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Phytochemical, antibacterial and antifungal properties of an aqueous extract of *Eucalyptus microcorys* leaves

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Australia is home to over 800 different species of *Eucalyptus* and traditionally, many *Eucalyptus* species have been utilised to heal wounds and treat fungal infections by the Indigenous people of Australia. In view of this, our study was designed to investigate the phytochemical, antibacterial and antifungal properties of crude aqueous extract of *E. microcorys* leaves. The freeze-dried powdered extract was prepared and the phytochemical profile was studied by analysing the total phenolic content (TPC), total flavonoid content (TFC), proanthocyanidins, antioxidants and saponins. The TPC, TFC and proanthocyanidin values found were: 501.76 ± 14.47 mg of gallic acid equivalents per g, 61.53 ± 0.83 mg of rutin equivalents per g and 10.76 ± 0.89 mg of catechin equivalents per g, respectively. The antioxidant values expressed in mg trolox equivalents per g of extract (mg TE/g) were: ABTS = 1073.13 ± 10.73 mg TE/g, DPPH = 1035.44 ± 65.54 mg TE/g and CUPRAC = 1524.30 ± 66.43 mg TE/g. The powdered extract was also evaluated for activity against three pathogenic bacterial strains (*Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus lugdunensis*); and three fungal strains (*Geotrichum candidum*, *Aspergillus brasiliensis* and *Candida albicans*) using the disc diffusion method and 96 well plate-based method with resazurin dye. The extract exhibited clear zones of inhibition against the tested bacteria and fungi. Minimum inhibitory concentration (MIC) values were demonstrated to be: *A. brasiliensis* = 2.44 μg/mL, *G. candidum* = 4.88 μg/mL, *S. lugdunensis* = 78 μg/mL, *E. coli* = 156.25 μg/mL, *E. aerogenes* = 312.5 μg/mL and *C. albicans* = 1250 μg/mL. These results reveal the significant potential of *E. microcorys* as a source of phenolics, antioxidants and antimicrobial agents and also highlight the necessity of further purification and characterisation of solitary bioactive compounds for their prospective applications in food, nutraceutical and pharmaceutical industries.

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1. Introduction

Over 800 different species of eucalypts are indigenous to Australia (Vuong et al., 2015a). They are extensively planted for wood products and considered as one of the most important plants in the world (Gilles et al., 2010; Topçu et al., 2011; Gharekhani et al., 2012; Gupta et al., 2013). Eucalypts are a storehouse of both volatile and non-volatile compounds with a wide spectrum of biological activities. However, the volatile compounds found in the essential oils from eucalypts have so far, mostly been exploited for their application in the pharmaceutical and food industries. The Indigenous people of Australia have used eucalypts as traditional bush medicine to heal wounds and treat fungal infections since time immemorial (Gilles et al., 2010). Even though, eucalypts are native to Australia, they have been introduced throughout the tropics and subtropics including the Africa, Americas, Europe, the Mediterranean basin, the middle east, China and the Indian subcontinent for production of timber and paper (Gharekhani et al., 2012; Gupta et al., 2013). A number of more recent scientific studies have reported on the compounds present in the essential oils from eucalypts and their role as antimicrobials (Cimanga et al., 2002; Vilela et al., 2009; Gilles et al., 2010; Mulyaningsih et al., 2011; Fratini et al., 2014). However, there is little information available emphasising the potential antimicrobial properties of crude extracts from eucalypts. In addition, *Eucalyptus microcorys* is one of the least exploited *Eucalyptus* species in terms of its phytochemical content and biological activity.

Development of multi-drug resistance pathogenic strains is one of the biggest concerns in today's world as it has adverse effects on the efficacy of medical and pharmaceutical treatments, as well as agricultural and food industries. Various factors such as overuse, inappropriate prescription, extensive agricultural use of antibiotics and availability of very few new antibiotics contribute to the development of drug resistance in microorganisms (Ventola, 2015). Hence, there is an urgent need to look for novel antimicrobials, especially from nature. Plant phenolics and antioxidants have been extensively reported in the scientific literature for their antimicrobial potential (Davidson and Branden, 1981;

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Tomás-Barberán et al., 1990; Nicholson and Hammerschmidt, 1992; Cowan, 1999; Zhu et al., 2004; Proestos et al., 2005; Estevinho et al., 2008; Süzgeç-Selçuk and Birteksöz, 2011; Stojkovic et al., 2013; Gyawali and Ibrahim, 2014; Guil-Guerrero et al., 2016). Therefore, the present study evaluated the phytochemical, antioxidant, antibacterial and antifungal properties of the aqueous crude extract from one of the least explored *Eucalyptus* species: *E. microcorys*.

2. Materials and methods

2.1. Plant materials

Fresh leaves of *E. microcorys* were collected from cultivated plants located at Ourimbah, Central Coast, NSW, Australia (latitude of 33.4° S, longitude of 151.4° E) on 2nd April, 2014. One of the authors (A.C.C.) authenticated the plant and a voucher specimen was deposited at the Don McNair Herbarium, The University of Newcastle (Accession number - 10499). The leaves were immediately transferred to the laboratory after collection and stored at −20 °C to avoid potential degradation of the phytochemicals. The leaves were then dried at 70 °C using a dry air oven for 5 h to constant weight (Vuong et al., 2015b). With the help of a commercial grade blender (Rio™ Commercial Bar Blender, Hamilton Beach), the leaves were then ground to a fine powder then sieved (≤1 mm) using a 1 mm EFL 2000 stainless steel mesh sieve (Endecotts Ltd., London, England) and packed in a sealed container and stored at −20 °C until required.

2.2. Extraction and preparation of crude extract powder

The solvent of choice in this study was water as it is the safest, cheapest and the most environmentally friendly solvent available and we have previously demonstrated its efficiency in terms of extracting phenolic compounds and antioxidants from *Eucalyptus* species using both conventional and modern techniques (Bhuyan et al., 2015, 2016, 2017). Conventional extraction using distilled water was carried out as follows. 5 g of powdered leaf sample was mixed with 100 mL of distilled water and heated at 85 °C for 15 min in a shaking water bath (Ratek instruments Pty Ltd., Boronia, VIC, Australia). The extract was immediately cooled on ice to room temperature (RT) and then filtered using Whatman® No.1 filter paper. Using a rotary evaporator (Buchi Rotavapor B-480, Buchi Australia, Noble Park, VIC, Australia) at 55 °C with reduced pressure, the filtrate was concentrated to one third of the initial volume. The concentrated extract was then immersed in liquid nitrogen and freeze-dried for 48 h in a freeze dryer (Thomas Australia Pvt., Ltd., Seven Hills, NSW, Australia) with drying chamber pressure of 2×10^{-1} mbar and cryo-temperature of −50 °C. The extract was resuspended in 5% dimethyl sulphoxide (DMSO) for performing the *in vitro* antimicrobial assays (Chandrasekaran and Venkatesalu, 2004).

2.3. Determination of total phenolic content (TPC)

TPC was measured according to the method described by Škerget et al. (2005). The extract was diluted up to 40 times to fit within the optimal absorbance range (0.1–1.0). The result was expressed as mg of gallic acid equivalents per g of dry weight (mg GAE/g) with gallic acid as a standard.

2.4. Determination of total flavonoid content (TFC)

Colorimetric assessment of TFC of the extract was performed as per the method described by Tan et al. (2014). Rutin was used as standard and TFC was expressed as mg of rutin equivalents per g of dry weight (mg RE/g).

2.5. Determination of proanthocyanidin content

The vanillin-HCl method as described by Broadhurst and Jones (1978) was employed to determine the proanthocyanidin content of the extract. Catechin was used as standard and the proanthocyanidin content was expressed as mg of catechin equivalents per g of dry weight (mg CAE/g).

2.6. Determination of antioxidant capacity

2.6.1. ABTS total antioxidant capacity (TAC)

The ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay as described by Bhuyan et al. (2015) was used to measure the TAC of the extract. To construct the calibration curve, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard and the result was expressed in mg trolox equivalents per g of dry weight (mg TE/g).

2.6.2. Free radical scavenging capacity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by Bhuyan et al. (2015) was employed to measure the free radical scavenging activity of the extract and the result was expressed in mg trolox equivalents per g of dry weight (mg TE/g).

2.6.3. Cupric reducing antioxidant capacity (CUPRAC)

The CUPRAC assay as described by Apak et al. (2004) was implemented to determine the cupric ion chelating capacity of the extract and the result was expressed in mg of trolox equivalents per g of dry weight (mg TE/g) with trolox as the calibration standard. Ascorbic acid was also included in all three antioxidant assays to make comparisons with the extract.

2.7. Determination of saponin content

To determine the saponin content of the extract, the method described by Hiai et al. (1976) was used. Aescin was used as standard with the result expressed as mg of aescin equivalents per g of dry weight (mg AE/g).

2.8. Antimicrobial assays

2.8.1. Microbial culture

Three bacterial strains: *Escherichia coli* (ATCC 10536), *Enterobacter aerogenes* (ATCC 13048) and *Staphylococcus lugdunensis* (ATCC 700328), two pathogenic fungal strains: *Geotrichum candidum* (ATCC 34614), *Aspergillus brasiliensis* (formerly known as *Aspergillus niger*) (ATCC 16404) and one yeast: *Candida albicans* (ATCC 10231) were used in this study. The microbial cultures were obtained from Thermo Scientific™ Oxoid™ in the form of Culti-Loops®. The stock bacterial and fungal cultures were maintained on nutrient agar medium and Sabouraud dextrose agar (SDA) medium, respectively at 4 °C.

2.8.2. Preparation of Inocula

Inoculums were prepared as per the method described by Chandrasekaran and Venkatesalu (2004) with some modification. Briefly, selected bacteria and yeast were grown for 24 h in nutrient broth (NB) at 37 °C and SDA at 28 °C, respectively and then mixed with sterile physiological saline and the turbidity was adjusted at a Mac Farland turbidity standard of 0.5 (10^6 colony forming units (CFU) per mL). The fungal isolates were subcultured on SDA and incubated at 28 °C for 7–14 days. The growth was aseptically macerated thoroughly in sterile distilled water and the absorbance of the fungal suspension was adjusted to 0.60 at 450 nm.

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