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Full Length Research Paper

Antibacterial activity of elder (*Sambucus nigra L.)* flower or berry against hospital pathogens

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An evidence-based scientific scrutiny of Irish traditional medicines for their antimicrobial potency is urgently required for combating antibiotic resistant common nosocomial pathogens. We now report our seminal findings on the major constituents including terpenes identified in native, historically significant herbal medicinal plant Elder (*Sambucus nigra* L.*)* flower and elder berry in particular and their concomitant strong antimicrobial effects exhibited on various nosocomial pathogens notably upon methicillin-resistant *Staphylococcus aureus* MRSA, recognised globally as a clinically significant pathogen, associated with skin and soft tissue infections.

Key words: Traditional Irish medicinal plants, MRSA, antibacterial activity.

INTRODUCTION

The World Health Organisation (WHO) along with other various national authorities, now recognizes antimicrobial resistance in both medicine and agriculture as a major emerging problem of public health importance. Over time, many nosocomial pathogens have become antibiotic resistant. For instance, community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) and healthcare- associated (HA)-MRSA are now described globally, as a clinically significant pathogen, particularly associated with simple skin and soft tissue infections, including abscesses, cellulitis and furunculosis. In order to minimise the potential development of further anti-microbial resistance "the Copenhagen recommendations: report from the invitational EU Conference on the microbial threat" (http://www.im.dk/publikationer/micro98 /index.htm), outlined the need for the development of "novel principles for treating or preventing infections in humans and animals." One way of meeting this objective is to take cue from the re-discoveries of antimicrobial properties of native plants used in herbal medicine in India, China and Africa.

Recent reviews on ethnobotany and medicine (e.g. Ballard, 2008; Allen and Hatfield, 2004) highlight the fact that traditional herbal cures and remedies have indeed played an important historical role in the treatment of a variety of illnesses and diseases in great Britain and Ireland for the last 300 years. Notable examples include the use of Meadowsweet (*Filipendula ulmaria*) for stomach problems, heartburn ulcers, pain reliever for sore joints and muscles); known since Roman times, the elderflower (Family: Caprifoliacea*; Sambucus nigra L*) for antiviral and immune-boosting properties, treating colds, flu and feverish symptoms; Buttercup (*Ranunculus repens*), a popular folk medicine for treating cancer and internally and externally for haemorrhoids (as mixed

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formulations) with honey from clover (*Trifolium repens*) blossoms and other herbs. Unfortunately, although these plants have strong associations with the local historical evidence base, there is very limited and mainly no formal publications in the medical/scientific evidence base, examining their scientific background and clinical efficacy.

From our previous investigations (Woods-Panzaru et al., 2009), aqueous or ethanol extracts of fresh meadow-sweet, buttercup or elder whole plants showed limited antimicrobial activities. However, elder plant extracts were the most promising of the native plants tested for antibacterial activity. In this study, we evaluated freeze-dried elder flowers or their berries normally used in herbal formulations. After TLC and concentration steps, elder extracts were subjected to a solid-phase extraction (Waters SEP-PAK C-18 cartridge) and eluted with methanol/water (v/v). The extracts were analysed via hyphenated methods such as HPLC-UV and GC-MS and the fractions were tested for efficacy against nosocomial pathogens.

MATERIALS AND METHODS

Bacteria tested

The clinical isolates of bacteria tested are enlisted in Table 1, and maintained as pure cultures using methods described in the northern Ireland public health laboratory food and environmental standard analytical procedures (Anon, 2001). In order to prepare the inocula for challenge, all organisms were cultured on Columbia blood agar (Oxoid CM0331) supplemented with 5% (v/v) defibrinated horse blood and incubated for 24 h at 37°C.

Preparation of the plant material

Fresh (*S. nigra* L.) flower or berry (Figure 1A) were collected from the grounds of the Ulster Folk and Transport Museum, Cultra, Co. Down, northern Ireland (54°39'05.23"N; 5°47'50.73"W] and freeze-dried. 10 g of the elder flower or berry freeze-dried sample was homogenised in 200 ml of solvent mixture (50:50 water: 96% ethanol), and stirred for 72 h at 4°C, filtered, centrifuged at 9000 x g for 10 min and the pelleted (plant residue) left behind was repeat extracted as above, at least 3 more rounds. The supernatants obtained each time was carefully combined, then filtered using Buckner flask and Sintered glass filter bed apparatus and packed up to 0.5 cm depth with a mixture of Celite (Merck) and activated charcoal (10:1 w/w) to minimise plant pigments. The filtered cold aqueous ethanol extract were collected over anhydrous sodium sulphate for drying, concentrated using a rotary evaporator (Buchi) and the resulting aqueous residues freeze-dried (Figure 1B).

Separation and identification of antimicrobial natural products

The elder plant flower or berry extracts were purified on a preparative TLC (Sigma-Aldrich, UK; 20 x 20cm size glass plate; 0.5 - 2.0 mm silica gel ODS (octadecylsilanization), developed in a solvent front mixture comprising 4 parts n-butanol, 1 part acetic acid and 5 parts water. The resulting bands (Figure 1C) were carefully re-constituted in ethanol and the rotary vacuum dried residues were re-dissolved in water and carefully freeze-dried. Extracts containing a known quantity of freeze-dried powder mixed with an equal weight/volume of sterile 0.1% (w/v) peptone saline (CM0733, Oxoid Ltd., Basingstoke, UK) was filter-sterilized through a 0.22 µ syringe filter (Millipore Inc., USA), before microbiological challenge and the potent band identified. Portions of the TLC-separated, freeze-dried corresponding fraction from elder flowers or berry were loaded into a waters SEP-PAK C-18 cartridge and a solid-phase extraction was carried out using methanol: water (v/v). The resulting fractions were concentrated. Freeze-dried portions suspended in methanol screened for aromatic and other macromolecules by hyphenated techniques depicted in a schematic diagram (Figure 1D) using the methods of LC-UV-MS (interfaced with mass spectrometry), modified from Hostettmann et al. (1996); and GC-MS (e.g. Cooper et al., 1999) and HPLC-UV (Jäger et al., 2009).

Antibacterial efficacy tests

For bacterial assay purposes, the procedures developed before (Hearst et al., 2009) a recorded weight of freeze dried powder of elder flower or berry extracts was reconstituted with an equal weight/volume of sterile 0.1% (w/v) peptone saline (PS, CM0733, Oxoid Ltd., Basingstoke, UK) to give a known concentration for each extract solution, filter-sterilised through a 0.22 µ syringe filter (Millipore Inc., USA), before microbiological challenge (Rao et al., 2009) and as detailed in Table 1. Sterile PS and antibiotic susceptibility disks containing 5 µg ciprofloxacin (Mast Diagnostics Ltd., Bootle, Merseyside, UK) were employed as a negative and positive control, respectively. In order to prepare the inocula for challenge, all organisms were cultured on Columbia blood agar (Oxoid CM0331) supplemented with 5% (v/v) defibrinated horse blood and incubated for 24 h at 37°C. An overnight bacterial broth culture (200 µls) plated to form a 'lawn' of the test bacterial growth on the agar surface. Sterile discs (Mast diagnostics, Ltd., Bootle, Merseyside, UK) impregnated with plant extract (20 µls) transferred aseptically to the relevant sectors of the petri dishes containing the microbial lawns and the plates incubated overnight at 30°C for facilitating bacterial growth. Assays also included an aqueous extract of elder leaf, a common practice of herbal medicine was also included at 10 fold of its strength. The zones of clearance recorded in mm indicated the antibacterial effects and the zone of inhibition (Figure 1E).

RESULTS AND DISCUSSION

The antimicrobial activity of elder plant extracts at 10 fold (aqueous extract of leaf) or 100 fold dilution of ethanol extracted, freeze-dried concentrates of flower or berry extracts against the 13 common nosocomial pathogens is shown in Table 1. Elder leaf aqueous extract supplied at 10 fold dilution was moderately active against the *Bacillus cereus* and *Serratia marcescens* (6.0 mm), but failed to inhibit any of the key nosocomial pathogens including MRSA. Interestingly, the aqueous elder extracts demonstrated a notable inhibition of *E. coli* 0157 growth (7 mm). The elder flower (*S. nigra* L.) extracts were most toxic to all the bacteria tested compared to the other extracts. It also exhibited a larger zone of inhibitions when challenged against a wide range of bacteria and the highest inhibitory activity was towards MRSA (17 mm) and the lowest being environmental pathogens (e.g. *Pseudomonas aeroginosa* (9 mm). The 'potent' TLC band-derived extracts from either Elder flower or berry

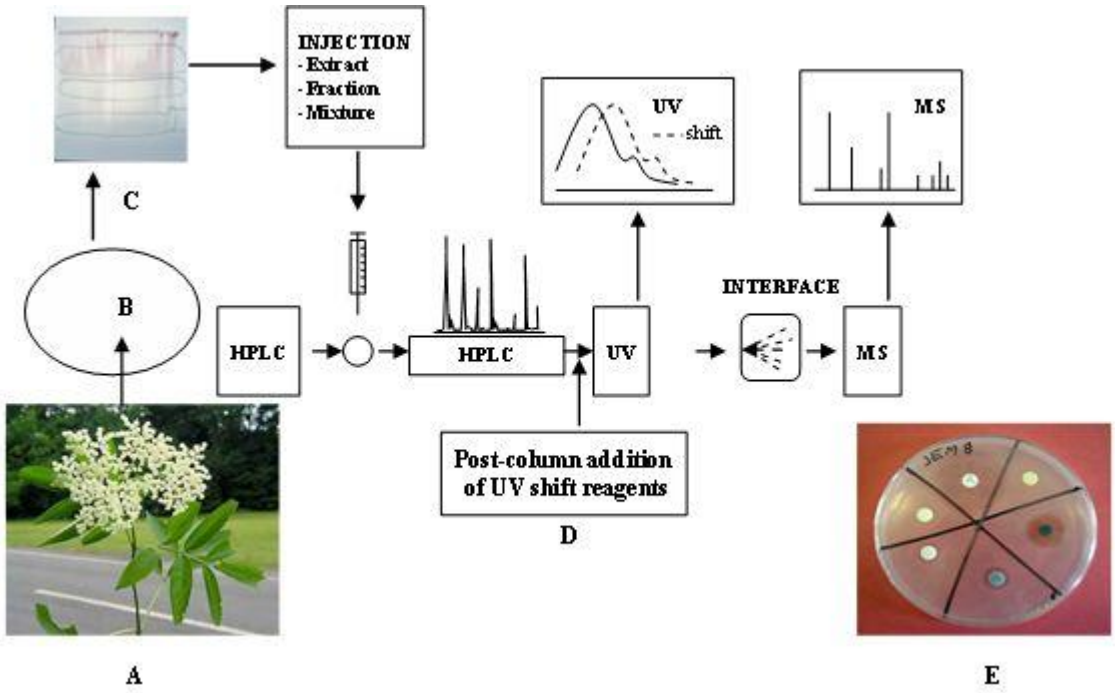

Figure 1. Schematic diagram of strategies adopted for separation, identification and examination of plant secondary metabolites from Elder (*Sambucus nigra*) flower and berry. Flowers or berry obtained from fresh Elder plants (**A**), were extracted, concentrated and freeze-dried (**B**). After initial separation on thin layer chromatography (**C**) as described by Cooper (2004), the Elder flower or berry extracts were analysed via well established hyphenated techniques of LC-UV-MS (**D**) for natural products incorporating shift reagents (post-column derivatives) to provide extra UV spectral information and/or authentication of the eluting metabolite (Modified from Hostettmann, et al, 1996). Fractions were tested for efficacy against nosocomial pathogens (**E**).

inhibited most of the bacteria, namely both gram positive (e.g. *Staphylococcus* sp, *B. cereus*) and Gram negative (e.g. *Salmonella poona, P. aeroginosa*) nosocomial pathogens. However, the inhibitory effects were expressed at subdued levels as evidenced by a drop in the zone of inhibitions down to ca. 5~14mm. Also, thepotent TLC band flower extracts altogether appear to have lost its potency against methicillin sensitive *S. aureus* (MSSA) indicating perhaps the 'interactive' metabolite(s) were either lost, and/or altered in their structural specificity or bacterial sensitivity/resistance conferring mechanisms may be critical for the corresponding mode of actions expressed by antibacterial metabolites in the TLC band. In the case of MRSA, the zone of inhibition exhibited by ciprofloxacin 5 µg disc is apparently larger (~22 mm) than the natural product extracts. However, it must be noted that the inhibitory elder flower extracts exhibited against MRSA is formidable (17 mm) despite being supplied at 100 fold dilutions of the extract. It was beyond the scope of this study to quantify individual metabolites in the elder flower or extracts.

Our preliminary HPLC-UV and GC-MS qualitative analyses of elderflower or berry extracts contain a wide range of bioflavonoids (unfragmented molecular ion peaks, M^+ corresponding to 222, 238, 240) indicating of the possible presence of flavones, flavonols and dihydroflavonols respectively as the most abundant aromatic constituents. Most flavonoids detected would normally show an M^+ with a loss of 1 mass unit (that is, with a loss of H). In structure activity relationships, most flavonoids are well documented to be normally associated with their anti-inflammatory, anti-oxidant and anti-cancer properties than for their antimicrobial effects. The flower derived fractions confirm the presence of flavonols (M^+ 238), the most abundant being quercetin and anthocyanins (M^+ main fragmentations, 225, 301, 316, 344) and a molecular ion peak at M^+342 (e.g. cyanidin 3-sambubioside) and a number of phenolic constituents such as gallic and cinnamic acid derivatives respectively occurred with structural and stereo-chemical variations.

In the case of elder berry, HPLC-UV shift characteristics in $AlCl_3$, $ZrCl_4$ salts, revealed the occurrence of Rutin, (M^+ 610) α-flavonols (M^+ 410) glycosylated flavonols (M^+ 480). Further derivatisations were necessary to detect polymeric flavans, catechin and anthocynanidins, isoquercitrin hyperosid (M^+ 464-glucoside), quercitrin (M^+ 464-galactoside), astralgin (M^+ 460) were the main components and in each case one would observe a mass ion peak with a loss of 18 mass units (that is, an ion peak corresponding to loss of H_2O).

It is interesting to note that among flavonols, the structure-anticancer activity relationships often rely on the 2', 5' hydroxyl substitutions which significantly reduce the anti-cancer potency (quercetin > morin > rutin); but does not appear to be so, in the case of antibacterial effects we tested. The tannins [(e.g. derivates of gallic acid (M^+ ~200), hydroxycinnamic acid (M^+ 174), caffeic acid (M^+ 180) and its ester with quinic acid] were the major components (that are normally ineffective anti-cancer agents regardless of their stereochemistry) and suggest being the likely antimicrobial constituents in this cluster.

Together with flavonoids, a number of 'new multi-functional antimicrobial components' of *S. nigra* such as triterpenoids were identified provisionally using GC-MS characteristics (mainly oleananes such as β-amyrin (M^+ 427), erythrodiol (M^+441), oleanoic acid M^+ 458), maslinic acid (M^+470); which elute at least 20 min after injection on HPLC-UV set at 210 nm and GC-MS ion traces corresponding to peaks > 40 min) and were present in elder flower while the berry extracts contained lupines such as lupeol (M^+ 424), betulin (M^+ 443), consistent with previous reports in bark or leaves of this plant species (e.g. Borchardt et al., 2008; Jäger et al., 2009). Other non-aromatics antimicrobials we observed in the elder flower or berry fractions using GC-MS traces comprise lectins, oligosaccharide fragments, peptides (data not shown) that are also potent inhibitors of transcription *in vitro*, microbial cell metabolism, targeting in particular the DNA-dependent synthesis of RNA (Karpova et al., 2007).

This study revealed that a number unidentified pentacyclic triterpenes present in elder flower and berries in addition to those previously reported in either leaves or bark of plants (Borchardt et al., 2008) and warrants urgent systematic identification and quantification. Terpenes is an attractive choice in that, their pharma-cological relevance in last 2 decades demonstrates their 'multi-target' properties such as wound (infection) healing, anti-inflammatory, anti-bacterial, antiviral, hepatoprotective and anti-tumoral effects while interestingly exert least mammalian cytotoxicity. Our approach has now revealed that the constituent natural products including a new array of terpenes with multi-functional new generation antibiotics can be tapped from simple flowers and berries of native species such as elder (*S. nigra* L.) show potency for combating hospital bacteria such as MRSA that have become resistant to several conventional antibiotics and may have other health benefits.

Insofar as macromolecules are concerned, honey has been a simplistic, yet strong contender in combating wound management and the antibacterial effects of Irish (flower) honey on CA-MRSA but has been investigated for their pros and cons in topical applications (e.g. Maeda et al., 2008). The constituents identified in elder flower or berry (*S. nigra* L.) was toxic to all nosocomial pathogens tested particularly, *S. aureus* (MRSA). Freeze-dried pow-der formulations in oily gels, emulsion or cream formats of products from medicinal plants have a distinct advantage over those of other sources as they inhibit the growth of persistent infectious bacteria. Previous studies on Italian medicinal plants (Quave et al., 2008) demonstrated that they may help reduce or even prevent 'biofilm formation' in antibiotic resistant organisms such as MRSA in soft skin tissue infections. The inhibitory activity against MRSA with herbal remedy phyto-chemicals particularly terpenes highlighted in this study is exciting and requires additional research for wound healing effects. A useful guidelines exhaustively summarising the available techniques for evaluation of medicinal plant products as antimicrobial agents has been recently reviewed (Das et al., 2010) and our recent approach to freeze-drying procedures (e.g. Hearst et al., 2009) have yielded metabolites not normally encountered in direct aqueous or solvent extractions. In terms of a suitable pharmaceutical delivery system, and treatment modalities for utilising the whole freeze-dried plant parts (such as *S. nigra* L. flower or berry extracts) as opposed to fresh plant extract recipes in herbal remedies may be useful for topical uses for skin infections.

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