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Novel R-plasmid conjugal transfer inhibitory and antibacterial activities of phenolic compounds from *Mallotus philippensis* (Lam.) Mull. Arg.

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ABSTRACT

Antimicrobial resistance severely limits the therapeutic options for many clinically important bacteria. In Gram-negative bacteria, multidrug resistance is commonly facilitated by plasmids that have the ability to accumulate and transfer refractory genes amongst bacterial populations. The aim of this study was to isolate and identify bioactive compounds from the medicinal plant *Mallotus philippensis* (Lam.) Mull. Arg. with both direct antibacterial properties and the capacity to inhibit plasmid conjugal transfer. A chloroform-soluble extract of *M. philippensis* was subjected to bioassay-guided fractionation using chromatographic and spectrometric techniques that led to the isolation of the known compounds rottlerin [5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene] and the red compound (8-cinnamoyl-5,7-dihydroxy-2,2,6-trimethylchromene). Both compounds were characterised and elucidated using one-dimensional and two-dimensional nuclear magnetic resonance (NMR). Rottlerin and the red compound showed potent activities against a panel of clinically relevant Gram-positive bacteria, including meticillin-resistant *Staphylococcus aureus* (MRSA). No significant direct activities were observed against Gram-negative bacteria. However, both rottlerin and the red compound strongly inhibited conjugal transfer of the plasmids pKM101, TP114, pUB307 and R6K amongst *Escherichia coli* at a subinhibitory concentration of 100 mg/L. Interestingly, despite the planar nature of the compounds, binding to plasmid DNA could not be demonstrated by a DNA electrophoretic mobility shift assay. These results show that rottlerin and the red compound are potential candidates for antibacterial drug lead development. Further studies are needed to elucidate the mode of inhibition of the conjugal transfer of plasmids.

1. Introduction

The problem of antimicrobial resistance continues to threaten the future of global health and healthcare systems [1]. Microbial infections caused by multidrug-resistant (MDR) Gram-positive bacteria such as *Staphylococcus aureus* as well as Gram-negative bacteria, including among others *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, represent an increasingly growing problem. Bacterial plasmids have become broadly recognised as a major contributor to the emergence and burden of antibiotic resistance, especially in Gram-negative bacteria, owing to their ease of mobility across and within bacterial species using highly efficient type IV secretion systems (T4SSs) during conjugation [2]. The T4SSs translocate DNA and protein substrates across the bacterial cell envelope and are widespread within Gram-negative bacteria [3]. Exemplary plasmids comprising different incompatibility (Inc) groups that are of clinical relevance include pUB307 [4], pKM101 [5], TP114 [6], R7K [7] and R6K [8]. Over the years, heterocyclic compounds, intercalators such as ethidium bromide and sodium dodecyl sulphate [9], acridine dyes, surface-active alkyl sulphates [10] and quinolones [11] have been reported as plasmid 'curing' agents. However, most of these compounds are

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associated with toxicity and mutation [12], thereby rendering them unsuitable as potential drug templates for anti-plasmid activity. Recent studies have shown that plant-derived drugs can interact with plasmids causing plasmid loss from the host cell or inhibiting T4SSs [13]. Furthermore, natural plant products are a rich source of bioactive chemical scaffolds that have yielded antimicrobial drug leads and have been exploited for various diseases. Natural compounds such as phenolics [14] and acylphloroglucinols [15] are capable of modifying the bacterial resistance phenotypes of meticillin-resistant *S. aureus* (MRSA). A novel and promising approach to deal with multidrug resistance and plasmid-encoded antibiotic resistance is to discover new antimicrobial hits that can complement the clinical efficacy of existing antibiotics.

Mallotus philippensis (Lam.) Mull. Arg. (Euphorbiaceae family), commonly known as 'kamala' (Fig. 1A), is a well-known medicinal plant from Asia and Australia producing a wide range of natural products including phenols, diterpenoids, steroids, flavonoids, cardenolides, triterpenoids, coumarins and isocoumarins [16]. The various compounds isolated from different parts of the plant, especially the 'red compound' and rottlerin, have shown antitumour, cytotoxic, antiviral, antileukaemic, antioxidant, anti-inflammatory and immunoregulatory activities [17], antibacterial activity against resistant *Helicobacter pylori* strains [18] and antitubercular activities. In this study, the antibacterial properties of the plant were further investigated, specifically to find agents that had potent activities against Gram-positive bacteria and had the capacity to act as anti-plasmid agents, preventing plasmid transfer between Gram-negative bacteria.

2. Materials and methods

2.1. Chromatographic and spectrometric methods

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AVANCE CP QNP 500 MHz instrument (Bruker UK Ltd., Coventry, UK). Chromatographic separations using thin layer chromatography (TLC), column chromatography and vacuum liquid chromatography (VLC) were carried out on silica gel GF_{254} (0.25 mm; Merck, Feltham, UK). The mass spectrum was recorded with a matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE Pro; Applied Biosystems, Warrington, UK) at the UCL School of Pharmacy (London, UK).

2.2. Plant material, extraction and isolation

Dried and powdered fruit was collected by two of the authors (VS and KS) from the premises of Poona College of Pharmacy (Pune, India) and a voucher specimen (No. Mat-001) was deposited at the herbarium in the UCL School of Pharmacy.

Red powder (500 g) was exhaustively extracted by cold agitation with solvents of increasing order of polarity (2 L of hexane, chloroform and methanol). The solutions were placed in an ultrasonic bath for 48 h. The resulting extracts were dried under vacuum on a rotary evaporator and were stored in a refrigerator for further analysis. Then, 3 g of the chloroform extract was fractionated using VLC with an increasingly polar gradient of 100% hexane to 100% ethyl acetate and finally 100% methanol, which yielded 21 fractions. These fractions were monitored by TLC using the solvent system containing hexane–ethyl acetate–formic acid (4:6:1). Spots on TLC were visualised by long (365 nm) and short (254 nm) wavelengths as well as being sprayed with 1% (w/v) vanillin–sulphuric acid and heated until a colouration was observed.

Using TLC profiling, similar fractions were pooled together into 10 fractions (F, G, H, I, I2, J, K, M, O and P) and were subjected to antibacterial activity determination against two bacteria (*S. aureus* SA1199B and *S. aureus* XU212) and were evaluated for anti-plasmid activity against *E. coli* harbouring the pKM101 or TP114 plasmids. Fraction K was active in the antibacterial assay with a minimum inhibitory concentration (MIC) of 8 mg/L against SA1199B and 2 mg/L against XU212, whilst fractions J and K were active in the anti-plasmid assay (data not shown). Fractions J and K were pooled to gain sufficient material (10.9 mg) and were subjected to column chromatography using a gradient from 100% toluene to 100% acetone. All fractions obtained were monitored by TLC, yielding a compound with an R_f value of 0.35 with a mixture of hexane–ethyl acetate–formic acid (4:6:1). The fractions that showed single spots were submitted for NMR analysis, which yielded pure compound **1**. The hexane-soluble extract showed antibacterial activity, which led to the bioassay-guided isolation of compound **2**. Both compounds **1** and **2** were then assessed for antibacterial and anti-plasmid activities.

2.3. Bacterial strains and plasmids

All bacterial strains and plasmids were cultured on nutrient agar slopes and were incubated for 24 h at 37 °C prior to MIC

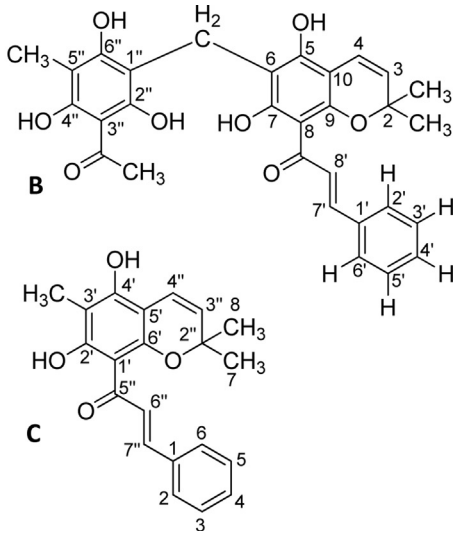

Fig. 1. (a) Photograph of *Mallotus philippensis* (Lam.) Mull. Arg. and (b, c) chemical structure of rottlerin (b) and the red compound (c).