C for 1-2 minutes. Vanillin-sulfuric acid reagent was prepared by mixing 6g vanillin with 2.5mL concentrated H2SO4 in 250mL ethanol. The vanillin-sulfuric acid stain indicated the presence of terpenes as blue, red or violet spots, phenols as pink, red or orange spots and steroids as red or blue spots [141-148].

Extraction and isolation of compounds

The EtOAc extract of *P. persica* (8g) was dissolved in methanol (10mL), mixed with silica gel (5g) and evaporated to dryness by rotary evaporation at 37°C. The sample was then applied to the top of a normal phase silica column (270g silica) prepared with 100% chloroform. The column was eluted with a gradient of chloroform: methanol (100:0 to 0:100%) [149]. 120 fractions (20mL each) were collected, spotted in duplicate on TLC plates and developed with chloroform: methanol: water solvent system in different proportions (1:8:1 to 8:1:1). One of the TLC plates was then stained with vanillin-sulfuric acid reagent; the other was used for overlay TLC bio autography testing against susceptible *S. aureus*. Those were then combined into three major sub-fractions according to similar TLC R_f values (chloroform: methanol: water 7:3:0.2 and 7:3:0.4) and TLC bio autography results to give EtOAc-1 [200mg, yellow solid, Rf 0.75-0.9 (chloroform: methanol: water 7:3:0.4), two spots on TLC plate, none active], EtOAc-2, [1.8g, orange solid, four spots of R_f 0.45, 0.60, 0.63 and 0.66 (chloroform: methanol: water

7:3:0.2), 2 inhibition zones merged R_f 0.63-0.66] and EtOAc-3 [3.0g, red solid, R_f baseline-0.45 (chloroform: methanol: water 7:3:0.4), four spots on TLC plate, two active] [149-152].

EtOAc-2 (1.8g) was dissolved in methanol, mixed with silica (1g) and rotary evaporated at 37 °C to dryness. The solid was applied on top of a normal phase silica column (200g silica) prepared with 100% chloroform. Elution with an increasing polarity gradient of 100% chloroform: 100% methanol yielded 30 sub-fractions, which were spotted in duplicate on TLC plates and developed, one being visualised with vanillin-sulfuric acid reagent, the other tested by TLC bio autography [153]. The fractions were combined based on their TLC R_f values and bio autography results into two sub-fractions: EtOAc-2a [800mg, red solid, two spots, R_f 0.63-0.66 of merged antibacterial activity (chloroform: methanol: water 7:3:0.2)] and EtOAc-2b [600mg, red solid, three spots, R_f 0.63, 0.66 and 0.45, spots of R_f=0.63, 0.66 showed very faint inhibition zones and spot of R_f 0.45 showed strong inhibition (chloroform: methanol: water 7:3:0.2)]. Crystallisation of EtOAc-2b with methanol gave ent- epiafzelechin-(2α→O→7, 4α→8)-(-)-*ent*-afzelechin (1) as a yellow solid (1mg). The same compound was later isolated from the EtOAc-3 fraction (5mg) [154].

Sub-fraction EtOAc-2a (800mg) was dissolved in methanol (0.25mL) and subjected to size exclusion column chromatography (Sephadex LH-20), eluting with methanol. This afforded three fractions, EtOAc-2a1 [220mg, yellow solid, two spots, base line-R_f 0.35 with faint activity and R_f 0.66, no activity ([chloroform: methanol: water 7:3:0.2)]. This sub fraction was further subjected to exclusion column chromatography eluting chloroform: methanol mixture (1:1) to afford two fractions base line-R_f 0.35 and R_f 0.66. NMR of those two fractions revelled the presence of more than one compound so the fractions were further purified on sephadex column eluting with methanol to afford two compounds: Caffeic acid phenylethyl ester (**3**) and Gallic acid (4). EtOAc-2a2 [200mg, white flecks, one spot, R_f 0.63, no activity (chloroform: methanol: water 7:3:0.2)], and EtOAc-2a3 [150 mg, white solid, two spots, no activity, R_f 0.63-0.66 (chloroform: methanol: water 7:3:0.2)] [155-161]. Sub-fraction EtOAc-2a2 was subjected to preparative thin layer chromatography (PTLC) to provide afzelechin (2) [2mg, Rf 0.63, no antibacterial activity (chloroform: methanol: water 7:3:0.2)].).

The *n*-hexane extract (1.5g) was dissolved in minimal methanol (appx 1mL) and subjected to size exclusion column chromatography (Sephadex LH-20) eluting with methanol. This afforded five fractions, Hex-1 (150mg, yellow oil, three spots, Rf ranging from 0.30 to 0.58, one active Rf 0.45 (petroleum ether:diethyl ether 3:7)], Hex-2 [130mg, white flecks, four spots, Rf 0.25-0.70, one active Rf 0.70 (petroleum ether: diethyl ether 3:7)], Hex-3 [60mg, beige solid, one spot, active, Rf 0.65 (petroleum ether: diethyl ether 3:7)] and fractions Hex-4 and Hex-5 (15mg and 8mg, yellow oils, three spot and four spots respectively, Rf=0.30-0.58 and 0.30-0.70, one active of Rf=0.45 [petroleum ether: diethyl ether 3:7]). Separate purification of Hex-1, Hex-4 and Hex-5 by Sephadex LH-20 column chromatography, eluting with methanol, yielded α-cyanobenzyl benzoate (3, 5mg, 1.5mg and 1mg, respectively). Re crystallisation with methanol of fraction Hex-3 yielded β-sitosterol (4, 50mg). Further purification of Hex-2 by Sephadex LH-20 column chromatography, eluting with methanol, yielded stigmast-4-en-3-one (**5**, 1mg).

Structure elucidation of isolated compounds

ent-Epiafzelechin-(2α→O→7',4α→8')-(-)-ent-afzelechin (1): yellow crystals. mp. Decomposition at 238 °C. UV (MeOH): λmax 273. IR (neat) υmax (cm^{-1}): 3391 (br, strong), 2918, 2849, 1700, 1364, 835. CD (MeOH) [θ]232–44.87[α]D–113.7° (MeOH). ESI-MS *m/z* 543 [M-H]-, HREI MS 567.1266 [M+Na] (calc. 567.1267), consistent with the formula C30H24O10 .^{1}H-NMR (600 MHz, CD3COCD3) δ 4.22 (1H, *d, J* 3.5 Hz, H-3), 4.24 (1H, *d, J* 3.5 Hz, H-4), 5.91 (1H, *d, J* 2.2 Hz, H-6), 6.06 (1H, *d, J* 2.2 Hz, H-8), 7.53 (2H, *d, J* 8.6 Hz, H-12, H-16), 6.85 (2H, *d, J* 8.6 Hz, H-13, H-15), 4.81 (1H, *d, J* 8.5 Hz, H-2'), 4.13 (1H, *m*, H-3'), 3.04 (1H, *dd, J* 5.5, 16.5 Hz, H-4'), 2.61 (1H, *dd, J* 5.5, 16.5 Hz, H-4'), 6.13 (1H, *s*, H-6'), 7.39 (2H, *d, J* 8.6 Hz, H-12', H-16'), 6.89 (2H, *d, J* 8.6 Hz, H-13', H-15'), 13C NMR (150MHz, CD3COCD3) δ 100.1 (C-2), 67.1 (C-3), 28.8 (C-4), 156.5 (C-5), 97.8 (C-6), 157.9 (C-7), 96.1 (C-8), 153.8 (C-9), 103.7 (C-10), 131.4 (C-11), 129.3 (C-12), 115.2 (C-13), 158.5 (C-14), 115.2 (C-15), 129.3 (C-16), 83.6 (C-2'), 67.7 (C-3'), 29.2 (C-4'), 155.5 (C-5'), 96.4 (C-6'), 151.8 (C-7'), 106.3 (C-8'), 150.6 (C-9'), 102.7 (C-10'), 129.8 (C-11'), 129.8 (C-12'),116.0 (C-13'), 158.5 (C-14'), 116.0 (C-15'), 129.8 (C-16').

Afzelechin (2): Creamish amorphous solid. UV (MeOH): λmax 273. ESI-MS *m/z* 273 $[M+H]^+$, 1H-NMR (400 MHz, CD3OD) δ 2.49 (1H, *dd*, *J* 8.5, 16.1 Hz, H-4), 2.87 (1H, *dd*, *J* 5.5, 16.1Hz, H-4), 3.97 (1H, *m*, H-3), 4.58 (1H, *d*, *J* 8.0 Hz, H-2), 5.83 (1H, *d*, *J* 2.1 Hz, H-6), 5.91(1H, *d, J* 2.1 Hz, H-8), 6.77 (2H, *d, J* 8.5 Hz, H-13, 15), 7.21 (2H, *d, J* 8.5 Hz, H-12, 16). 13CNMR (75 MHz, CD3OD) δ 82.77 (C-2), 68.87 (C-3), 29.15 (C-4), 157.88 (C-5), 95.48 (C-6), 157.8 (C-7), 96.41 (C-8), 157.28 (C-9), 100.8 (C-10), 131.5 (C-11), 129.58 (C-12), 116.21 (C-13), 156.86 (C-14), 116.21 (C-15), 129.58 (C-16)

Gallic acid (3): white needles. ESI-MS *m/z* 169.1 $[M-H]^-$,^{1}H-NMR (400 MHz, CD3COCD3) δ 6.46 (2H, *s*, H-1,5). ^{13}C NMR (75 MHz, CD3COCD3) δ 167.90 (C-7), 145.85 (C-2,4), 138.43(C-3), 109.16 (C-1,5).

Caffeic acic phenylethyl ester (4): Creamish amorphous solid. ESI-MS *m/z* 283 [M-H]-.

α-***Cyanobenzyl benzoate*** (5): yellow oil. EI-MS *m/z* 51, 77, 105, 115, 237. 1H-NMR (600MHz, CD3OD) δ 6.71 (1H, *s*, H-7), 7.43 (5H, *m*, *J* 16.2 Hz, H-1', 2', 3', 4', 5'), 7.58 (3H, *m*, *J* 17.4 Hz, H-2, 3, 4), 7.96

(2H, *d*, *J* 7.6 Hz, H-1, 5). ^{13}C NMR (150 MHz, CD3OD) δ 64.8 (C-7), 117.6 (C-8), 128.8 (C-2, C-4), 129.6 (C-3'), 129.8 (C-5, C-1), 130.3 (C-2', C-4'), 130.8(C-1', C-5'), 131.3 (C-6), 133.7 (C-3), 135.2 (C-6'), 165.9 (C-9).

β-***Sitosterol*** (6): White needle-like crystals, mp. 138°C (lit. 136-139°C) [15]. IR (neat) υmax (cm^{-1}): 3400 (br strong), 2955, 2918, 2850, 1735, 1463, 1377,1057. EI-MS *m/z* 55, 57, 81, 95, 105, 119, 133, 145, 159, 173, 185, 199, 213, 231, 241, 255,273, 288, 303, 315, 329, 341, 354, 367, 381, 396, 414, fragmentation pattern identical with the authentic sample (Sigma-Aldrich). HREI-MS *m/z* 414.3855 (calc. 414.3862 for C29H50O). ^{1}H- NMR (600MHz CDCl3) δ 0.65 (3H, *s*, H-18), 0.79 (3H, *d*, *J* 8.6 Hz, H-26), 0.81 (3H, *d*, *J* 8.6Hz, H-19), 0.83 (3H, *d*, *J* 8.6 Hz, H-29) 0.89 (3H, *d*, *J* 6.5 Hz, H-21), 0.91 (1H, *m*, H-9), 0.91(1H, *m*, H-24), 0.94 (1H, *m*, H-17), 0.94 (1H, *m*, H-14), 0.98 (3H, *s*, H-27), 1.13 (2H, *m*, H-23), 1.13 (2H, *m*, H-1), 1.24 (2H, *m*, H-28), 1.29 (2H, *m*, H-22), 1.33 (1H, *m*, H-20), 1.47 (2H, *m*, H-11), 1.49 (2H, *m*, H-7), 1.55 (2H, *m*, H-15) 1.64 (1H, *m*, H-25), 1.81 (2H, *m*, H-2), 1.97(2H, *m*, H-12), 1.97 (H, *m*, H-8), 2.21 (1H, *m*, H-4), 2.27 (1H, *m*, H-4), 3.50 (1H, *m*, H-3), 5.33 (1H, *d*, *J*=5.3 Hz, H-6). ^{13}C NMR (150 MHz, CDCl3) δ 37.22 (C-1), 31.64 (C-2), 71.80 (C-3), 42.27 (C-4), 140.73 (C-5), 121.72 (C-6), 31.87 (C-7), 31.89 (C-8), 50.09 (C-9), 36.48 (C-10), 21.06 (C-11), 39.74 (C-12), 42.30 (C-13), 56.74 (C-14), 24.28 (C-15), 28.23 (C-16), 56.01(C-17), 11.84 (C-18), 19.81 (C-19), 36.13 (C-20), 18.76 (C-21), 33.91 (C-22), 26.01 (C-23),45.79 (C-24), 29.10 (C-25), 19.00 (C-26), 19.38 (C-27), 23.03 (C-28), 11.96 (C-29).

Stigmast-4-en-3-one (7): White solid, EI-MS *m/z* 55, 95, 107, 124, 135, 147, 207, 229, 271,288, 327, 370, 397, 412; ^{1}H-NMR (600 MHz, CD3COCD3) δ 075 (3H, *m*, H-18), 0.83 (3H, *m*, H-27), 0.84 (3H, *m*, H-26), 0.93 (1H, *m*, H-9), 0.96 (1H, *m*, H-24), 1.01 (1H, *m*, H-7), 1.05(1H, *m*, H-17), 1.15 (1H, *m*, H-14), 1.22 (3H, *m*, H-19), 1.27 (1H, *m*, H-12), 1.48 (1H, *m*, H-11), 1.56 (1H, *m*, H-11), 1.65 (1H, *m*, H-1), 1.68 (1H, *m*, H-25), 1.87 (1H, *m*, H-7), 2.02 (1H, *m*, H-1) 2.03 (1H, *m*, H-12), 2.18 (1H, *m*, H-2), 2.26 (1H, *m*, H-6), 2.40 (1H, *m*, H-2), 2.45(1H, *m*, H-6), 5.62 (1H, *s*, H-4). ^{13}C NMR (150 MHz, CD3COCD3) δ 198.6 (C-3), 171.5 (C-5),124.2 (C-4), 56.6 (C-14), 56.1 (C-17), 54.9 (C-9), 46.7 (C-24), 43.2 (C-13), 40.6 (C-12), 39.3(C-10), 37.0 (C-20), 36.4 (C-22), 36.1 (C-1), 34.7 (C-8), 34.6 (C-2), 33.4 (C-6), 33.0 (C-7),29.0 (C-16), 29.0 (C-25), 26.8 (C-23), 24.8 (C-28), 21.8 (C-11), 20.1 (C-26), 19.4 (C-27), 19.2(C-21), 17.6 (C-19), 12.3 (C-18), 12.3 (C-29).

All spectral data were in agreement with the published results.

GS-MS analysis

Analytical gas chromatography (GC) was carried out on a Shimadzu GC17A gas chromatograph with a BP-5 column (30m×0.25 mm×25μm) that was programmed from 35- 250 °C at 3 °C/min with helium as the carrier gas. The injector and detector were both programmed at 220 °C. GC integrations were performed on a SMAD electronic integrator. GC-MS was carried out on a Shimadzu GCMS-QP5000 mass spectrometer operating at 70eV ionisation energy [162]. Mass spectra were recorded in electron impact (EI) mode at 70eV, scanning the 41-450*m/z* range. Compounds were identified by their matching GC retention indices relative to *n*-alkanes and by comparison of their mass spectra with either known compounds or published spectra.

Results and Discussion

Selection of Plants for Antimicrobial Testing

We have previously reported the customary uses by Chungtia villagers of 38 plants for the topical treatment of skin related ailments of a likely microbial aetiology. Following an extensive literature review on the antimicrobial activities and anti microbially active extracts/chemical constituents of these plants (Table 1), it was found that extracts from 21 of the plants, including relevant plant parts used by the Chungtia villagers, have previously been analysed for their antimicrobial properties, 12 were reported for possessing antimicrobial activity in a different plant part and compounds with antimicrobial activities have been isolated from fourteen of them. These findings strongly support the customary uses of these plants by the Chungtia villagers. Five Chungtia plants, namely *Albizia lucidior*, *Begonia picta*, *Cassia floribunda*, *Holboellia latifolia* and *Maesa indica* have no prior studies on their antimicrobial properties, while only fruit of *Prunus persica* have been previously studied for antimicrobial activity, with no reports on the roots used by the Chungtia villagers. These six plants were therefore selected for antimicrobial studies [163,164].

Table 1: Reported antimicrobial activities and anti microbially active extracts/chemical constituents from plants used by Chungtia villagers for skin related conditions of possible microbial aetiology.

Plant and parts used by Chungtia villagers		Antimicrobial activity (Plant part: microbe against which active)		
		Reported active	Reported not active	(Plant part: compound: microbe)
1	Albizia lucidior, Roots	None found	None found	None found
2	Allium chinense, Bulbs	Bulbs *S. aureus*; Whole plant: *S. aureus* [102,170]	None found	No active compounds isolated

3	Artocarpus heterophyllus, Sap 69596 WR Leaves: *S. aureus, MRSA, E. coli, S. typhimurium, P. aeruginosa, P. mirabilis, B. subtilis, B. cereus, L. monocytogenes, S. enterica, E. faecalis, A. flavus* [50,161]; seeds: *S. aureus, E. coli, P. aeruginosa, B. megaterium, B. subtilis, B. cereus, M. luteus, S. epidermitis, S. faecalis, S. pneumoniae, C. freundii, E. aerogenes, K. pneumoniae, N. gonorrhoeae, P. mirabilis, P. vulgaris, S. marcescens* [120]; Stem, root heart- wood, leaves, fruits, seeds: *P. aeruginosa, E. coli, S. aureus, C. albicans, S. typhymurium, S. typhi, S. pyogenes, B. cereus, B. coagulans,B. megaterium, B. subtilis, M. luteus, M. roseus, S. albus, S. epidermidis, S. faecalis, S. pneumoniae, C. freundii, E. aerogenes, K. pneumoniae, N. gonorrhoeae, P. mirabilis, P. vulgaris, S. marcences* [50,98,146,147]; whole plant: *MRSA, E.coli, P. aeruginosa* [146]; seeds: E. coli, S. typhi [148]	None found	Fruit: cycloartenone: *E. coli, S. aureus, K. pneumoniae, A. niger, A. flavus* [93], cycloartenol: *E. coli, P. aeruginosa* (Ragasa et al. 2004, Momo et al. 2011), artocarpesin: *S. aureus, E. coli, P. aeruginosa, S. typhi* [100,108]; wood: cycloartocarpin: *S. aureus, E. coli, P. aeruginosa, S. pyogenes [147], artocarpin: S. aureus, MRSA, E.coli, P. aeruginosa* [68,146], artocarpanone: *S. aureus* [68], cyanomaclurin: *S. aureus, E. coli, S. pyogenes* [147] artocarpin: MRSA, *E.coli, P. aeruginosa* [146]	
4	Asclepias curassavica, Leaves69617	Leaves, seeds: *P. aeruginosa* [103]; fruit: *S. aureus* [156]	Leaves, seeds: *S. aureus, E. coli, B. subtilis* [103]; aerial parts: *S. aureus, E. coli, P. aeruginosa* [74, 156]; aerial parts: *S. aureus, S. pyogenes, C. albicans* [123], Kavitha and Satish 2016); roots: *S. aureus, P. aeruginosa, C.* albicans [101];	No active compounds isolated
5	Begonia picta, Leaves69642	None found	None found	None found
6	Calotropis gigantea, Leaves 69691	WR Flowers: *S. aureus, E. coli, P. aeruginosa, S. typhimurium, B. subtilis, B. megaterium, S. Lutea S. sonnei, S. shiga, S. dysenteriae, C. albicans* [80], [55]; stem, roots, leaves, seed: *S. aureus, E. coli, S. typhimurium, S. typhi, V. cholera, P. aeruginosa* [135];	Roots, leaves: *S. aureus, E. coli, P. aeruginosa* [138]; flowers: *B. megaterium*	Flowers: anhydrosophoradiol-3-acetate: *S. aureus, E. coli, S. typhi, S. typhimurium, P. aeruginosa, B. subtilis, S. lutea, S. sonnei, S. shiga, S. dysenteriae, A. flavus*, di-(2-ethylhexyl) phthalate: *S. aureus, E. coli, S. lutea*[80,171];
7	Cassia floribunda, Leaves69535	None found	None found	None found
8	Celosia cristata, Leaves 69520	Flowers, seeds: *S. aureus, C. albicans, B. subtilis* [170]; aerial parts: *S. aureus, E. coli* [38]; whole plant: *B. subtilis* [38];	Whole plant : *K. pneumoniae, S. aureus, E. coli, S. typhi, P aeruginosa, C. albicans* [38];	No active compounds isolated

9	Chrysanthemum indicum, Leaves 69529	WR Flowers: *S. aureus, E. coli, B. subtilis, P. vulgaris, S. typhi, K. pneumoniae, E. faecalis, P. mirabilis, Candida sp* [151]; aerial parts: *S. aureus, S. pyogenes, E. coli, S. epidermidis, S. mutans, S. anginosus, S. gordoniiP. gingivalis* [94]	Flowers: *S. aureus, E. coli, Candida sp* [151];	Seeds: Gallic acid: *S. aureus. E. coli, P. aeruginosa, S. typhi, C. albicans* [56]; caffeic acid : *P. aeruginosa, E. coli*[88]; Luteolin-7-O-β-D-glucoside *S. aureus*: [115];α-pinene, Camphor, 1,8-Cineole, Terpinen-4-ol, Borneol, β- Caryophyllene: *S. aureus, S. pyogenes, E. coli, S. epidermidis, S. mutans, S. anginosus, S. gordonii, P. gingivalis* [94]
10	Cyclea peltata, Leaves 69650	WR Tuber: *E. coli, S. pyogenes, P. vulgaris, P. mirabilis* [22]; whole plant: *E. coli, P. aeruginosa, S. aureus, S. pyogenes, K. pneumoniae, P. vulgaris* [36]; [152]	Tuber: *S. aureus, S. typhi* [22]	No active compounds isolated
11	Dendrocnide sinuata, Stem 69508	Leaves: *E. coli, P. aeruginosa, E. aerogenes* [157]; roots: *S. aureus, E. coli, B. subtilis, S. typhi* [155]	Leaves: *S. aureus, B. subtilis, M. luteus, C. albicans* [157]	No active compounds isolated
12	Drymaria cordata, Whole plant 69530	Aerial parts: *S. aureus, E. coli, P. aeruginosa, B. subtili, B. pumilis* [134,114,125]	None found	Aerial parts: cerebroside: *S. aureus, E. coli, M. tuberculosis, C. albicans* [54]; monogalactosyl diacylglycerol: *S. aureus, MRSA, E. coli, S. Enteric E. faecalis, M. tuberculosis* [109]
13	Duabanga grandiflora, Stem bark 69686	Whole plant: *S. aureus, E. coli, S. epidermidis, B. cereus, B. subtilis [127]; leaves: MRSA* [144]	None found	No active compounds isolated
14	Elsholtzia blanda, Leaves 69524	Aerial parts, leaves: MRSA [34,172] Whole plant: *S. aureus*	Whole plant: E. coli Li et al. 2008	Aerial parts: essential oils - 1,8-cineole, R-(+)-α-phellandrene, bornyl acetate, camphene, linalool, α- terpinene, α-pinene: *S. aureus, E. coli* [75,79]
15	Erythrina stricta, Stem bark 69629	Bark: *S. aureus, MDRSA, MRSA, C. albicans, E.coli* [39]; *S. aureus* [149]; stem bark: *S. aureus, E. coli, P. aeruginosa, C. albicans* [89]		Roots: maackiaflavanone *B, alpinumisoflavone, lupalbigenin, cristacarpin, erystagallin A, erythrabyssin: S. aureus, E. coli, P. aeruginosa* [89,143]; whole plant: ethanol extract [149]; bark: erynone, wighteone, alpinum isoflavone, luteone, obovatin, erythrinassinate, isovanillin: *S. aureus, MRSA, MDRSA* [39]
16	Eupatorium odoratum, Leaves 69523	WR Whole plant: *MRSA, S. aureus* [60]; leaves: *S. aureus, E. coli, C. albicans* [90,107,153]	Whole plant: *S. Aureus* [60]	No active compounds isolated
17	Euphorbia royleana, Sap 69684	Whole plant: *S. aureus, C. albicans* [150]; rhizomes: *S. aureus, E. coli, K. pneumoniae, B. subtilis, P. aeruginosa, S. marcenses, S. typhi, Shigella sp* [133]	None found	Whole plant: ent-11-hydroxyabieta-8(14),13(15)-dien-16,12R-olide, helioscopinolide A, helioscopinolide B: *S. aureus* [150, 165]
18	Eurya acuminata, Leaves 69612	Leaves, stem: *S. aureus* [78]	Leaves, stem: *E. coli, S. cerevisiae, F. oxysporum* [78]	No active compounds isolated
19	Ficus elastica, roots 69619	Leaves: *P. aeruginosa, B. cereus* [41]; bark of aerial roots: *S. aureus, E. coli* [110]	Leaves: *S. aureus* [131]	Leaves: emodin: *S. aureus, E. coli* [62], morin: *S. aureus, MRSA, E. coli, P. aeruginosa, B. cereus* [99,130,45], rutin: S. *aureus, E, coli, S. typhimurium, MRSA, P. aeruginosa, B. cereus* [42,45,105]; bark of aerial roots: ficusamide: *E. coli, S. aureus* [69,110,159], elasticoside: *E. coli, S. aureus*

20	Gmelina arborea, Drupe 69518	WR Fruits: *S. aureus, P. aeruginosa, B. subtilis* [121]; Roots: *S. aureus, E. coli, P. aeruginosa, E. faecalis, A. baumiannii, C. freundii E. aerugenes, K. pneumoniae, P. mirabilis* [112]; leaves, stem bark: *E. coli, K. pneumoniae, P. mirabilis, S. dysenteriae, S. typhi* [73]; Bark: *E. coli* [61]	None found	No active compounds isolated
21	Hedyotis scandens, Leaves 69539	Stem: *S. aureus, E. coli, K. pneumoniae, P. vulgaris* [154]	None found	Leaves: Rosmarinic acid: *S. aureus, P.aeruginosa* [35,46]
22	Holboellia latifolia, Leaves 69500	None found	None found	None found
23	Ipomoea nil, Flowers and leaves 69678	Whole plant, seeds, leaves: *S. aureus, E. coli, B. pumilus, S. pneumoniae, C. freundii, K. pneumoniae,A. niger, C. albicans* [88,91];	None found	Leaves: hydroxybenzoic acid: *S. aureus, E. coli, P. aeruginosa, S. typhimurium, C. albicans* Cho et al. 1998
24	Kalanchoe pinnata, Leaves 69515	WR Leaves: *S. aureus, E. coli, P. aeruginosa, C. albicans* [71,66]; whole plant: *P. aeruginosa,* [116]; leaves, stem: *S. aureus, E. coli, P. aeruginosa, B. subtilis,S. dysenteriae, V. Cholera* [53]	WR Leaves: *S. aureus, E. coli, P. aeruginosa, C. albicans* [167]; whole plant: *S. aureus, E. coli, S. pneumoniae, B. cereus* [116]	Leaves: Quercetin: E. coli [124,163]; rutin: *E. coli* [163]; aerial parts: caffeic acid, p-coumaric acid: *E. coli, S. aureus, protocatechuic acid: E. coli* [48,86,164], 4-hydroxy-3-methoxycinnamic acid: *E. coli, S. aureus* [86]
25	Lagenaria siceraria, Leaves 69521	WR Peel and mesocarp: *S. aureus, E. coli, P.aeruginosa, B. subtilis, C. albicans [58]; seeds: S. aureus, P. aeruginosa, E. coli, S pyogenes, B. megaterium, E. aerogenes, S. Typhi* [58]; leaves: *S. aureus. E. coli, P. aeruginosa, C. albicans [49,118,141]; Fruits: P. aeruginosa, S. pyogenes, S. aureus* [65,118,141]	WR Fruits: *E. coli; leaves: E. coli* [76]	Seeds: palmitic acid: *S. aureus, S. typhi* [58]
26	Lasia spinosa, Leaves and stem 69655	Leaves: *S. aureus, E. coli, P. aeruginosa, B. cereus, B. subtilis, S. lutea, S. paratyphi, S. typhi, S. boydii, S. dysenteriae, V. mimicus, V. parahemolyticus, C. albicans, A. niger* [77]		No active compounds isolated
27	Maesa indica, Leaves 69514	None found	None found	None found
28	Melastoma malabathricum Fruits and leaves 69652	Leaves: *S. aureus, P. aeruginosa* [47]; Leaves and flowers: *S. aureus, E. coli, P. aeruginosa, B. cereus, B. subtilis, S. typhi, K. pneumoniae* [168]; Whole plant: *S. aureus, S. agalactiae* [43];	Whole plant: *E. coli, K. Pneumonia* [44]	
30	Mikania cordata, Leaves and stem 69534	Stem: *S. aureus, E. coli, S. dysenteriae* [161]; Leaves: *S. aureus, S. epidermidis, S. pyogenes, P. aeruginosa, E. coli, S. typhi, S. sonnie, C. albicans* [81,122];	None found	No active compounds isolated
31	Mussaenda roxburghii, Leaves 69502	Leaves: *S. aureus, E. coli, P. aeruginosa, B. cereus, B. megaterium, B. subtilis, S. lutea, S. paratyphi, S. typhi, S. boydii, S. dysenteriae, V. mimicus, V. parahemolyticus* [87]; aerial parts: *S. aureus, E. Coli*[67];	None found	Aerial parts: shanzhiol: S. aureus, E. coli [67]
32	Myrica esculenta, Fruits and leaves 69522	WR Stem bark: *S. aureus, E. coli, P. aeruginosa, B. pumilis, S. epidermidis, A. niger, S. cerevisiae, C. albicans*, (Mahato and Chaudhary 2005, Agnihotri et al. 2012); leaves: *S. aureus, E. coli, S. epidermidis* (Bamola et al. 2008)	None found	No active compounds isolated

33	Nephrolepis cordifolia, Tubers 69623	Fronds, whole plant: *E. coli, P. aeruginosa, S. typhimurium, P. mirabilis, E. aerogenes, K. pneumoniae, B. subtilis, B. cereus, S. faecalis* Aerial parts: *S. aureus, S. pneumoniae, E. faecalis, S. typhimurium, K. pneumoniae, S. flexneri, P. vulgaris* [72,139];	None found	No active compounds isolated
34	Piper betel, Leaves 69697	WR Leaves: *S. aureus, E. coli, P. aeruginosa, K. pneumoniae, B. cereus, S. typhimurium, B. subtilis, P. vulgaris, P. morganii, A. faecalis, E. aerogenes, K. pneumoniae, C. freundii, C. tropicalis, C. Albicans* [145,162];	Leaves: E. coli, P. Aeruginosa [145]	No active compounds isolated
35	Polygonum hydropiper, Leaves 69641	WR Roots: *S. aureus, E. coli, P. aeruginosa, B. subtilis, B. megaterium, E. aerogenes, S. typhi, S. sonnei, C. albicans* [84]; leaves: *S. aureus, E. coli, P. aeruginosa* [70,166]	None found	Leaves: Confertifolin: S. aureus, E. coli, P. aeruginosa, K. pneumoniae, S. epidermidis, B. subtilis, E. faecalis, P. vulgaris [70,166]
36	Prunus persica, Roots69503	Fruit: *S. aureus, E. coli, C. albicans* [24]	None found	No active compounds isolated
37	Stereospermum chelonoides, Stem69610	Stem bark, whole plant: *S. aureus, E. coli, P. aeruginosa, C. albicans, S. typhimurium, B. cereus, B. megaterium, B. subtilis, S. lutea, S. paratyphi, S. typhi, S. boydii, S. disenteriae, V. mimicus, V. parahemolyticus, A. niger, S. cerevacae* [82,129]	None found	Stem bark: stereochenol A, stereochenol B, caffeic acid: E. coli [83,96]
38	Tagetes erecta, Whole plant 69670	Whole plant: MRSA [60]; roots: *S. aureus, E. coli, P. aeruginosa, C. albicans* [57,60] leaves: *S. aureus, E. coli, P. aeruginosa* [64]; flowers: *S. aureus, E. coli* [92,128]		No active compounds isolated

WR - widely referenced. Bacteria: *E. coli - Escherichia coli, P. aeruginosa - Pseudomonas aeruginosa, S. typhimurium - Salmonella typhimurium, S. aureus - Staphylococcus aureus,* MRSA - methicillin resistant Staphylococcus aureus, MDRSA - multi drug resistant Staphylococcus aureus, *S. pyogenes - Streptococcus pyogenes*, Fungi: *C. albicans- Candida albicans.*

Antimicrobial Activity of Selected Plants

The 70% aqueous ethanolic extracts of *A. lucidior* (roots), *B. picta* (leaves), *C. floribunda* (leaves), *H. latifolia* (leaves), *M. indica* (leaves) and *P. persica* (roots) were analysed by disc diffusion assay against susceptible and resistant strains of *S. aureus* and *E. coli*, Susceptible strains of *P. aeruginosa*, *S.* typhimurium and *S. pyogenes* as well as fungal strain of *C. albicans* using 2mg of extract per disc and a microbial density of 1.5×106 CFU/mL. This is consistent with the ideal concentration of crude plant material for the disc diffusion assay is being between 1-5mg per disc and the recommended microbial density being 1.5×106 cfu/mL. For interpretation of the results, zones of inhibition larger than 15mm in diameter (including the 6 mm disc) were considered as having high activity, between 10 and 15 mm as having moderate activity and less than 10mm as having weak activity. The *P. persica* extract showed very good activity against all three strains of *S. aureus*. The other plant extracts were all inactive in the disc diffusion assay against all microbial strains.

The crude extracts of the six plants were also assayed against test microbes susceptible as well as drug resistant strains of *S. aureus* and *E. coli* and drug susceptible strains of *S.* typhimurium, *P. aeruginosa* and *S. pyogenes* using the MTT microdilution assay. For interpretation of the results, partitions with MIC values <312µg/mL were considered as having good activity, between 612µg/mL and 1250µg/mL as having moderate activity and >1250µg/mL as having poor activity. Unlike in the disc diffusion assays, all of the plants showed activity against the susceptible *S. aureus* strain as well as the resistant MRSA and MDRSA strains (Table 2). The *P. persica* extract showed the strongest inhibitory activity against the MRSA and MDRSA strains (MIC 156µg/mL), and its inhibition was more potent than that of the control antibiotic vancomycin (MIC 312µg/mL for MRSA and MDRSA). *H. latifolia* exhibited high antimicrobial potential against the susceptible *S. aureus* strain (MIC 156µg/mL) and moderate activity against the MRSA and MDRSA strains (MIC 1250µg/mL for both strains). *H. latifolia* also moderately inhibited the growth of *S. typhimurium* with an MIC value of 625µg/mL. *M. indica* inhibited the growth of all *S. aureus* strains (MICs 312-2500µg/mL). This plant extract also weakly inhibited *S. typhimurium* with an MIC of 2500µg/mL. *B. picta* showed moderate activity against the susceptible and MRSA *S. aureus* strains with MIC values of 612µg/mL and 1250µg/mL, respectively. *A. lucidior* was moderately active against both susceptible and MRSA *S. aureus* with MIC values of 1250µg/mL against both strains. *C. floribunda* showed moderate to weak activity against all the bacterial strains (MIC 612-2500µg/mL). None of the plant extracts exhibited activity against *C. albicans* (Table 2).

Table 2: MTT microdilution assay results.

Plant name	Yields	MIC (µg/mL)			ST	E.C β-	E.C β+	PA	SP
		SA	MRSA	MDRSA					
M. indica leaves	0.9 g	625	625	625	2500	na	na	na	na
H. latifolia leaves	0.5 g	156	1250	1250	625	na	na	na	na
P. persica roots	2.3 g	156	156	156	na	na	na	na	na
B. picta leaves	1.5 g	612	1250	na	na	na	na	na	na
A. lucidior roots	2 g	1250	1250	na	na	na	na	na	na
C. floribunda leaves	2 g	2500	2500	2500	2500	1250	2500	1250	612
Kanamycin		156	nt	nt	nt	nt	nt	nt	nt
Gentamycin		nt	nt	nt	312	156	312	312	156
Vancomycin		nt	312	312	nt	nt	nt	nt	nt

na - not active at 2500µg/mL, nt - not tested. SA – *Staphylococcus aureus,* MRSA – *methicillin resistant S. aureus,* MDRSA – *multi drug resistant S. aureus*, ST – *Salmonella typhimurium,* E.C β- - *Escherichia coli* β lactamase negative, E.C β+ - *Escherichia coli* β lactamase positive, PA – *Pseudomonas aeruginosa,* SP – *Streptococcus pyogenes.* MIC values were determined as the wells with the lowest sample concentration that showed no change from yellow to dark blue color after addition of MTT.

While all the tested plant extracts showed activity against at least two microorganisms in the MTT micro dilution assay, only the *P. persica* extract was active when tested using the disc diffusion method. The discrepancy in results between these two methods is not uncommon. While the disc diffusion assay is a commonly used method for the antimicrobial screening of medicinal plants, the activity measured as the zone of inhibition is influenced by numerous factors including the size and polarity of the compounds present. Moreover, What man filter paper discs, which are commonly used and were utilised in this study, can also influence results. Paper discs are composed of cellulose, which possesses many free hydroxyl groups which render the surface of the discs hydrophilic Burgess 1999. Therefore, polar compounds can absorb to the surface of the discs and not diffuse into the medium. As a consequence, some polar compounds that possess antimicrobial activity may not show a zone of inhibition in the disc diffusion assay. Non-polar compounds would not be influenced by the hydroxyls on the surface of the paper, but because of their hydrophobic nature may not diffuse through the aqueous medium resulting in false negatives. Large molecules also often diffuse poorly. Thus, some antimicrobial compounds may not be identified using a disc diffusion assay [165]. On the other hand, the accuracy of the MTT micro dilution assay can be compromised by samples that are coloured (such as plant extracts), redox active and/or samples that are not soluble in the medium, which is predominantly aqueous. Although more toxic solvents such as methanol or acetone can be used for water- insoluble compounds (no more that 2% final concentration), DMSO is a popular alternative due to its comparatively lower toxicity. Regardless of the solvent used, some of the compounds might still precipitate which will reduce interaction between the sample tested and the bacteria and as a result limit the sample activity. Therefore, a combination of the disc diffusion assay with at least one other assay is often preferred for screening.

Isolation and antimicrobial activity of extracts and compounds of *P. persica*

As the extract of the roots of *P. persica* demonstrated good activity in both the disc diffusion and MTT micro dilution assays, it was selected for further chemical analyses. The Chungtia villagers consume the liquid from fresh roots of *P. persica* soaked in water to treat typhoid and the seed endosperm is eaten to treat dysentery and diarrhoea. The liquid from the roots and aqueous decoctions of the leaves are also used to treat skin related infections [166]. Except for the roots, all plant parts from this species have been reported for various pharmacological properties, such as antioxidant, anti-inflammatory activities, and hepato- and cardio-protective properties. To the best of our knowledge, the only antibacterial activity reported of this species is for the roots, in a recent study [167].

The 70% aqueous ethanolic extract of *P. persica* was re suspended in water and successively partitioned with *n*-hexane, DCM and EtOAc. These partitions were then tested using the MTT micro dilution assay (Table 3). The EtOAc fraction was found to be the most active with MIC values of 312µg/mL against all tested strains of *S. aureus*. The *n*-hexane partition MIC value was 625µg/mL against the susceptible strain of *S. aureus* and 312µg/mL against both drug resistant strains. The EtOAc and *n*-hexane extracts were therefore deemed worthy of further analysis. None of

the partitions were active against the strains of *E. coli*, *P. aeruginosa, S. typhimurium, S. pyogenes* and *C. albicans.* Bioassay guided isolation of the *n*-hexane and EtOAc partitioned fractions were performed, using a combination of normal phase silica gel column chromatography, size exclusion column chromatography (Sephadex LH-20) and re crystallization, along MTT micro dilution and TLC bio autography assays against susceptible and resistant strains of *S. aureus*. This afforded *ent*- epiafzelechin-(2α→O→7', 4α→8')-(-) *ent*-afzelechin (1) and afzelechin (2) from the EtOAc partition and from the n-hexane fraction, α-cyanobenzyl benzoate (3), stigmast-4-en-3-one (4) and β-sitosterol (5). The antimicrobial activities of compounds 1-5 were tested against susceptible as well as resistant strains of *S. aureus* and *E. coli*, susceptible strains of *P. aeruginosa*, *S. typhimurium*, *S. pyogenes* and the fungus *C. albicans* by the MTT microdilution assay. α-Cyanobenzyl benzoate (**3**) showed excellent antimicrobial properties (MIC 78μg/mL) against all tested *S. aureus* strains, good activity against β lac- *E. coli* (MIC 312μg/mL) and moderate activity against *P. aeruginosa* (MIC 612μg/mL). The antimicrobial properties of this compound are reported here for the first time. Stigmast-4-en-3-one (4) showed very good antimicrobial properties against susceptible and resistant *S. aureus* (MIC 156μg/mL), good antimicrobial activity against antibiotic susceptible *E. coli* (MIC 312μg/mL) and moderate antimicrobial properties against *P. aeruginosa* (MIC 1250μg/mL) and *S. typhimurium* (MIC 612μg/mL). *ent*-Epiafzelechin-(2α→O→7',4α→8')-(-)*ent*-afzelechin (1) was found to possess very good antibacterial activity against the susceptible (MIC 156μg/mL) and resistant strains of *S. aureus* (MIC=312μg/mL) and weak activity against the susceptible strains of *E. coli*, *S. typhimurium* and *P. aeruginosa*.

Table 3: Antimicrobial activity of *P. persica partitions* and compounds by MTT microdilution assay.

Fractions and compounds	Minimum inhibitory concentration (MIC, μg/mL) and minimum bactericidal concentration (MBC, μg/mL)									
	SA		MRSA		MDRSA		EC β-	ST	PA	CA, SP, EC β+
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MIC	MIC	MIC
n-Hexane	625	625	312	625	312	312	na	na	na	na
3	78	nt	78	nt	78	nt	312	na	625	na
4	2500	nt	2500	nt	2500	nt	2500	625	1250	na
5	156	nt	156	nt	156	nt	312	625	1250	na
DCM	625	625	625	625	625	625	na	na	na	na
EtOAc	312	625	312	625	312	625	na	na	na	na
1	156	nt	312	nt	312	nt	2500	2500	2500	na
2	na	nt	na	nt	na	nt	na	na	na	na
Water	1250	na	1250	na	1250	na	na	na	na	na
Vancomycin	156	nt	312	nt	312	nt	nt	nt	nt	nt
Gentamycin	nt	nt	nt	nt	nt	nt	156*	156	156	156*, 312**
Fluconazole	nt	nt	nt	nt	nt	nt	nt	nt	nt	312***

MIC values were determined as the wells with the lowest concentration of the samples that displayed no yellow to blue change of the MTT colour. *MIC values for *E. coli (β-, β+), P. aeruginosa and S. typhimurium.* **MIC value for S. pyogenes. ***MIC value for C. albicans. NT: not tested; na: not active; DCM: dichloromethane; EtOAc: ethyl acetate; MeOH: methanol; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration

This compound was inactive against the resistant strain of *E. coli* as well as the antibiotic susceptible strains of *S. pyogenes* and *C. albicans*. This is the first report of compound 1 possessing antimicrobial properties against the antibiotic susceptible as well as MDR bacterial strains. Afzelechin (2) showed no antibacterial activity in the MTT microdilution assay [168]. Compound 2 is a structural sub-unit of *ent*-epiafzelechin- (2α→O→7',4α→8')-(-) *ent*-afzelechin (1), so it is possible that either other compounds were responsible for the activities and were lost during purification process or this compound arises from the degradation of 1 which could explain the loss in activity seen as fraction EtOAc-2 was purified. All compounds were previously isolated and the spectral data was in agreement with the results previously reported [26-29]. In the bio autography method clear zones were observed for compounds 1, 3, 4 and **5** against susceptible as well as resistant strain of *S. aureus*. Compound **2** was not active by the TLC bio autography assay against all the tested strains of *S. aureus*. This was despite spots at its Rf value (0.63) having shown activity for the partly purified compound 2 in the TLC bio autography assay [169].

Phytochemical and GS-MS analyses of n-Hexane extracts

Following its promising antibacterial activity, the *n*-hexane partition of *P. persica* was selected for further analysis to identify components responsible for its bioactivity. *p*-Anisaldehyde treatment of the TLC chromatogram of the *n*-hexane partition indicated the possible presence of terpenes Wagner 1996. GC-MS analysis of the

extract was done using DB-5 column. Eight phytochemicals were identified (Table 4) and (Figure 1) by comparing their GC retention times relative to *n*-alkanes (C5-C26) and also by comparison of their mass spectra (*m/z* values) with either known compounds or published [170]. The major constituents identified were hexadec-1-ene, octadec-1-ene, palmitic acid, ethyl palmitate, linoleic acid, oleic acid, stearic acid and ethyl stearate. According to the literature, palmitic acid [171], linoleic acid and oleic acid possess good activity against *S. aureus*. The antimicrobial activity of the *n*-hexane partition could therefore be at least partly ascribed to the presence of these bioactive compounds [172].

Table 4: GS-MS analysis of P. persica n-hexane partition using DB-5 column.

Compound	Retention time (min)	m/z value	Reference
Hexadec-1-ene	15.433	224	[126]
Octadec-1-ene	17.8	252	[126]
Palmitic acid	19.617	256	[2]
Ethyl palmitate	19.933	284	[132]
Linoleic acid	21.333	280	[2]
Oleic acid	21.383	282	[2]
Stearic acid	21.6	284	[2]
Ethyl stearate	21.867	312	[132]

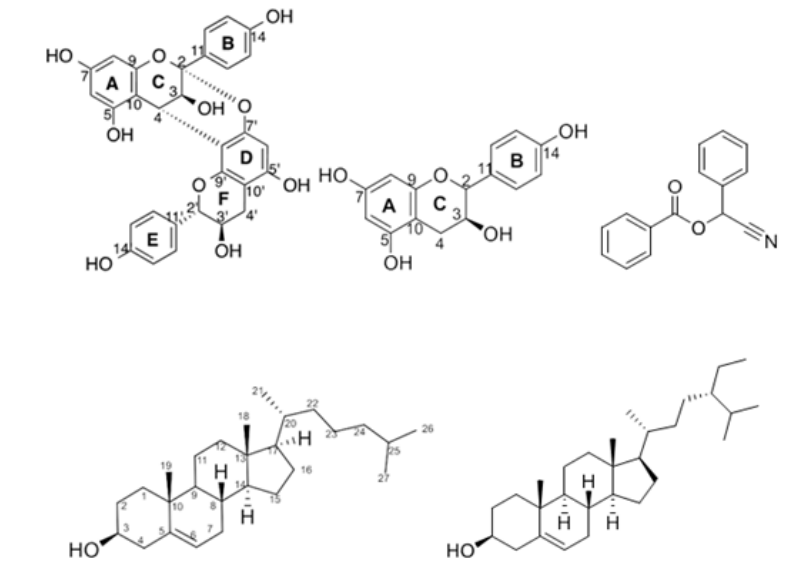

Figure 1: The structures of compounds 1-5.

Conclusion

Antimicrobial screening of Chungtia medicinal plants usedf customarily for skin related ailments, i.e. *Albizia lucidior* (roots), *Begonia picta* (leaves), *Cassia floribunda* (leaves), *Holboellia latifolia* (leaves), *Maesa indica* (leaves) and *Prunus persica* (roots) against dermatologically relevant microorganisms showed that all of the plants possess various levels of antibacterial activity. This supports the customary use of these species. Investigation of the *n*-hexane and EtOAc fractions of the root extract of *P. persica* using bioassay-guided isolation techniques led to the isolation of *ent*-epiafzelechin-(2α→O→7',4α→8')-(-)*ent*-afzelechin (1, *S. aureus* MIC 156μg/mL, MRSA and MDRSA MIC 312μg/mL, *E. coli* β-, *S. typhimurium* and *P.aeruginosa* MIC 2500μg/mL), afzelechin (2, not active), α-cyanobenzyl benzoate (3, *S.aureus*, MDRSA and MRSA 78μg/mL, *E. coli* β- MIC 312μg/mL and *P. aeruginosa* MIC 625μg/mL), β-sitosterol (4, *S. aureus*, MDRSA, MRSA and *E.coli* β-MIC 2500μg/mL, *S. typhimurium* MIC 625μg/mL, *P. aeruginosa* MIC 1250μg/mL) and stigmast-4-en-3-one (5, *S. aureus*, MDRSA and MRSA MIC 156μg/mL, *E. coli* β- MIC 312, *S. typhimurium* MIC 625μg/mL and *P. aeruginosa* MIC 1250μg/mL). Very good antimicrobial activity of the isolated compounds 1 and 3 and good activity of compound 4 against a range of dermatologically relevant bacteria supports the traditional application of this plant to treat skin related ailments. This is the first report of the isolated compounds 1 and 3 possessing antimicrobial activitiesas well as the first report of *ent*-epiafzelechin-(2α→O→7',4α→8')-(-)*ent*-afzelechin and α-cyanobenzyl benzoate being isolated from genus Prunus.

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