

# Evaluation of the antibacterial activity and toxicity of *Myrciaria cauliflora* methanolic leaf and fruit extracts

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## Abstract

Methanol extracts from *M. cauliflora* leaves and fruit were tested for antimicrobial activity and toxicity in vitro. *M. cauliflora* leaf extract inhibited the growth of 9 of the 14 bacteria tested (64%) whilst the fruit extract inhibited the growth of 11 of the 14 bacteria tested (79%). Both Gram-positive and Gram-negative bacterial growth were inhibited by *M. cauliflora* leaf and flower extracts. 7 of the 10 Gram-negative bacteria (70%) and 2 of the 4 Gram-positive bacteria (50%) tested had their growth inhibited by *M. cauliflora* leaf extract whereas the fruit extract inhibited 7 of the 10 Gram-negative bacteria (70%) and 100% of the Gram-positive bacteria tested. *M. cauliflora* leaf extract proved to be toxic in the *Artemia franciscana* bioassay with 24, 48 and 72h LC<sub>50</sub>'s of 232.9 ± 36.3 µg/ml, 169.1 ± 11.5 µg/ml and 128.1 ± 5.6 µg/ml respectively, making it more toxic than Mevinphos (24, 48 and 72h LC<sub>50</sub>'s 1346.0 ± 78.2 µg/ml, 505.3 ± 37.7 µg/ml, 103.9 ± 12.8) at all time points except 72h but less toxic than potassium dichromate (24, 48 and 72h LC<sub>50</sub>'s 86.3 ± 5.1 µg/ml, 80.4 ± 4.3 µg/ml, 77.9 ± 4.9). *M. cauliflora* fruit extract was non-toxic in the *Artemia franciscana* bioassay indicating its potential as an antibacterial agent for medicinal use.

## INTRODUCTION

Bacterial resistance to currently used antibiotics is becoming a concern to public health (Monroe and Polk, 2000). The development of bacterial super resistant strains is resulting in currently used antibiotic agents failing to end many bacterial infections. For this reason the search is ongoing for new antimicrobial agents, either by the design and synthesis of new agents, or through the search of natural sources for as yet undiscovered antimicrobial agents (Bhavnani and Ballou, 2000). The antiseptic qualities of medicinal plants have been long recognised. Recently there has been a revival of interest in herbal medications (Chariandy et al., 1999) due to a perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals.

*Myrciaria cauliflora* (Mart.) O.Berg. (commonly known as jaboticaba) is a small evergreen tree of the family Myrtaceae. It is native to Brazil and the West Indies. *M. cauliflora* is grown extensively in Minas Gerais region near Rio de Janeiro in Southern Brazil for the small (3-4cm diameter) tough skinned purple grape-like fruit it grows along its branches. The fruit has a thick purple skin covering a sweet, white or rosy pink gelatinous flesh containing 1 – 4 seeds. The fruit have a pleasant flavour and are commonly eaten fresh or used to produce jams, strong wines, and

liqueurs. *M. cauliflora* also has a history of use as a traditional medicine for the treatment of numerous ailments. The skin is used to produce an astringent decoction which has been used in the treatment of a wide variety of ailments including diarrhoea, asthma and haemoptysis (Morton, 1987). Gargling the decoction has also been used to relieve chronic inflammation of the tonsils (Morton, 1987).

Despite its range of traditional medicinal uses, the phytochemistry and therapeutic potential of *M. cauliflora* has not been extensively studied. *M. cauliflora* has been reported to contain tannins (Morton, 1987), cyanidin 3-glucoside (Einbond et al., 2004) and peonidin 3-glucoside and its aglycone (Trevisan et al., 1972). In his recent doctoral thesis, Reynertson (2007) identified a novel depside (jaboticabin) and 16 other known compounds from crude methanolic extracts from *M. cauliflora* fruit. This report highlighted the two depsides jaboticabin and 2-O-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid as being of particular therapeutic potential due to their ability to inhibit cytokine production, their cytotoxicity towards HT29 and HCT116 colon cancer cell lines and their antioxidant activity.

Surprisingly, the antiseptic properties of *M. cauliflora* remain largely unstudied. The antibacterial and antifungal

properties of other members of the Myrtaceae family are well known. Eucalypts (Cock, 2008b; Sartorelli, 2007; Delaquis et al., 2002; Oyedele et al., 1999), Callistemons (Sanjai and Charu, 2006), Leptospermums (Cock 2008a; Davis and Ward, 2003; Weston et al., 2000; Setzer et al., 2000), Melaleucas (Carson et al., 2006; Papadopoulos et al., 2006) and Syzygiums (Cock 2008a; Arora and Kaur, 2007; Park et al., 2007; Duraipandiyar et al., 2006; Djip et al., 2000) are all known to have antimicrobial activities. However, we are unaware of any scientific studies into the antibacterial potential of *M. cauliflora*. Therefore, the current study reports on the antibacterial properties of *M. cauliflora* fruit and leaf extracts as well as examining their toxicity to determine their potential as antibiotic agents.

## **MATERIALS AND METHODS**

### **PLANT MATERIAL**

#### **COLLECTION OF PLANT SAMPLES**

*Myrciaria cauliflora* leaves and fruit were a gift from Mervyn Cooper of the Queensland Tropical Fruit Association. Samples were obtained from a single tree, washed in deionised water and processed within 4 hours of collection.

#### **PREPARATION OF CRUDE EXTRACTS**

*Myrciaria cauliflora* leaves and fruit were dried separately in a Sunbeam food dehydrator. The dried leaf material was ground to a coarse powder and the fruit was finely diced. 1 g of each of the dried plant materials was extracted extensively in 50 ml methanol (Ajax, AR grade) for 24 hours at 4 °C with gentle shaking. The extract was filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellet was dissolved in 10 ml 20 % methanol. The extract was passed through 0.22 µm filter (Sarstedt) and stored at 4 °C.

### **ANTIBACTERIAL SCREENING**

#### **TEST MICROORGANISMS**

All microbial strains were obtained from Michelle Mendell and Tarita Morais, Griffith University, Australia. Stock cultures of *Aeromonas hydrophila*, *Alcaligenes feacalis*, *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.

### **EVALUATION OF ANTIMICROBIAL ACTIVITY**

Antimicrobial activity of all plant extracts was determined using a modified Kirby-Bauer (Bauer et al, 1966) disc diffusion method. Briefly, 100 µl of the test bacteria were grown in 10 ml of fresh media until they reached a count of approximately 10<sup>8</sup> cells/ml. 100 µl of microbial suspension was spread onto nutrient agar plates.

The extracts were tested using 5 mm sterilised filter paper discs. Discs were impregnated with 10 µl of the 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 feacalis*, *Aeromonas hydrophila*, *Bacillus cereus*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Serratia marcescens*, were incubated at 30 °C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Escherichia coli*, *Salmonella newport*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones 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 study. 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. cauliflora* extract was 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 TOXICITY**

## SCREENING

Potassium dichromate ( $K_2Cr_2O_7$ ) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/ml solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia 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 serially diluted in artificial seawater for use in the bioassay.

## NAUPLII TOXICITY SCREENING

Toxicity was tested using the *Artemia franciscana* nauplii lethality assay developed by Meyer et al (1982) for the screening of active plant constituents with the following modifications. *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. 400 µl of seawater containing approximately 42 (mean 41.6, n = 150, SD 17.8) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were diluted to 2 mg/ml in seawater for toxicity testing, resulting in a 1 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 (Finney, 1971).

## RESULTS

### ANTIBACTERIAL ACTIVITY

1 g of powdered dried *M. cauliflora* leaves and 1 g of finely diced *M. cauliflora* fruit was extensively extracted with methanol and dried under vacuum, resulting in 90 mg and 633 mg of dried extracted material respectively. Resuspension of the dried fractions in 10 ml of 20 % methanol resulted in the crude test extract concentration of 9.0 mg/ml (leaf extract) and 63.3 mg/ml (fruit extract). The extracts were diluted to 5 mg/ml for testing for antimicrobial activity. 10 µl of extract (50 µg) was tested in the disc diffusion assay against 14 bacteria (table 1). *M. cauliflora* leaf extract inhibited the growth of 9 of the 14 bacteria tested (64.3 %). In comparison, *M. cauliflora* fruit extract inhibited the growth of 11 of the 14 bacteria tested (78.6 %).

**Figure 1**

Table 1: Antibacterial activity of leaf and fruit extracts measured as zones of inhibition (mm).

	<i>Myrciaria cauliflora</i>		Antibacterial controls	
	leaf	fruit	Amp	Chl
<b>Gram negative rods</b>				
<i>A. faecalis</i>	16.2 ± 0.4	13.0 ± 0.7	15.2 ± 1.2	6.3 ± 0.6
<i>A. hydrophilia</i>	17.6 ± 0.9	10.1 ± 1.4	12.0 ± 1.0	28.7 ± 1.6
<i>C. freundt</i>	12.2 ± 1.3	10.4 ± 0.5	8.3 ± 0.6	15.7 ± 1.2
<i>E. coli</i>	9.5 ± 0.5	14.6 ± 0.3	14.7 ± 0.6	17.3 ± 0.6
<i>K. pneumoniae</i>	13.1 ± 0.9	10.2 ± 0.8	10.3 ± 0.6	21.3 ± 1.5
<i>P. mirabilis</i>	16.4 ± 0.8	13.8 ± 1.3	17.3 ± 0.6	8.7 ± 0.6
<i>P. fluorescens</i>	12.4 ± 0.9	8.6 ± 0.5	18.2 ± 0.5	21.2 ± 1.2
<i>S. newport</i>	-	-	18.7 ± 0.6	20.3 ± 0.6
<i>S. marcescens</i>	-	-	-	14.7 ± 0.6
<i>S. sonnei</i>	-	-	14.0 ± 0	14.3 ± 0.6
<b>Gram positive rods</b>				
<i>B. cereus</i>	9.0 ± 0	10.6 ± 0.3	26.7 ± 0.6	13.3 ± 1.2
<b>Gram positive cocci</b>				
<i>S. aureus</i>	9.3 ± 0.6	9.6 ± 0.3	11.7 ± 2.1	16.0 ± 1.0
<i>S. epidermidis</i>	-	8.0 ± 0	26.3 ± 1.5	12.3 ± 0.6
<i>S. pyogenes</i>	-	13.3 ± 1.2	17.0 ± 1.0	24.0 ± 1.0

Numbers indicate the mean diameters (mm) of inhibition of triplicate experiments ± standard deviation. – indicates no growth inhibition. Chloramphenicol (10 µg) and ampicillin (2 µg) were used as the positive controls.

Both Gram-positive and Gram-negative bacteria were affected by the *M. cauliflora* leaf extract although the Gram-negative bacteria appeared more susceptible. Of the 10 Gram-negative bacteria tested, 7 (70%) were inhibited by *M. cauliflora* leaf extract. The leaf extract also inhibited the growth of 2 (*B. cereus* and *S. aureus*) of the 4 Gram-positive

bacteria tested (50 %). In contrast, Gram-positive bacteria were more susceptible to *M. cauliflora* fruit extract. 7 of the 10 Gram-negative bacteria tested (70%) were inhibited by *M. cauliflora* fruit extract compared to 100 % of the Gram-positive bacteria tested.

The relative level of antibacterial activity was evaluated by determining the MIC values for each extract against the bacteria 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 (Gaudreau et al., 2007). Both extracts were particularly effective at inhibiting the growth of *C. freundii* and *B. cereus* as seen by minimum inhibitory concentration (table 2). The leaf extract was also particularly effective against *P. fluorescens*.

Figure 2

Table 2: Minimum inhibitory concentrations (µg/ml) of extracts against susceptible bacteria.

	MIC (µg/ml)									
	<i>A. faecalis</i>	<i>A. hydrophila</i>	<i>C. freundii</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>P. fluorescens</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>S. epidermidis</i>
<i>M. cauliflora</i> (L)	1000	384.6	137.4	800	797.1	840.3	125	105.3	358.5	-
<i>M. cauliflora</i> (F)	1062	1015	167.8	923.3	650.8	814.8	714.3	166.8	266.6	636.4

Numbers indicate the mean MIC values of at least triplicate determinations. - indicates no growth inhibition.

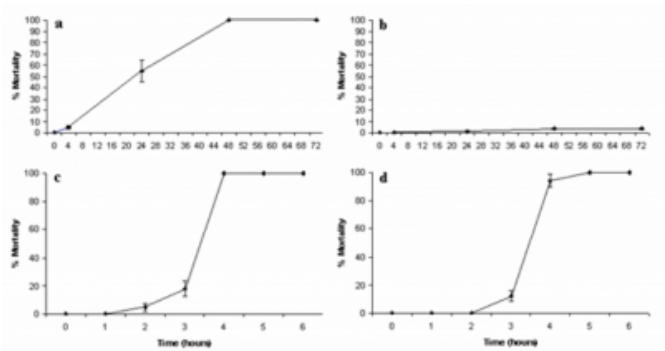
QANTIFICATION OF TOXICITY

The *M. cauliflora* leaf (figure 1a) and fruit (figure 1b) extracts were diluted to 2000 µg/ml in artificial seawater for toxicity testing, resulting in 1000 µg/ml concentrations in the *Artemia franciscana* lethality bioassay. For comparison, the reference toxins potassium dichromate (800 µg/ml) (figure 1c) and Mevinphos (2000 µg/ml) (figure 1d) were also tested in the *Artemia franciscana* lethality bioassay. The potassium dichromate and Mevinphos reference toxins were much more rapid in their onset of mortality than either of the *M. cauliflora* extracts at the concentrations tested. For both reference toxins, the induction of mortality was seen within the first 3 hours of exposure. 100% mortality was evident following 4 hours of exposure. In contrast, mortality due to *M. cauliflora* leaf extract exposure was evident within 24

hours and 48 hours was required to kill 100% of the brine shrimp. *M. cauliflora* fruit extract did not induce mortality above the levels seen for seawater controls at any time.

Figure 3

Figure 1: Brine shrimp lethality of (a) leaf extract (1000 µg/ml), (b) fruit extract (1000 µg/ml), (c) potassium dichromate (800 µg/ml), (d) Mevinphos (2000 µg/ml). All bioassays were performed in at least triplicate and are expressed as mean ± standard deviation.



To determine the effect of toxin concentration on the induction of mortality, the LC<sub>50</sub> values of the extracts was determined by testing across the concentration range 1000 µg/ml to 15µg/ml in the *Artemia* nauplii bioassay. For comparison, potassium dichromate and Mevinphos were tested across the same concentration range. Table 3 shows the LC<sub>50</sub> values of *M. cauliflora* extracts and the control toxins towards *A. franciscana*. No LC<sub>50</sub> values are reported for *M. cauliflora* fruit extract as no increase in mortality above the seawater controls was seen for this extract. The *M. cauliflora* leaf extract was substantially more toxic than Mevinphos at 24 and 48 hours with LC<sub>50</sub> values of 232.9 ± 36.3 µg/ml and 169.1 ± 11.5 µg/ml respectively compared to 1346.0 ± 78.2 µg/ml and 505.3 ± 37.7 µg/ml for Mevinphos. Potassium dichromate was substantially more toxic at both 24 hours (24 h LC<sub>50</sub> 86.3 ± 5.1) and at 48 hours (48 h LC<sub>50</sub> 80.4 ± 4.3 µg/ml). The leaf extract and both reference toxins showed similar toxicity at 72 hours as seen by their LC<sub>50</sub> values.



**Figure 4**

Table 3: LC (95% confidence interval) for brine shrimp nauplii exposed to leaf and fruit extracts, the reference toxins potassium dichromate and Mevinphos and a seawater control.

	LC50 (µg/ml)		
	24 h	48 h	72 h
<i>Myrciaria cauliflora</i> leaf	232.9 ± 36.3	169.1 ± 11.5	128.1 ± 5.6
<i>Myrciaria cauliflora</i> fruit	-	-	-
Potassium dichromate	86.3 ± 5.1	80.4 ± 4.3	77.9 ± 4.9
Mevinphos	1346.0 ± 78.2	505.3 ± 37.7	103.9 ± 12.8
Seawater control	-	-	-

- denotes values that were not obtained as ≥ 50 % mortality was not obtained at this time point.

## DISCUSSION

The current study reports on the antimicrobial activity and toxicity of *M. cauliflora* leaf and fruit extracts. The ability of *M. cauliflora* leaf and fruit extracts to inhibit the growth of both Gram-positive and Gram-negative bacteria is in agreement with previous reports of the antibacterial activity of other members of the Myrtaceae family. The antiseptic properties of the Eucalypts (Cock, 2008b; Sartorelli, 2007; Delaquis et al., 2002; Oyediji et al., 1999), *Leptospermums* (Cock 2008a; Davis and Ward, 2003; Weston et al., 2000; Setzer et al., 2000) and *Melaleucas* (Carson et al., 2006; Papadopoulos et al., 2006) 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-positive bacteria to be more susceptible to *M. cauliflora* fruit extract. The greater susceptibility of Gram-positive bacteria towards the fruit extract seen in this study is in agreement with previously reported results for South American (Paz et al., 1995), African (Kudi et al., 1999; Vlietinck et al., 1995) and Australian (Palombo and Semple, 2001) plant extracts. Results within this laboratory (Cock, 2008a) have also confirmed the greater susceptibility of Gram-positive bacteria towards other Australian plant extracts. The Gram-negative bacterial cell wall outer membrane is thought to act as a barrier to many substances including antibiotics (Tortora et al., 2001). The uptake of the *M. cauliflora* extract antibiotic agents by Gram-negative bacteria is presumably affected by the cell wall outer membrane of some bacteria. In contrast, Gram-negative bacteria appeared more susceptible to the *M. cauliflora* leaf extract than Gram-positive bacteria although this may be due to the small sample of Gram-positive bacteria tested.

Individual *M. cauliflora* leaf and fruit components responsible for the extracts antiseptic potential were not identified in the current study. However, reports have identified various bioactive components of other members of the Myrtaceae family (*Eucalypts* (Brophy et al., 1985; Foley and Lassak, 2004), *Leptospermum* (Porter and Wilkins, 1999; Carr, 1998), *Melaleucas* (Russell and Southwell, 2003; Carson and Riley, 1995)). These plants all contain terpenes including 1, 8-cineole, terpinen-4-ol,  $\alpha$ -pinene and  $\beta$ -pinene. Both 1, 8-cineole and terpinen-4-ol have antimicrobial activity (Simić et al., 2005; Mondello et al., 2006). Recent studies have also reported on the antibacterial activities of the *Callistemons* (Sanjai and Charu, 2006; Saxena and Gomber, 2006) and *Syzygiums* (Arora and Kaur, 2007; Park et al., 2007; Duraipandiyar et al., 2006; Shafi et al., 2002). It has been postulated that terpene components may also be responsible for the antiseptic properties of these plants (Jirovetz et al., 1997). Whilst the phytochemistry of *M. cauliflora* has not been extensively studied, a variety of tannins (Morton, 1987), flavanols, phenolic acids anthocyanins, purocyanin B, depsides and ellagic acid (Reynertson et al., 2006) have been identified. Recent studies have identified cyanidin 3-glucoside (Einbond et al., 2004), peonidin 3-glucoside and its aglycone (Trevisan et al., 1972) as well as the depsides Jaboticabin and 2-O-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid (Reynertson, 2007) as being of particular interest. Jaboticabin and 2-O-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid have the ability to inhibit cytokine production, are cytotoxic towards HT29 and HCT116 colon cancer cell lines and have antioxidant activity and thus warrant more extensive investigation (Reynertson, 2007).

The findings reported here also indicate that *M. cauliflora* leaf extract display toxicity towards *Artemia franciscana*. This would impact on the usefulness of the extract as a medicinal antiseptic agent. Toxicity towards *A. franciscana* has previously been shown to correlate well with toxicity towards human cells for some toxins (McLaughlin et al., 1998). However, future studies need to determine whether this is also true for the *M. cauliflora* leaf extract. Toxic antibacterial extracts may be useful as non-medicinal antibacterial agents (eg surface disinfectants). Likewise, toxic plant extracts may also still have medicinal potential even if they are not antimicrobial. McLaughlin et al (1998) have demonstrated that toxicity in the *A. franciscana* bioassay may indicate anti-cancer potential. Toxic extracts

such as the *M. cauliflora* leaf extracts should also be tested against human cancer cell lines to determine their potential as anticancer drugs.

In conclusion, the results of this study indicate that *M. cauliflora* leaf and fruit extracts are worthy of further study due to their antibacterial activity. 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 antimicrobial agents, caution is needed before these compounds can be applied to medicinal purposes and as food additives to inhibit spoilage. In particular, further toxicity studies using human cell lines are needed to determine the suitability of these extracts for these purposes.

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