SUPPLEMENTARY MATERIAL

Antimicrobial flavonoids isolated from Indian medicinal plant *Scutellaria oblonga* inhibit biofilms formed by common food pathogens

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Scutellaria oblonga Benth., a hitherto phytochemically unexplored Indian medicinal folklore plant was extracted with acetone and subjected to chromatography to yield nine flavonoids, for the first time from this plant. Antimicrobial assays were performed against eleven food borne pathogens, and three molecules (Techtochrysin, Negletein and Quercitin-3-glucoside) depicted significant activity. These molecules were assessed for their rate of antibacterial action using time kill curves which depicted complete inhibition of most of the bacteria within 12-16 h. The significant biofilm reducing capability was exhibited by these three molecules formed a significant finding of the current study. In most of the experiments, a 90-95% reduction in biofilms was observed. Thus, flavonoids as natural molecules from *S. oblonga* could be further researched to be used as potent antimicrobial and anti-biofilm agents.

Key words: *Scutellaria oblonga*, food pathogens, flavonoids, biofilm inhibition

Supporting Information

Experimental Section

Collection of plant material and extraction

Leaves of *Scutellaria oblonga* were obtained from tropical evergreen forests of Western Ghats, India. The plant was authenticated by Dr. Jayendran, Department of Botany, Government Arts College, Ootacamund, India. A voucher specimen (JDB1498) was deposited in Government Arts College, Ootacamund, India. The leaves were shade dried and ground to fine powder and used for extraction. Extracts were prepared by soaking 1 kg of plant material in Hexane, acetone and Methanol successively at room temperature for 24 h and repeated thrice with the residue. The extract was filtered through Whatman No.1 filter paper, and then all the filtrates were pooled up successively and concentrated under vacuum by Rotary evaporator (Buchi® Rotavap R-210).

Chemicals and Instrumentation

All reagents were purchased from Sigma-Aldrich. TLC was monitored with silica gel-precoated aluminum sheets (Type 60 F254, Merck, Darmstadt, Germany) and the spots were visualised in the ultraviolet light chamber, Iodine chamber, 5% MeOH-H₂SO₄ mixture. Elemental analyses were carried out on an automatic Flash EA 1112 Series, CHN Analyser (Thermo). All melting points are measured using Buchi-545. ¹H NMR and ¹³C NMR spectra were determined on a Bruker-400 NMR spectrometer with DMSO-d₆ and chemical shifts were expressed as part per million against TMS as internal reference. Mass spectra were recorded on Agilent 1200 (Liquid Chromatography), Agilent 6320 (Quadrupole Mass Analyser) spectrophotometer.

Fractionation and compound isolation

The acetone extract (25g) obtained after defatting with hexane from *S. oblonga* was taken for column chromatography with silica gel (60-120 mesh) (150 g) packed in a glass column of 4 x 45 cm with bed height of 30 cm. Elution was started with Hexane, followed by increasing ethyl acetate (EA)- hexane combinations (5,10,20,40 and 80% EA in hexane) and finally with EA followed by MeOH. The column elution was monitored by TLC and fractions were pooled based on similar TLC profiles. In total, 9 fractions (F1-F9) were collected and were concentrated under reduced pressure in a Rotary evaporator. Fraction F3 and F4 had similar TLC profiles and therefore were subsequently pooled up which afforded to produce a precipitate. The precipitate was centrifuged, separated from supernatant and evaporated to dryness which yielded Baicalein (160 mg). The supernatant was further subjected to column chromatography with silica (100-200 mesh). Elution was initially with hexane followed by increasing ethyl acetate (EA) - hexane combinations (5, 10, 20, 50 and 75% EA in hexane) and finally with EA followed by MeOH. This column yielded 6 fractions. Fractions F₂1 and F₂2 yielded Tectochrysin (60 mg), F₂3 and F₂4 yielded Chrysin (52 mg), Both F₂5 and F₂6 were pooled up and a flash column chromatography with Silica gel (230-400mesh) with increasing ethyl acetate (EA) - hexane combinations (flow rate of 50 ml/min) yielded 5 fractions. Fractions F₃1 - F₃3 yielded Naringenin (63 mg) and F₃4 - F₃5 yielded Negletein (45 mg).

Fraction F5 obtained from column 1 profusely precipitated to yield Pinostrobin (450 mg). Fraction F6-F8 were subjected to column chromatography with Silica gel (100-200 mesh) eluted initially with

chloroform followed by increasing concentrations of Methanol-chloroform combinations (2, 5, 10, 15, 20, 40, 50%) and finally with pure methanol. 5 fractions were finally obtained from which Fraction 1 yielded Quercitin (35 mg), Fractions F₄₂ - F₄₃ yielded Quercitin-3-Glucoside (45 mg) and fractions F₄₄- F₄₅ yielded Naringin (32 mg). The compounds were carefully separated, evaporated to dryness and characterised for structural elucidation.

Antimicrobial studies

Pathogens and antibiotics used

Staphylococcus aureus (MTCC 96), *Bacillus subtilis* (MTCC 441), *Enterococcus faecalis* (MTCC 439), *Listeria monocytogenes* (MTCC 657), *Salmonella enterica* (MTCC 9844), *Escherichia coli* (MTCC 723), *Vibrio cholera* (MTCC 3904), *Klebsiella pneumonia* (MTCC 432), *Shigella dysenteriae* (ATCC 23513), *Pseudomonas aeruginosa* (MTCC 1688) were used for the antimicrobial studies. Commercial antibiotic (Ciprofloxacin) was purchased from Sigma-aldrich, India All pathogens were maintained on nutrient agar slants at 4°C.

Inoculum preparation

All procedures for determination of Antimicrobial activity were done and inoculum size was standardised according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 1993). Mueller Hinton Broth (MHB; HiMedia, Mumbai, India) was used to prepare inoculum and grown in incubator orbital shaker at 37°C for 4-8 h until the cultures attained turbidity of 0.5 McFarland Unit. Inoculum size was adjusted and standardised to 5×10^5 CFU ml⁻¹ throughout the experiments.

Determination of antibacterial efficacy

The tests to find minimum inhibitory concentrations (MICs) for purified isolated compounds were carried out in triplicate using Resazurin Microtitre Assay (REMA) (Martin et al., 2003) with some modifications. Stock solutions of samples at 500µg/ml were prepared by dissolving the samples in 10% Dimethyl sulphoxide (DMSO). The test samples were diluted in MH broth. The concentration range was 3.9-500 µg/ml. In 96-well microtiter plates, 100 µl of each of the compound dilutions was added to a mixture of 90 µl of MHB and 10 µl of bacterial inoculum. The negative control consisted of 100µl of 10% DMSO, 90 µl of MHB and 10 µl of cell suspension; the positive control had the

addition of ciprofloxacin (3.9-500 µg/ml). Upon the incubation of the test plates at 30°C for 24 h, cell viability was determined by the addition of 15 µl of a 0.01% (wt/vol) Resazurin solution to each of the wells, following an extra incubation period of 2 h at 30°C. Viable microorganisms reduced the blue dye to a pink color, which was detected by fluorescence scanning using a microfluorimeter (FLX-800 fluorimeter, BioTek, Winooski, VT) set to an excitation/emission profile of 530 nm/590 nm.

The MIC plates after incubation were taken for determination of minimum bactericidal concentrations (MBCs) which is the lowest concentration of agent that kills 99.9% of the test bacteria. This was done by plating out aliquots onto each appropriate agar plate. Ciprofloxacin was used as control.

Time kill curves

To assess the rate of growth inhibition of isolated bioactive compound against pathogens, bactericidal action was evaluated using time-kill curves. Tubes containing isolated compound at MBC were inoculated with a suspension of pathogens yielding the final bacterial concentration in broth at $4.5-5.5 \times 10^5$ CFUs. The tubes were thereafter incubated at 37°C and viable counts were performed at 0, 0.5, 1, 2, 3, 4, 5, 6, 12, 16, 20 and 24 h after the addition of isolated compound. Aliquots of culture broth were taken, serially diluted and spread over agar plates and incubated for up to 48 h at 37°C. Repeated washing and centrifugation was done to minimize antibiotic carry over. Colony counts were done in triplicate and data represented in arithmetic mean.

Anti-biofilm activity

To assess the efficacy of Techtochrysin, Negletein and Quercetin-3-glucoside in inhibiting biofilms caused by certain bacteria, it was tested for anti-biofilm activity against four food associated bacteria which usually form biofilms. The inhibition of biofilm formation was tested based on crystal violet staining as described by Christena et al., 2015. Briefly, overnight cultured cells of *Staphylococcus aureus* and *Bacillus subtilis* (gram positive); *Pseudomonas aeruginosa* and *Escherichia coli* (Gram negative) were diluted (1:100) and inoculated into 96 well microtitre plates. These plates contained the compounds (1 – 64µg) in dilute (1:100) Tryptic Soy Broth kept for incubation (18-24 h). Then, the plates were washed with PBS, dried (60°C) and stained with 1 % crystal violet for 15-20 min. Further, it was followed by washing (PBS), drying (60°C) and the stain was extracted using 30% acetic acid

for 1-20 min. The colour developed was analysed by measuring the absorbance at 595 nm using a microplate reader. The experiment was performed in triplicates and the mean $\pm$ SD was recorded.

Figures

Fig S1 Flavonoids isolated from *S. oblonga*

Techtochrysin (1), Chrysin (2), Baicalein (3), Naringenin (4), Negletein (5), Pinostrobin (6), Quercetin (7), Quercetin-3-glucoside (8), Naringin (9).

Fig S2 Proton and carbon Nuclear Magnetic Resonance (NMR) of Techtochrysin.

Fig S3 Proton and carbon Nuclear Magnetic Resonance (NMR) of Negleitein.

Fig S4 Proton and carbon Nuclear Magnetic Resonance (NMR) of Quercetin-3-glucoside.

Fig S5 Time kill curves for Techtochrysin, Negletein and Quercitin-3-glucoside

References

- National Committee for Clinical Laboratory Standards. 1993. Methods for Dilution in Antimicrobial Susceptibility Test, Approved Standard M2–M5. Villanova, PA: *National Committee for Clinical Laboratory Standards*.
- Martin A, Camacho M, Portaels F, Palomino JC. 2003. Resazurin microtiter assay plate testing of *Mycobacterium tuberculosis* susceptibilities to second-line drugs: rapid, simple, and inexpensive method. *Antimicrob Agents Chemother.* 47: 3616–3619.
- Christena LR, Mangalagowri V, Pradheeba P, Ahmed KBA, Shalini BIS, Vidyalakshmi M, Anbazhagan V, Sai Subramanian, N. 2015. Copper nanoparticles as an efflux pump inhibitor to tackle drug resistant bacteria. *RSC Advances.* 5:12899-12909.