

Research Letter

Evaluation of the potential of *Macadamia integriflora* extracts as antibacterial food agents

Boyer H^{a,b} and Cock IE^{a,c*}

^aEnvironmental Futures Centre, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia

^bEcole Supérieure d'Ingénieurs en Développement Agroalimentaire Intégré, Université de la Réunion, Parc Technologique, 2 rue Joseph Wetzell, 27490 Sainte Clotilde, Ile de La Réunion

^cBiomolecular and Physical Sciences, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Queensland 4111, Australia

ABSTRACT: **Background:** *Macadamia integriflora* (family Proteaceae) is an endemic Australian plant traditionally used by Australian Aborigines as a food. Its nuts are known to keep well, raising the possibility that they may contain antimicrobial compounds and therefore may have value as a functional food to retard spoilage and prevent food poisoning, as well as potential medicinal antibiotic uses. **Methods:** The antimicrobial activity of *M. integriflora* was investigated by disc diffusion assays against a panel of bacteria and fungi. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** All *M. integriflora* extracts displayed antimicrobial activity in the disc diffusion assay. The flower methanol extract had the broadest specificity, inhibiting the growth of 7 of the 14 bacteria tested (50%) and all 3 (100%) of the fungi tested. All other extracts inhibited the growth of 6 (42.9%) of the bacterial species tested and up to 2 (66.6%) of the fungi tested. All extracts were more effective at inhibiting the growth of Gram-negative bacteria than Gram-positive bacteria. Indeed, only the flower methanol extract was capable of inhibiting the growth of any of the Gram-positive bacteria, inhibiting the growth of only 1 (*B. cereus*) of the 4 Gram-positive bacteria tested (25%). All *M. integriflora* extracts were non-toxic in the *Artemia franciscana* bioassay, with no significant increase in mortality induction above that of the negative control. **Conclusions:** The lack of toxicity of the *M. integriflora* extracts and their inhibitory bioactivity against a panel of bacteria and fungi demonstrate their potential as food additives to inhibit bacterial spoilage and food borne illnesses without the need for chemical preservative additives. Furthermore, *M. integriflora* extracts also have promise as antimicrobial agents for medicinal purposes.

KEYWORDS: *Macadamia integriflora*, Proteaceae, Australian plants, functional food, antibacterial, antifungal

INTRODUCTION

Antimicrobial food spoilage and microbial induced food poisoning are major concerns to the food production industry. Incidences of food-borne illnesses were estimated at 76 million cases annually in the USA alone in a 1999 study, with at least 5000 deaths annually directly attributed to food poisoning.^[1] The major method of controlling food borne microbes and thereby reducing spoilage and food toxin production is currently by the

addition of chemical preservatives during the food production process. Commonly used chemical food preservatives include butylhydroxyanisole (BHA), butylated hydroxytoluene (BHT), calcium propionate, nitrates, nitrites, sulphur dioxide (SO₂) and sulfites (SO₃).^[2] The effectiveness of these chemical preservatives is dependent on the type of microbial flora and the physical and chemical characteristics of the food.^[2,3] Of concern, the safety of many of the chemical preservatives used in food has yet to be determined and in some cases these preservatives have been linked with serious health problems. Studies have indicated that chemical preservatives may cause respiratory problems,^[4] aggravate attention deficit hyperactivity disorder (ADHD)^[5] and cause anaphylactic shock in susceptible individuals.^[4]

Due to greater consumer awareness and the negative perceptions of artificial preservatives, consumers are increasingly avoiding foods containing preservatives of chemical

*Correspondence

Dr. IE Cock,

Biomolecular and Physical Sciences, Nathan Campus,
Griffith University, 170 Kessels Rd, Nathan, Queensland 4111,
Australia

E-mail: I.Cock@griffith.edu.au

DOI: 10.5530/pc.2013.3.10

origin. Natural antimicrobial alternatives are increasingly being sought to increase the shelf life and safety of processed foods.^[6] Plant extracts and oils are candidates for antimicrobial agents that would be more acceptable to consumers due to their natural origin and consumer perception of safety. In addition, many plants have well established antimicrobial activity.

Macadamia (family Proteaceae) is a genus of 9 species of flowering plants. Seven *Macadamia* species are native to eastern Australia, 1 species is native to Indonesia (*Macadamia hildebrandii*) and 1 species is native to New Caledonia (*Macadamia neurophylla*). *Macadamia integriflora* (Figure 1) (commonly known as macadamia nut, Queensland nut, bauple nut) is an Australian species, native to the state of Queensland in north-eastern Australia, although it has been introduced and is grown commercially in many tropical and subtropical regions internationally. Hawaii is a major producer of macadamia nuts (second in production only to Australia), with Brazil, Bolivia, Columbia, Costa Rica, Guatemala, Israel, Kenya, Malawi, New Zealand and South Africa all producing significant quantities commercially. *M. integriflora* nuts are a valuable food crop. Indeed, the macadamia nut is the only Australian native food plant which is currently produced and exported on a large scale.

M. integriflora nuts are high in fats, especially beneficial monosaturated fats and omega-7 palmitoleic acid,^[7] which have been shown to decrease plasma cholesterol levels.^[8] The high palmitoleic acid levels and the oxidative stability of *M. integriflora* nut oil make it a desirable component of cosmetics and skin care products.^[7,9] The nuts also contain vitamins A1, E, B1, B2 and niacin in significant quantities.^[7]



Figure 1. *Macadamia integriflora* leaves and flowers (photographed accessed from Wikipedia Commons 21 June 2012 and is reproduced here with the relevant permissions).

Despite the reported beneficial phytochemistry of the nuts, no reports were found in the published literature of Australian Aborigines using *M. integriflora* in traditional medicinal practices. However, this does not preclude their usage as traditional medicinal agents as much of the Aboriginal ethnobotanical knowledge was passed by word of mouth. As Australian Aborigines have assimilated into mainstream Australian society and have an increased reliance on allopathic medicines, much of this knowledge has been lost irretrievably.

Limited scientific studies have examined the therapeutic potential of *M. integriflora*. A recent study has reported antibacterial activity of methanolic extracts of *M. integriflora* leaves and flowers against a panel of bacteria.^[10] Other studies have reported that MiAMP1 protein which has been detected in macadamia nuts, is also present in some other plant species with antimicrobial activities.^[11,12] The current study was undertaken to test *M. integriflora* extracts for the ability to inhibit microbial growth/contamination against a variety of bacteria involved in food spoilage and/or food poisoning and against some important fungi. Through examining the antibacterial capability of *M. integriflora* extracts, we aim to assess their potential as additives to foods to retard spoilage and to potentially reduce food poisoning in processed foods.

METHODS

Plant collection and extraction

M. integriflora leaves and flowers were collected from a single verified tree on Logan campus of Griffith University, Australia on 18 September 2007 as previously described.^[10] Voucher specimens (leaf, 1L1E07; flower, 1F1E07) have been deposited in the School of Biomolecular and Physical Sciences, Griffith University, Australia. The leaves and flowers were cut up and dried in a Sunbeam food dehydrator and subsequently coarsely ground and stored at -30°C until use.

1 g of plant material was weighed into each of five tubes and five different extracts were prepared by adding 50 ml of methanol, water, ethyl acetate, chloroform, or hexane respectively. All solvents were obtained from Ajax and were AR grade. The ground *M. integriflora* material was extracted in each solvent for 24 hours at 4°C with gentle shaking. The extracts were filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant dry extracts were weighed and redissolved in 10 ml deionised water.

Qualitative phytochemical studies

Phytochemical analysis of the *M. integriflora* extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.^[13–15]

Antimicrobial screening

Test microorganisms

All bacterial and fungal strains were obtained from Michelle Mendell and Tarita Morais, Griffith University, Australia. Stock cultures of *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Bacillus cereus*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were subcultured and maintained in nutrient broth at 4 °C. *Aspergillus niger*, *Candida albicans*, and *Saccharomyces cerevisiae* were subcultured and maintained in Sabouraud media at 4 °C.

Evaluation of antimicrobial activity

Antimicrobial activity of each plant extract was determined using a modified Kirby-Bauer disc diffusion method.^[16–18] Briefly, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 10⁸ cells/ml for bacteria, or 10⁵ cells/ml for fungi. 100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained.

The extracts were tested using 5 mm sterilised filter paper discs. Discs were impregnated with 10 µl of test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4 °C for 2 hours before incubation with the test microbial agents. Plates inoculated with *Alcaligenes faecalis*, *Aeromonas hydrophila*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Yersinia enterocolitica*, *Candida albicans* and *Saccharomyces cerevisiae* were incubated at 30 °C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella Salford* and *Staphylococcus aureus* were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones were measured. *Aspergillus niger* inoculated plates were incubated at 25 °C for 48 hours then the zones of inhibition were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values are reported in this report. Standard discs of ampicillin (2 µg) and chloramphenicol (10 µg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial

activity. Filter discs impregnated with 10 µl of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the *M. integriflora* extracts were determined by the disc diffusion method across a range of doses. The plant extracts were diluted in deionised water across a concentration range of 5 mg/ml to 0.1 mg/ml. Discs were impregnated with 10 µl of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

Toxicity screening

Reference toxins for biological screening

Potassium dichromate (K₂Cr₂O₇) (AR grade, Chem-Supply, Australia) was prepared as a 2 mg/ml solution in distilled water and was serially diluted in synthetic seawater for use in the *A. franciscana* nauplii bioassay. Mevinphos (2-methoxycarbonyl-1-methylvinyl dimethyl phosphate) was obtained from Sigma-Aldrich as a mixture of cis (76.6%) and trans (23.0%) isomers and prepared as a 4 mg/ml stock in distilled water. The stock was diluted in artificial seawater for use in the bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.^[19–21] *Artemia franciscana* Kellogg cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/l distilled water were prepared prior to use. 2 g of *A. franciscana* cysts were incubated in 1 L synthetic seawater under artificial light at 25 °C, 2000 Lux with continuous aeration. Hatching commenced within 16–18 h of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10 h of hatching. Nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii rich water closest to the light was removed for biological assays. Seawater (400 µl) containing approximately 46 (mean 46.3, n = 174, SD 17.8) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were diluted to 5 mg/ml in seawater for toxicity testing, resulting in a 2.5 mg/ml concentration in the bioassay. 400 µl of diluted plant extracts and the reference toxins were transferred to the wells and incubated at 25 ± 1 °C under artificial light (1000 Lux). A negative control (400 µl seawater) was run in at least triplicate for

each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 72 h all nauplii were sacrificed and counted to determine the total number per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Statistical analysis

Data are expressed as the mean \pm SEM of at least three independent experiments.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried plant material with the various solvents yielded dried plant extracts ranging from 37.8 mg to 310.3 mg (Table 1). Chloroform gave the highest yield of extracted material for both leaf and flower extracts (310.3 ± 22.8 mg and 279.4 ± 24.6 mg respectively). Methanol (leaf, 75.0 ± 12.2 mg; flower, 97.8 ± 18.7 mg), water (leaf, 102.7 ± 14.7 mg; flower 121.6 ± 18.4 mg) and hexane (leaf, 131.0 ± 16.3 mg; flower, 144.8 ± 15.2 mg) also extracted relatively high levels of material. In contrast, ethyl acetate extracted the lowest mass (leaf, 37.8 ± 10.4 mg; flower, 41.0 ± 11.2 mg). The dried extracts were

resuspended in 10 ml of deionised water, resulting in the extract concentrations shown in Table 1.

Phytochemical studies (Table 1) show that methanol and water extracted the widest range and largest amount of phytochemicals in this study. The methanol and water extracts of both leaves and flowers for both plants showed moderate to high levels of phenolics, flavonoids and tannins. Similar classes of phytochemicals were detected in the ethyl acetate, chloroform and hexane extracts, although generally at lower levels. The chloroform and hexane extracts showed only low levels of any class of extracted phytochemicals tested. Alkaloids were not detected in any extract of either the leaves or flowers.

Antibacterial activity

Aliquots (10 μ l) of each extract was tested in the disc diffusion assay against a panel of 14 bacteria and 3 fungi (Table 2). All extracts displayed broad spectrum antibacterial activity, being capable of inhibiting the growth of between 6 (35%) and 10 (59%) of the 17 microbial species tested (Table 2). The methanolic extracts displayed the broadest antibiotic specificity, inhibiting the growth of 6 (42.9%) and 7 of the 14 bacteria tested (50.0%) for the leaf and flower extracts respectively. The leaf and flower methanolic extracts also inhibited the growth of 2 (66.7%) and 3 (100%) of the fungi tested respectively. The methanolic extracts were particularly potent against *A. hydrophilia*, *P. mirabilis* and *P. fluorescens* as determined

Table 1: The mass of dried extracted material, the concentration of extracts after resuspension in deionised water and qualitative phytochemical screenings of solvent extractions

	Extract	Mass of Dried Extract (mg)	Resuspended Extract Concentration (mg/ml)	Total Phenolics	Water Soluble	Water Insoluble	Cardiac Glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Meyer test)	Alkaloids (Wagners test)	Flavanoids	Tannins	Free Anthraquinones	Combined Anthraquinones
Leaf	Methanol	75.0 \pm 12.2	7.5 \pm 1.2	+++	++	+	-	-	-	-	-	-	++	++	-	-
	Water	102.7 \pm 14.7	10.3 \pm 1.5	+++	++	+	-	-	-	-	-	-	++	++	-	-
	Ethyl Acetate	37.8 \pm 10.4	3.8 \pm 1.0	++	+	+	-	-	-	-	-	-	-	-	-	-
	Chloroform	310.3 \pm 22.8	31.0 \pm 2.3	+	-	+	-	-	-	-	-	-	-	-	-	-
	Hexane	131.0 \pm 16.3	13.1 \pm 1.6	+	-	+	-	-	-	-	-	-	-	-	-	-
Flower	Methanol	97.8 \pm 18.7	9.8 \pm 1.9	+++	++	+	-	-	-	-	-	-	++	++	-	-
	Water	121.6 \pm 18.4	12.2 \pm 1.8	+++	++	+	-	-	-	-	-	-	++	++	-	-
	Ethyl Acetate	41.0 \pm 11.2	4.1 \pm 1.1	++	+	+	-	-	-	-	-	-	-	-	-	-
	Chloroform	279.4 \pm 24.6	27.9 \pm 2.5	+	-	+	-	-	-	-	-	-	-	-	-	-
	Hexane	144.8 \pm 15.2	14.5 \pm 1.5	+	-	+	-	-	-	-	-	-	-	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

Table 2: Antibacterial activity of *M. integriflora* solvent extracts measured as zones of inhibition (mm)

	<i>M. Integriflora</i> leaf					<i>M. Integriflora</i> flowers					Positive Controls	
	Methanol extract	Water extract	Ethyl acetate extract	Chloroform extract	Hexane extract	Methanol extract	Water extract	Ethyl acetate extract	Chloroform extract	Hexane extract	Ampicillin	Chloramphenicol
Gram negative rods												
<i>A. faecalis</i>	–	–	–	–	–	–	–	–	–	–	9.8 ± 0.8	6.3 ± 0.6
<i>A. hydrophilia</i>	8.3 ± 0.4	6.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3	7.6 ± 0.6	6.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3	9.5 ± 1.4	28.7 ± 1.6
<i>C. freundii</i>	6.5 ± 0.7	6.3 ± 0.3	6.5 ± 0.7	6.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3	6.3 ± 0.3	9.8 ± 0.8	13.2 ± 1.2
<i>E. coli</i>	7.5 ± 0.5	7.8 ± 0.2	8.2 ± 0.2	8.2 ± 0.2	8.0 ± 0.4	7.8 ± 0.6	8.0 ± 0.4	8.2 ± 0.2	8.2 ± 0.2	8.2 ± 0.2	13.7 ± 1.6	20.0 ± 1.8
<i>K. pneumoniae</i>	–	–	–	–	–	–	–	–	–	–	9.3 ± 0.6	21.3 ± 1.5
<i>P. mirabilis</i>	8.2 ± 0.2	6.5 ± 0.7	6.3 ± 0.3	7.7 ± 0.6	6.3 ± 0.3	7.5 ± 0.5	7.8 ± 0.2	8.2 ± 0.2	8.2 ± 0.2	8.0 ± 0.4	14.6 ± 0.7	8.7 ± 0.6
<i>P. fluorescens</i>	8.3 ± 0.4	6.3 ± 0.3	6.8 ± 0.4	7.5 ± 0.5	6.3 ± 0.3	–	–	–	–	–	8.2 ± 0.5	12.2 ± 1.2
<i>S. newport</i>	–	–	–	–	–	–	–	–	–	–	9.5 ± 1.4	10.3 ± 1.8
<i>S. marcenscens</i>	7.2 ± 0.6	7.3 ± 1.0	6.6 ± 0.6	7.7 ± 0.6	6.7 ± 0.2	7.8 ± 0.6	7.3 ± 1.0	6.3 ± 0.3	8.0 ± 0	6.7 ± 0.2	–	11.7 ± 1.8
<i>S. sonnei</i>	–	–	–	–	–	7.8 ± 0.2	–	–	–	–	14.0 ± 0	14.3 ± 0.6
Gram positive rods												
<i>B. cereus</i>	–	–	–	–	–	7.2 ± 0.3	–	–	–	–	16.7 ± 2.0	11.3 ± 1.2
Gram positive cocci												
<i>S. aureus</i>	–	–	–	–	–	–	–	–	–	–	11.7 ± 2.1	18.6 ± 0.6
<i>S. epidermidis</i>	–	–	–	–	–	–	–	–	–	–	26.3 ± 1.5	12.3 ± 0.6
<i>S. pyogenes</i>	–	–	–	–	–	–	–	–	–	–	7.0 ± 1.0	10.3 ± 0.7
Fungi												
<i>A. niger</i>	–	–	–	–	–	6.3 ± 0.3	–	6.6 ± 0.6	–	–	–	–
<i>C. albicans</i>	6.5 ± 0.7	–	6.5 ± 0.7	–	6.3 ± 0.3	7.7 ± 0.6	–	7.9 ± 0.6	–	6.3 ± 0.3	8.3 ± 0.4	12.2 ± 1.2
<i>S. cerevisiae</i>	6.8 ± 0.4	–	–	–	–	7.2 ± 0.3	–	–	–	–	8.3 ± 0.4	13.0 ± 1.2

Numbers indicate the mean diameters of inhibition ± SEM (mm) of at least triplicate experiments. – indicates no growth inhibition. Ampicillin (2 µg) and Chloramphenicol (10 µg) were used as the positive controls. Deionised water was included as a negative control.

from the zone of inhibition. *M. integriflora* ethyl acetate and hexane extracts also displayed good antifungal specificity. The *M. integriflora* flower ethyl acetate extract inhibited the growth of 2 of the 3 fungi tested (66.7%). Similarly, the *M. integriflora* leaf ethyl acetate and the leaf and flower hexane extracts inhibited the growth of 1 of the 3 fungi tested (33.3%). Neither the water nor chloroform (fruit and leaf) extract displayed any antifungal activity.

The flower methanol extract was the only extracts capable of inhibiting the growth of any Gram-positive bacteria, inhibiting 1 (*B. cereus*) of the 4 Gram-positive bacteria tested (25%). All *M. integriflora* extracts inhibited the growth of 6 of the 10 Gram-negative bacteria tested (60%). The most susceptible bacteria to the *M. integriflora* extracts were *A. hydrophilia*, *P. mirabilis* and *P. fluorescens* (as seen from the zone of inhibition).

The relative level of antibacterial activity was further evaluated by determining the MIC values for each extract against the bacterial and fungal species which were shown to be susceptible by disc diffusion assays. MIC's were evaluated in the current studies by disc diffusion across a range of concentrations. This has previously been determined to be a valid method of MIC determination as MIC values determined by disc diffusion correlate well with those determined by broth dilution assays.^[22]

All extracts tested proved to be effective bacterial growth inhibitors at low concentrations, with MIC values against all microbial species which they inhibited at <25 µg/ml (<0.25 µg impregnated in the disc). The methanol, water, ethyl acetate and chloroform extracts all displayed MIC values in the range of 2.4–7.9 µg/ml. The lowest MIC of the tested extracts was seen

Table 3: Minimum inhibitory concentrations ($\mu\text{g/ml}$) of *M. integriflora* extracts against susceptible bacteria

		Gram-Negative Bacteria					Gram-Positive Bacteria		Fungi		
		<i>A. hydrophila</i>	<i>C. freundii</i>	<i>E. coli</i>	<i>P. mirabilis</i>	<i>P. fluorescens</i>	<i>S. marcescens</i>	<i>B. cereus</i>	<i>A. niger</i>	<i>C. albicans</i>	<i>S. cerevisiae</i>
Fruit	Methanol	4.6	7.2	5.3	2.9	4.7	5.6	–	–	7.1	6.7
	Water	6.8	7.9	7.3	5.7	6.5	7.7	–	–	–	–
	Ethyl Acetate	4.8	7.4	5.7	5.3	5	7.6	–	–	7.3	–
	Chloroform	5.8	6.8	6	5.8	5.5	7.1	–	–	–	–
	Hexane	18.8	16.7	19.9	16.2	10.3	12.1	–	–	9.7	–
Leaf	Methanol	6.3	6.8	6.5	2.4	4.3	5.3	5.8	7.7	6.8	6.5
	Water	7.1	7.4	7.4	5	5.7	7.7	–	–	–	–
	Ethyl Acetate	4.8	7	5.5	4.8	5.2	7.5	–	7.2	6.5	–
	Chloroform	5.6	6.6	6	5.1	4.9	7.2	–	–	–	–
	Hexane	22.1	18.3	21.3	15.9	12.3	10.8	–	–	8.9	–

Numbers indicate the mean MIC values of at least triplicate determinations. – indicates no growth inhibition at any concentration tested.

for the flower methanol extract against *P. mirabilis* (2.4 $\mu\text{g/ml}$). In contrast, the hexane extracts generally had lower efficacy, with MIC values in the range 10.3–22.1 $\mu\text{g/ml}$ for all bacteria tested. Interestingly, the MIC values against the 1 fungal species inhibited by the hexane extracts (*C. albicans*) was slightly better (<10 $\mu\text{g/ml}$).

Quantification of toxicity

M. integriflora extracts were diluted to a concentration of 2000 $\mu\text{g/ml}$ in artificial seawater for toxicity testing, resulting in 1000 $\mu\text{g/ml}$ concentrations in the *Artemia* nauplii lethality bioassay. For comparison, the reference toxins potassium dichromate (1000 $\mu\text{g/ml}$) and Mevinphos (2000 $\mu\text{g/ml}$) were also tested in the bioassay. Figure 2 shows the % mortality induced by each of the leaf extracts and by the controls at various times. The potassium dichromate (Figure 2G) and Mevinphos (Figure 2H) reference toxins were rapid in their onset of mortality. Both reference toxins induced mortality within the first 3 hours of exposure and 100% mortality was evident following 4–5 hours. In contrast, all of the *M. integriflora* leaf extracts (Figures 2A–E) displayed mortality rates similar to those of the artificial seawater negative control (Figure 2F) at 24, 48 and 72 h. It was not possible to accurately determine an LC_{50} for any extract as the mortality never exceeded 50% for any extract at any time tested.

Similarly, the *M. integriflora* flower extracts were also found to be nontoxic in the *Artemia* nauplii bioassay. Figure 3

shows the % mortality induced by each of the fruit extracts and by the controls at various times. All of the *M. integriflora* flower extracts (Figures 3A–E) displayed mortality rates similar to those of the artificial seawater negative control (Figure 2F) at 24, 48 and 72 h. It was not possible to accurately determine an LC_{50} for any extract as the mortality did not exceed 50% for any extract at any time tested.

DISCUSSION

There is increasing consumer demand to find alternatives for chemical based artificial preservatives as consumers become more aware of the potential for chemical induced health problems. Edible plants could potentially provide a source of inhibitory substances for food borne pathogens and bacteria associated with food spoilage. The current study reports on the antibacterial activities of various *M. integriflora* extracts and on their toxicity. The ability of *M. integriflora* extracts to inhibit the growth of a wide variety of bacteria is in agreement with previous reports of the antibacterial activity of other Australian native plants. The antiseptic properties of the Eucalypts,^[23,24] Leptospermums,^[10,25] Callistemons,^[26] Melaleucas^[27,28] and Acacias^[29] have been extensively studied and shown to inhibit the growth of a wide variety of both Gram-positive and Gram-negative bacteria.

The current study shows Gram-negative bacteria to be much more susceptible to *M. integriflora* extracts

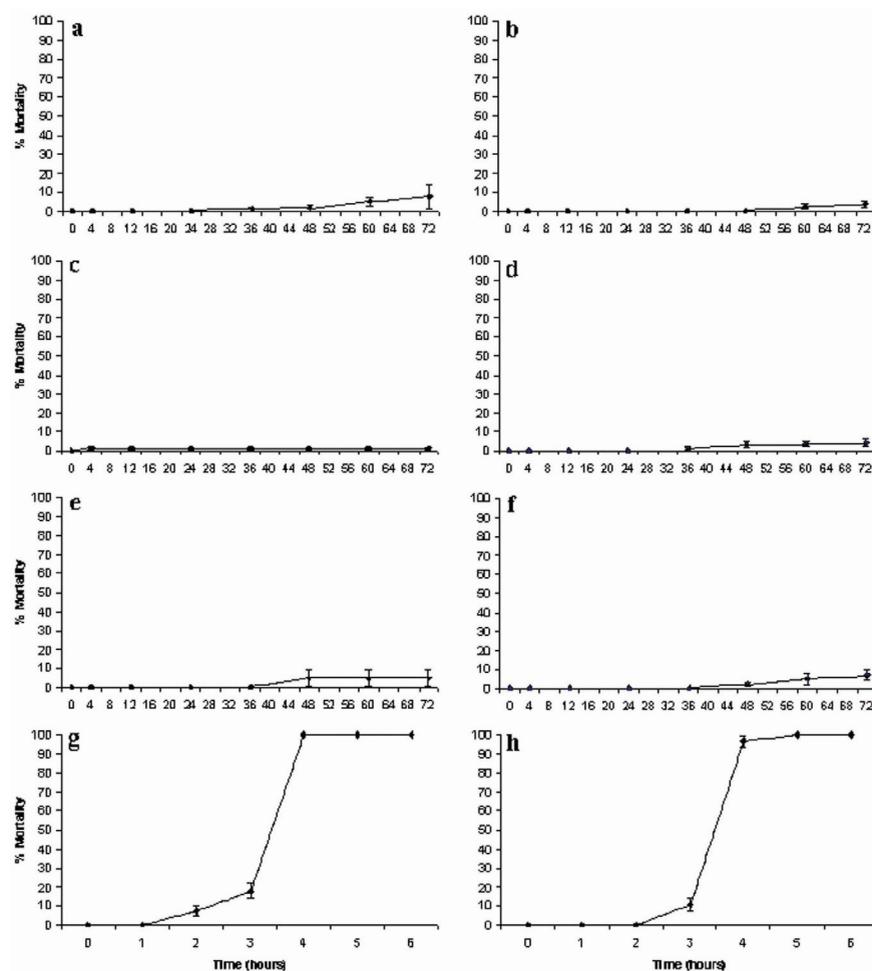


Figure 2. Brine shrimp lethality of (a) *M. integriflora* leaf methanol extract (1000 µg/ml), (b) *M. integriflora* leaf water extract (1000 µg/ml), (c) *M. integriflora* leaf ethyl acetate extract (1000 µg/ml), (d) *M. integriflora* leaf chloroform extract (1000 µg/ml), (e) *M. integriflora* leaf hexane extract (1000 µg/ml), (f) artificial seawater negative control, (g) potassium dichromate (1000 µg/ml), (h) Mevinphos (2000 µg/ml). All bioassays were performed in at least triplicate and are expressed as mean \pm SEM.

than Gram-positive bacteria. Indeed, only the flower methanolic extract was capable of inhibiting any Gram-positive bacteria, albeit inhibiting the growth of only 1 (*B. cereus*) of the 4 Gram-positive bacteria tested (25%). The greater susceptibility of Gram-negative bacteria observed in this study is in contrast to previous studies which have reported a greater susceptibility of Gram-positive bacteria towards solvent extracts for South American,^[30] African^[31,32] and Australian^[33] plant extracts. Results within this laboratory^[10,34-36] have also confirmed the greater susceptibility of Gram-positive bacteria towards many other Australian plant extracts, although examples of Australian plants having a greater effect on Gram-negative bacteria have also been reported.^[13,14]

The bacteria examined in this study were chosen because they are all important bacteria in food spoilage and/or food poisoning/intoxication. Staphylococcus spp. (especially *S. aureus*) is one of the most common sources of food

borne diseases worldwide.^[2] *S. pyogenes* contamination (especially of dairy products and salads) can cause pharyngitis as well as gastroenteritis and diarrhoea.^[37] *B. cereus*,^[38] *E. coli*,^[39] *C. freundii*,^[40] *K. pneumoniae*^[40] and *S. sonnei*^[41] all produce toxins and other proteins that induce gastroenteritis and diarrheal diseases. Many of these toxins are heat stable and are not destroyed by heat treatments/pasteurisation. Therefore, control of these bacteria in food is particularly important. Similarly, *P. mirabilis* releases factors that stimulate histamine production resulting in gastrointestinal, neurological (palpitations, headaches, itching), cutaneous (hives, rash) and hypertension symptoms.^[42] Whilst storage of food at refrigerated temperatures inhibits the growth of many of these pathogenic bacteria, the inclusion of antibacterial food components would further enhance food safety.

Of the pathogenic/toxic bacteria tested in this study, the Staphylococcus spp. and *S. pyogenes* were unaffected by any of the *M. integriflora* extracts. This is likely related

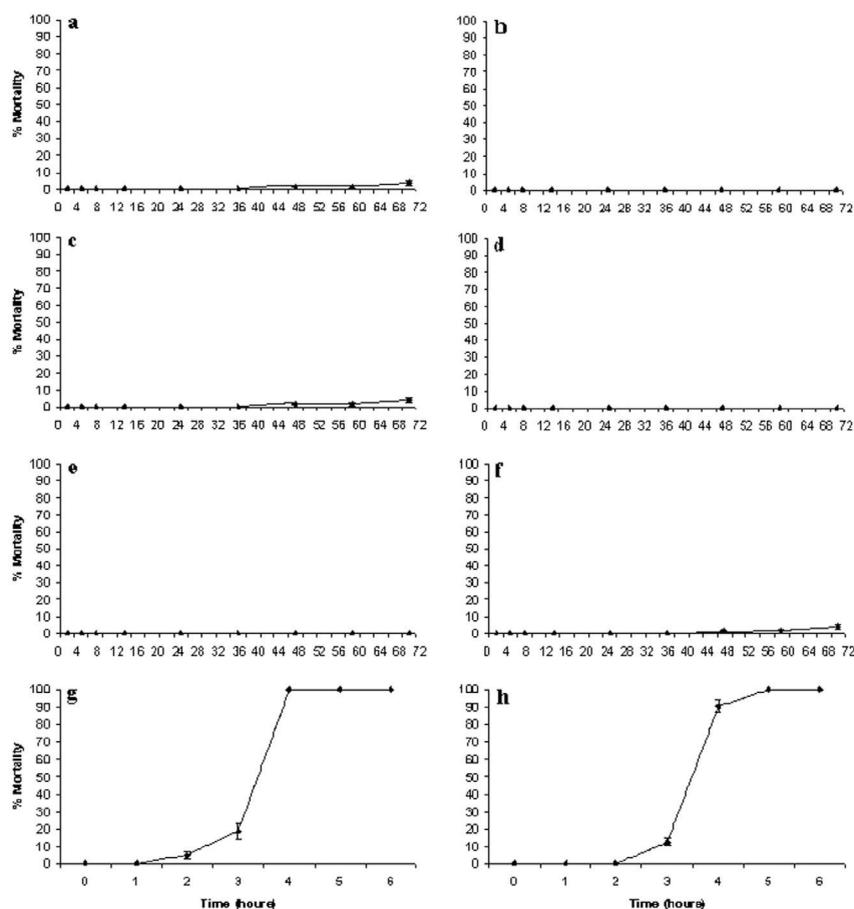


Figure 3. Brine shrimp lethality of (a) *M. integriflora* flower methanol extract (1000 $\mu\text{g/ml}$), (b) *M. integriflora* flower water extract (1000 $\mu\text{g/ml}$), (c) *M. integriflora* flower ethyl acetate extract (1000 $\mu\text{g/ml}$), (d) *M. integriflora* flower chloroform extract (1000 $\mu\text{g/ml}$), (e) *M. integriflora* flower hexane extract (1000 $\mu\text{g/ml}$), (f) artificial seawater negative control, (g) potassium dichromate (1000 $\mu\text{g/ml}$), (h) Mevinphos (2000 $\mu\text{g/ml}$). All bioassays were performed in at least triplicate and are expressed as mean \pm SEM.

to the fact that these are Gram-positive coccus bacteria. As previously noted, Gram-positive bacteria were less susceptible to the *M. integriflora* extracts tested in this study. The only susceptible Gram-positive bacteria examined (*B. cereus*) was only susceptible to the flower methanolic extract. As *B. cereus* is a Gram-positive rod, it would be of interest to test the susceptibility of other Gram-positive rod bacteria in future studies. Of the Gram-negative bacteria tested, only *A. faecalis*, *K. pneumoniae* and *S. newport* were resistant to all of the *M. integriflora* extracts. All other bacteria were inhibited by at least 1 of the extracts tested. Indeed, *A. hydrophilia*, *C. freundii*, *E. coli*, *P. mirabilis*, *P. fluorescens* and *S. marcescens* were inhibited by all extracts, demonstrating the potential of *M. integriflora* inclusion in processed foods for controlling food borne diseases.

Also particularly interesting was the ability of the *M. integriflora* extracts to inhibit the growth of psychotropic bacteria. Many foods are stored below 5 °C in refrigerators to retard bacterial growth. These foods are expected to have long shelf lives, in some cases up to 50 days or

more. Between processing and consumption, foods may become temperature abused to 10 °C or higher allowing psychotropic bacteria (eg. *A. faecalis*, *A. hydrophilia*, *B. cereus* and *P. fluorescens*) to cause spoilage. Some pathogenic bacteria are also psychotropic (eg. *B. cereus* and some strains of *C. freundii*, *E. coli* and *K. pneumoniae*).^[38–40] Therefore, food based antibacterial agents with inhibitory activity against psychotropic bacteria are especially useful. With the exception of *A. faecalis* which was not inhibited by any extract in this study, all of the psychotropic bacteria tested were inhibited by at least one *M. integriflora* extract. Indeed, of the psychotropic bacteria associated with spoilage, *A. hydrophilia* and *P. fluorescens* growth was inhibited by all extracts. Of the psychotropic bacteria associated with food poisoning, *C. freundii* and *E. coli* were also susceptible to all of the *M. integriflora* extracts.

Individual *M. integriflora* extract components responsible for the antibacterial potential of the solvent extracts were not identified in the current study. Previous reports have identified various bioactive components of other

Australian medicinal plants (Eucalypts,^[43] *Leptospermum*,^[44] *Melaleucas*^[45]). These plants all contain terpenes including 1, 8-cineole, terpinen-4-ol, α -pinene and β -pinene. Both 1, 8-cineole and terpinen-4-ol have antimicrobial activity.^[46,47] Recent studies have also reported on the antibacterial activities of the *Callistemons*^[26,48] and *Syzygiums*.^[49–51] It has been postulated that terpene components may also be responsible for the antiseptic properties of these plants.^[52] The phytochemistry of the *M. integriflora* extracts investigated in the current study was not examined. Further studies are required to identify which phytochemical(s) is/are responsible for the recorded bioactivities of these extracts.

The findings reported here also demonstrate that none of the *M. integriflora* extracts displayed significant toxicity towards *Artemia franciscana*. Indeed, none of the extracts tested induced mortality above that of the negative control at the doses tested (1000 $\mu\text{g/ml}$). Previously, compounds with an LC_{50} of greater than 1000 $\mu\text{g/ml}$ towards *Artemia nauplii* have been defined as being non-toxic.^[53] It was therefore determined that all *M. integriflora* extracts were non-toxic towards *Artemia nauplii*.

In conclusion, the results of this study demonstrate the antibacterial potential of *M. integriflora* in food preparation and indicate that *M. integriflora* extracts are worthy of further study. Further evaluation of the antibacterial properties of these extracts against a more extensive panel of microbial agents is warranted. Likewise, purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents. Whilst the extracts examined in this report are promising as antibacterial agents, caution is needed before these compounds can be applied to functional food and medicinal purposes. In particular, further toxicity studies using human cell lines are needed to verify the suitability of these extracts for these purposes.

ACKNOWLEDGEMENTS

The authors are grateful to Shimony Mohanty for assistance with technical aspects. Financial support for this work was provided by School of Biomolecular and Physical Sciences, Griffith University.

REFERENCES

- Mead PS, Slutsker L, Dietz V, McCaig LF, Breese JS, Shapiro C, Griffin PM, Tauxe RV. 1999. Food related illness and death in the United States. *Emerging Infectious Diseases* 1999; 5:607–25.
- Ray B, Bhunia A. *Fundamental Food Microbiology*, 4th edition 2008; CRC Press, Boca Raton, USA.
- Cherry JP. Improving the safety of fresh produce with antimicrobials. *Food Technology* 1999; 53:11, 54–9.
- Simon RA. Adverse reactions to food additives. *Current Allergy and Asthma Research*. 2004; 3(1):62–6.
- McCann D, Barrett A, Cooper A, Crumpler D, Dalin L, Grimshaw K, Kitchen E, Lok K, Porteous L, Prince E, Sonuga-Barke E, Warner JO, Stevenson J. Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial, *Lancet*. 2007; 370:1560–7.
- Vesoul J, Cock IE. The potential of Bunya nut extracts as antimicrobial functional food agents. *Pharmacognosy Communications*. 2012; 2(1):72–9.
- Akhtar N, Ahmad Madni A, Bakhsh. Evaluation of the basic properties of Macadamia nut oil. *Gomal University Journal of Research*. 2006; 22(1):21–7.
- Garg ML, Blake RJ, Wills RBH. Macadamia nut consumption lowers plasma total and LDL cholesterol levels in hypercholesterolemic men. *The Journal of Nutrition*. 2003; 133:1060–3.
- Ken K. Kukui and Macadamia nut oil. *Cosmetics and Toiletries*. 1991; 160(11):87–90.
- Cock IE. Antibacterial activity of selected Australian native plant extracts. *The Internet Journal of Microbiology*. 2008; 4, 2.
- McManus AM, Neilsen KJ, Marcus JP, Harrison SJ, Green JL, Manners JM, Craik DJ. MiAMP₁, a novel protein from *M. Integriflora* adopts a Greek key beta-barrel fold unique amongst plant antimicrobial proteins. *Journal of Molecular Biology*. 1999; 293:629–38.
- Marcus JP, Goulter KC, Green JL, Harrison SJ, Manners JM. Purification, characterisation and cDNA cloning of an antimicrobial peptide from *M. Integriflora*. *European Journal of Biochemistry*. 1997; 244(3):743–9.
- Cock IE, Kukkonen L. 2011, An examination of the medicinal potential of *Scaevola spinescens*: Toxicity, antibacterial, and antiviral activities. *Pharmacognosy Research*. 2011; 3(2):85–94.
- Vesoul J, Cock IE, 2011, An examination of the medicinal potential of *Pittosporum phylliraeoides*: Toxicity, antibacterial, and antifungal activities. *Pharmacognosy Communications*. 1(2):8–17.
- Kalt FR, Cock IE. GC-MS analysis of bioactive *Petalostigma* extracts: Toxicity, antibacterial and antiviral activities.
- Mohanty S, Cock IE. Bioactivity of *Syzygium jambos* methanolic extracts: Antibacterial activity and toxicity. *Pharmacognosy Research*. 2009; 2(1):4–9.
- Cock IE. Antibacterial activity of *Allocasuarina littoralis* methanolic leaf extracts. *The Internet Journal of Microbiology*. 2008; 5, 2.
- Cock IE. Antibacterial activity of *Aloe barbadensis* Miller leaf gel components. *The Internet Journal of Microbiology*. 2008; 4, 2.
- Mpala L, Chikowe G, Cock IE. No evidence of antiseptic properties and low toxicity of selected *Aloe* species, *Journal of Pharmaceutical Negative Results*. 2010; 1(1):10–16.
- Cock IE, Ruebhart, DR. Assessment of the toxicity of selected Australian native plant extracts using the *Artemia franciscana* nauplii bioassay. *The Internet Journal of Toxicology*. 2008; 5, 2.
- Cock IE, Ruebhart DR. Comparison of the brine shrimp nauplii bioassay and the ToxScreen-II test for the detection of toxicity associated with *Aloe vera* (*Aloe barbadensis* Miller) leaf extract. *Pharmacognosy Research*. 2009; 1(2):102–8.
- Gaudreau C, Girouard Y, Ringuette L, Tsimiklis C. Comparison of disc diffusion and agar dilution methods for erythromycin and ciprofloxacin susceptibility testing of *Campylobacter jejuni* subsp. *Jejuni*. *Antimicrobial Agents and Chemotherapy*. 2007; 1524–6.
- Cock IE. Antimicrobial activity of *Eucalyptus major* and *Eucalyptus baileyana* methanolic extracts. *The Internet Journal of Microbiology*. 2008; 6, 1.
- Sartorelli P, Marquiere AD, Amaral-Baroli A, Lima MEL, Moreno PRH. Chemical composition and antimicrobial activity of the essential oils from two species of *Eucalyptus*. *Phytotherapy Research*. 2007; 21:231–3.
- Weston RJ, Brocklebank LK, Lu Y. Identification of quantitative levels of antibacterial components of some New Zealand honeys. *Food Chem*. 2000; 70:427–35.
- Cock IE. Antimicrobial activity of *Callistemon citrinus* and *Callistemon rigidus* methanolic extracts. *Pharmacognosy Communications* 2012; 2(3):50–7.
- Carson CF, Hammer KA, Riley TV. *Melaleuca alternifolia* (Tea Tree) Oil: a Review of Antimicrobial and Other Medicinal Properties. *Clin Microbiol Rev*. 2006; 19(1):50–62.
- Papadopoulos CJ, Carson CF, Hammer KA, Riley TV. 2006. Susceptibility of pseudomonads to *Melaleuca alternifolia* (tea tree) oil and components. *J Antimicrob Chemoth*. 2006; 58:449–51.

29. Cock IE. Antimicrobial activity of *Acacia aulacocarpa* and *Acacia complanta* methanolic extracts. *Pharmacognosy Communications*. 2012; 2(1):66–71.
30. Paz EA, Cerdeiras MP, Fernandez J, Ferreira F, Moyna P, Soubes M, Vazquez A, Vero S, Zunino L. Screening of Uruguayan medicinal plants for antimicrobial activity. *J Ethnopharmacol*. 1995; 45:67–70.
31. Kudi AC, Umoh JU, Eduvie LO, Gefu J. Screening of some Nigerian medicinal plants for antibacterial activity. *J Ethnopharmacol*. 1999; 67:225–8.
32. Vlietinck AJ, van Hoof L, Totte J, Lasure A, Vanden Berghe D, Rwangabo PC, Mvukiyumwani J. Screening of a hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *J Ethnopharmacol*. 1995; 46:31–47.
33. Palombo EA, Semple SJ. 2001. Antibacterial activity of traditional Australian medicinal plants. *J Ethnopharmacol*. 2001; 77:151–7.
34. Kalt FR, Cock IE, The medicinal potential of Australian native plants from Toohey Forest, Australia, *The South Pacific Journal of Natural and Applied Sciences*. 2010; 28:41–7.
35. Cock IE, Mohanty S. Evaluation of the antibacterial activity and toxicity of *Terminalia ferdinandia* fruit extracts. *Pharmacognosy Journal*. 2011; 3 (20):72–9.
36. Cock IE. Antibacterial and Antifungal Activity of *Buckinghamia celsissima* leaf extracts. *The Internet Journal of Microbiology*. 2009; 6, 2.
37. Stiles ME. Less recognised or presumptive food borne pathogenic bacteria. In *Food borne Bacterial Pathogens 1989*; Doyel, MP (ed), Marcel Dekker, NY.
38. Granum PE. *Bacillus cereus*, In *Food borne Pathogens: Microbiology and Molecular Biology*, Fratamico PM, Bhunia AK, Smith JL, Caister Academic Press 2005; Norfolk, UK.
39. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*, *Clin Microbiol Rev*. 1998; 11(1):142–201.
40. Twedt RM, Boutin BK. Potential public health significance of non-*Escherichia coli* coliforms in food. *J Food Prot*. 1979; 42: 161–3.
41. Sansonetti PI. Shigella plays dangerous games. *ASM News*. 1999; 65:611–17.
42. Bean NH, Griffin PM, Goulding JS, Ivey CB. Food borne disease outbreaks, 5 year summary, 1983–1987. *J. Food Prot*. 1990; 53:711–28.
43. Foley W, Lassak E. The potential of bioactive constituents of Eucalyptus foliage as non-wood products from plantations. Publication no. 04/154, 2004; Rural Industries and Development Corporation, Australia.
44. Porter NG, Wilkins A. Chemical, physical and antimicrobial properties of essential oils of *Leptospermum scoparium* and *Kunzea ericoides*. *Phytochemistry*. 1999; 50(3):407–15.
45. Russell MF, Southwell IA. Monoterpenoid accumulation in 1,8-cineole, terpinolene and terpinene-4-ol chemotypes of *Melaleuca alternifolia* seedlings. *Phytochemistry*. 2003; 62:683–9.
46. Simić N, Palić R, Randjelović V. Composition and antibacterial activity of *Achillea clypeolata* essential oil. *Flavour Frag*. 2005; 20:127–30.
47. Mondello F, De Bernardis F, Girolamo A, Cassone A, Salvatore G. In vivo activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* Cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic *Candida* species. *BMC Infect Dis*. 2006; 6:158.
48. Sanjai S, Charu G. Antimicrobial potential of *Callestemon rigidus*. *Pharm Biol*. 2006; 44(3):194–201.
49. Park MJ, Gwak KS, Yang I, Choi WS, Jo HJ, Chang JW, Jeung EB, Choi IG. Antifungal activities of the essential oils of *Syzygium aromaticum* (L.) Mer. Et Perry and *Leptospermum petersonii* Bailey and their constituents against various dermatophytes. *J Microbiol*. 2007; 45(5):460–5.
50. Shafi PM, Rosamma MK, Jamil K, Reddy PS. Antibacterial activity of *Syzygium cumini* and *Syzygium tra_ancoricum* leaf essential oils. *Fitoterapia*. 2002; 73:414–16.
51. Cock IE. Antimicrobial activity of *Syzygium australe* and *Syzygium leuhmannii* methanolic extracts. 2012 *Pharmacognosy Communications*. 2012; 2(2):71–7.
52. Jirovetz L, Fleischhacker W, Buchbauer G, Ngassoum MB. Analysis of the essential oils of *Callistemon rigidus* (Myrtaceae) from Cameroun by GC/FID and GC/MS. *Scientia Pharmaceutica*. 1997; 65:315–19.
53. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med*. 1982; 45:31–4.