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Characterisation of proanthocyanidins and related phenolics for inhibition of gastrointestinal tract microorganisms (Phase I)

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Characterisation of proanthocyanidins and related phenolics for inhibition of gastrointestinal tract microorganisms

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Executive Summary

The general public is becoming increasingly aware of problems from the over-use and misuse of traditional antibiotics. Coupled with this view is the general consensus among the medical profession that the use of antibiotics by the poultry industry has the potential for creating "superbugs", which are microorganisms highly resistant to clinical antibiotics. As a result of this concern, antibiotics have currently been restricted, and a total ban has been in place, in Europe since January 2006. Therefore European poultry producers are faced with the challenge to produce poultry products without antibiotics with consequent losses in feed efficiency, performance and increased in production costs. There is a general acceptance that production of meat without the use of antibiotics is likely to increase within the Asia Pacific Region during the next decade. It is expected that Australia will follow this trend due to the economic benefits obtained as a leading "clean green" image country. The increasing demand for 'natural' and drug-free meat production throughout the more affluent countries of the world reinforces the need for the evaluation of plant antimicrobial compounds as alternatives to antibiotics.

The main objectives of this study were to: i) assess the antimicrobial activity of isolated, fractionated and purified compounds from grape and olives residues; ii) isolate, fractionate, purify and characterise condensed tannins and related phenolics from olive pomace; and iii) identify and characterise specific polyphenolic fractions from grapes and olive pomace able to inhibit microorganisms such as *Clostridium* spp., *Campylobacter* spp. and *Salmonella* spp. under *in vitro* conditions.

First evaluation

Initially, Tarac Technologies processed and provided seven seed extracts, grape seeds tannins and red grape marc. These extracts were labelled as 136-2005, 178-2005F, 178- 2005AXA, oligomeric GSE, polymeric acid, N05010 and monomeric GSE. The University of Queensland provided pure reference compounds such as caffeic acid, p-courmeric acid, syringic acid and vanillic acid. The compounds were evaluated at ARI under world accepted methods for *in vitro* conditions using disc diffusion and agar-based minimal inhibitory concentration (MIC).

The *in vitro* techniques used evaluated the extracts against *Campylobacter jejuni* ATCC 33560, the strain recommended by the National Committee Clinical Laboratory Standards. Additional reference strain for *Campylobacter coli* ATCC 33559 was used. *C. jejuni* isolates – C338, C627, C838, C858 and C1209 were added to the evaluations. The isolates were selected to represent the five most common genotypes seen in broilers in south-east Queensland. *Clostridium perfringens* isolates were also evaluated (being part of the QDPI&F collection unique in Australia).

The results of the initial evaluation indicated that *in vitro* methodologies for antimicrobial activity of grape extracts were established and validated. Most of the grape extracts showed no inhibitory activity at the dilutions tested. However, the extract termed polymeric acid did show inhibitory activity in *C. jejuni* ATCC 33560 and three of the four field isolates were inhibited at a concentration of 1 mg/ml of this extract. Polymeric acid was also affecting *Clostridium perfringens*. *C. coli* ATCC 33559 and one of the field isolates of *C. jejuni* were not inhibited by this concentration of this extract. Skin extract inhibited all types of microorganisms, although sulphur dioxide (SO_2) present in the skin extract solution may have affected the results. Of the four pure reference compounds tested, three (p-courmeric acid, syringic acid and vanillic acid) inhibited the two reference strains and the four field isolates at 1 and 0.5 mg/ml. Caffeic acid inhibited the two reference strains and the four field isolates at all three concentrations tested (1, 05 and 0.25 mg/ml). The *Campylobacter jejuni* ATCC 33560 strain gave the correct result for tetracycline of 2 μg/ml – confirming the acceptability of the assays. Therefore it was recommended to increase the concentration of polymeric acid fraction in the study using 10, 5, 2.5, and 1.25 dilutions. To ensure SO_2 did not affect results, it was recommended that more skin extract free of SO_2 was needed for the second evaluation. The initial samples provided by

Tarac exhibited large amounts of proanthocyanidins (condensed tannins) which are mainly in the form of polymers and oligomers. It was also shown that Cathechins, Epicatechin gallate and others dominate the monomeric fractions. It was established that liquid chromatography mass spectrometry (LCMS) analysis provided molecular weight of peaks that assisted in the identification of some of the compound present. It was indicated the need for further purification steps on relevant fractions to improve resolution. It was indicated that to the SO_2 free extract polymeric fraction would be further purified and fractionated using ethyl acetate (EA). This will allow us to check effectiveness on microbial inhibition by fractionating it into two portions, the EA extractable phenolics and the non-EA extractable (including condensed tannins) to produce at least three new fractions to test, the original extract, the EA fraction and the no-EA extractables.

Second evaluation

Following the meeting held on 9 May 2007, it was agreed that Tarac would supply additional quantities of the Polymeric fraction that had shown some anti-microbial activity in the first tests as well as a Red skin extract that was free of SO_2 to eliminate this as the compound responsible for some anti-microbial activity demonstrated in the first tests. The University of Queensland fractionated the polymeric fraction to remove the small quantity of phenolics present in the material from the bulk of the condensed tannins for further testing and fractionated the SO_2 free Red skin extract into various fractions for testing. **The Polymeric fraction** was fractionated and cleaned into phenolics and condensed Tannins (CT) followed UQ procedures.

The Red skin extract was fractionated, acidified and extracted with ethyl acetate. Phenolics were removed with $NaHCO_3$ to yield the flavonoid fraction. The $NaHCO_3$ fraction was then re-acidified, extracted with ethyl acetate, vacuum evaporated to yield the phenolic fraction. The results of the fractionation steps showed that the polymeric fraction comprised 93.7% CT with the remainder as phenolics. The Red skin extract had a flavonoid content of 2.8 mg/mL and a phenolics content of 8 mg/mL. The crude flavonoid fraction obtained previously was then subjected to Size Exclusion Chromatography (SEC) to further fractionate the classes of compounds present. This process provided two groups of compounds classified as purified flavonoids and CT. A portion of the purified flavonoid fraction was then subjected to acid hydrolysis to provide the free aglycones for analysis. HPLC analysis of the flavonoid fraction showed the presence of quercetin and myricetin as the free aglycones in the crude fraction. However, fractionation of this by SEC and HPLC analysis of the methanol eluent where the flavonoids were expected to be present did not show the presence of quercetin or myricetin. Acid hydrolysis of the methanol fraction to break the glycosidic bonds and release the free aglycones did show the presence of a small peak that was identified as quercetin. HPLC analysis of the crude flavonoid fraction obtained by solvent extraction with ethyl acetate, and previously tested to show antimicrobial activity, indicated the presence of the two common flavonoids quercetin and myricetin as well as other unidentified peaks. However, fractionation of this by SEC that separates the flavonoids from the CT did not show the presence of these two flavonoids in the expected methanol eluent fraction despite many large peaks being present in the chromatogram. Acid hydrolysis of this did reveal a small peak for quercetin indicating that it was present as a glycosidic form, not as the free form present in the original extract. The acetone eluent fraction expected to represent the CT did show a positive response with the butanol/HCl colorimetric test, but not a strong colour. This suggests that these flavonoids were strongly retained and not eluted until the mobile phase was changed to acetone/water. A subsequent test on the Toyopearl column with quercetin as a marker compound showed that it was retained at the head of the column and did not elute until the solvent was changed to acetone/water, when the brown colored condensed tannins also eluted. Therefore, for the second anti-microbial testing the following fractions were produced:

1) Polymeric fraction as supplied;
2) Condensed tannin fraction from polymeric fraction;
3) Phenolic fraction from polymeric fraction;
4) Red skin extract as supplied (nil SO_2);

5) Water fraction of Red skin extract after acetone solvation and filtering and extraction with ethyl acetate;
6) First ethyl acetate extract fraction (phenolics + flavonoids);
7) Phenolic fraction and;
8) Flavonoid fraction.

The olive pomace fractions were prepared by The University of Adelaide. Four samples (1 g each) of phenolic extracts from olive-derived materials were received for antimicrobial testing. The material was described as follows:

1) OLI-2322-1B - Mixture of total phenolics from olive leaf;
2) OL2-2322-1B - purified oleuropein;
3) OW1-2322-1B - Mixture of total phenolics from olive mill pomace;
4) OW2-2322-1B - Mixture of total phenolics from olive mill black water.

For the second evaluation, the grape extracts compounds provided by the University of Queensland and the University of Adelaide were tested against the same set of *Campylobacter jejuni/coli* strains and *Clostridium perfringens* isolates used in the initial screening.

The Tarac Red Skin, Flavonoid fraction inhibited all strains of *Campylobacter* at 0.5 and 0.25 mg/ml concentration. The next most effective compound was the Tarac Red Skin, Flavonoid and Phenolics fraction with most, but not all, strains being inhibited at the highest dilution (0.25 mg/ml) tested.

The most active of the olive extracts was the OL1-2322-1B fraction, although the level of activity was less than the best performed of the Red skin extracts.

For *Clostridium perfringens*, the only compound to show any major activity was the Tarac Red Skin, Flavonoid fraction which inhibited all strains of *Clostridium perfringens* at 1.0 and 0.5 mg/ml concentrations and 2/7 strains at the 0.25 mg/ml concentration.

Based on the above results, it would be logical to obtain more of the Tarac Red Skin, Flavonoid fraction and complete the dilution work (*i.e.*, test greater dilutions until an end point is reached (minimum inhibitory concentration) with all strains of both *Campylobacter* and *Clostridium perfringens*.

Third evaluation

Previous work has demonstrated that the only compound that has shown any major activity on all bacteria tested was the Tarac Red Skin, Flavonoid fraction which inhibited all strains of *Campylobacter* (at concentrations 0.25, 0.5 and 1 mg/ml) and *Clostridium perfringens* (at 1.0 and 0.5 mg/ml concentrations and 2/7 strains at the 0.25 mg/ml). It was suggested that further testing using Red skin flavonoid fraction at lower dilutions was needed using greater dilutions until an end point is reached with both *Campylobacter* and *Clostridium perfringens* isolates used in the initial screening. As well, a collection of *Salmonella enterica* isolates (each a different serovar from a different broiler farm) would be tested against this promising flavonoid fraction.

The University of Queensland prepared the larger volumes of Red skin extract to produce phenolic acid and flavonoid fractions using the method described previously. The obtained material was: named combined fraction, phenolic fraction and Flavonoid fraction.

The results of the third *in vitro* evaluation showed that the Flavonoid fraction inhibited all strains of *Campylobacter* at 0.25 mg/ml concentration. No other tested compound showed any activity except that the anthocyanine fraction inhibited one *Campylobacter* at 0.5 mg/ml. The Flavonoid fraction also inhibited most strains (6/7) of *Clostridium perfringens* at the 0.5 mg/ml concentration. No other tested

compound showed any activity. The Flavonoid fraction inhibited most strains (4/6) of Salmonella at the 0.5 mg/ml concentration. No other tested compound showed any activity.

These results are consistent with the previous results recorded for the *Campylobacter* and *Clostridium* strains in our earlier reports. The Flavonoid fraction was the only fraction to demonstrate marked anti-bacterial activity, inhibiting all strains of *Campylobacter* at the 0.25 mg/ml concentration, most strains (6/7) of *Clostridium perfringens* and most strains (4/6) of *Salmonella* and *E. coli* at the 0.5 mg/ml concentration. This current round of testing has established the formal minimal inhibitory concentration (MIC) of this compound for all strains used in the study. The Flavonoid fraction has been shown to be active against *Campylobacter* with typical MIC of 0.25 mg/ml as well as *Clostridium perfringens* and *Salmonella* and *E. coli* with most strains showing an MIC of 0.5 mg/ml. To complement the work undertaken in this study, further work is required to:

i) identify the antimicrobial compounds in the Flavonoid fraction of Tarac Red skin extract by using high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LCMS) analysis;

ii) (from the obtained flavonoid fraction, made of several fractions), search for a more active fraction by further fractionation to obtain individual flavonoid fractions which then need to be testied for antimicrobial activity.

Fourth evaluation

In order to provide sufficient material for each fraction, a fresh bulk of Red skin extract was supplied by Tarac Technologies for processing. This material solvent was extracted and separated into two groups of phenolic acids and crude flavonoids, then concentrated and run in a series of batches on the Toyopearl column. The eluate was collected on a Fraction Collector and then divided into a group of eight fractions, concentrated and finally dried under vacuum. The dried material was then submitted for antimicrobial testing.

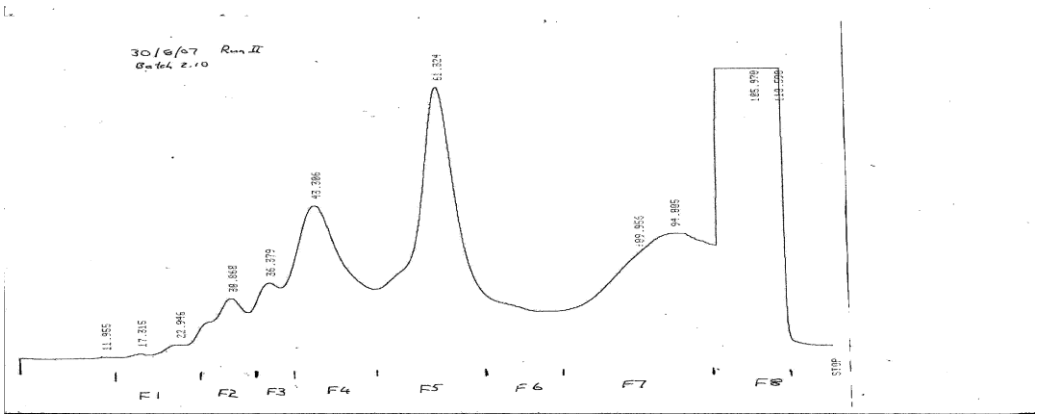

Figure 1. Chromatogram of crude flavonoids separated on a Toyopearl HW-40F column 2.6 x 30 cm column eluted with 40% methanol till 70 min then 70% acetone until 110 min at a flow rate of 5 mL/min.

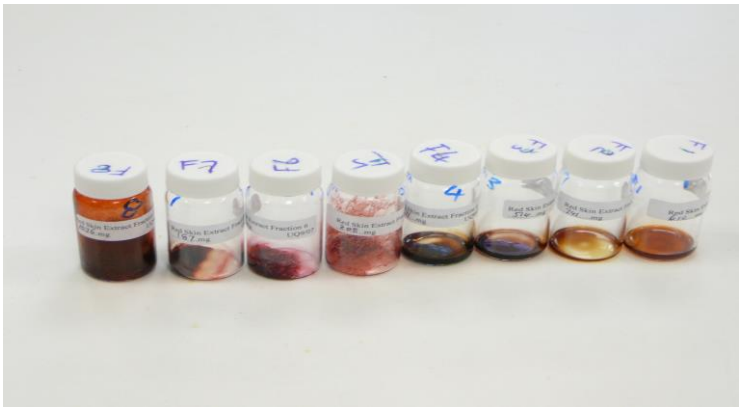

Figure 2. Rotary and vacuum evaporated flavonoids fractions

Fractions 4, 5 and 6 showed an ability to inhibit all strains of *Campylobacter* at the 0.5 mg/ml concentration. Fraction 4 also inhibited all of the strains at the 0.25 mg/ml concentration. No other tested compound showed any activity except that Fraction 1 inhibited the *C. coli* reference strain at the 0.5 mg/ml concentration.

Fraction 4 inhibited most of the strains (6/7) of *Clostridium perfringens* at the 0.5 mg/ml concentration. No other tested compound showed any activity.

Comments on the Red skin material and fractions obtained:-

- Solvent extraction of the Red skin material by ethyl acetate showed that the antimicrobial activity was in the ethyl acetate phase and not in the remaining aqueous phase;
- Fractionation of the ethyl acetate phase into phenolic acids and crude flavonoids showed that the antimicrobial activity was only in the crude flavonoid fraction and not associated with the phenolic acid fraction;
- Fractionation of the crude flavonoids by Size Exclusion Chromatography on Toyopearl HW-40F into two groups showed that the antimicrobial activity was associated with the compounds eluted initially with methanol/water and not with the retained fraction subsequently eluted with acetone/water;
- Antimicrobial testing showed that the test flavonoids quercetin and rutin did not posess antimicrobial activity;
- Subsequent testing on the Toyopearl column showed that quercetin was retained on the column along with condensed tannins and not eluted until the solvent was changed to acetone/water;
- Macro scale fraction on a Toyopearl HW-40F column into seven fractions of the methanol/water eluent showed that the antimicrobial activity was associated with the major peaks eluting at the low molecular weight time window;
- The antimicrobial activity originally demonstrated in the Red skin extract is not associated with the major flavonoids, quercetin and myricetin, present in wine grape skin, nor the phenolic acids or the condensed tannins, but with an as yet unidentified group of compounds.

Initial LCMS identification of active antimicrobial fractions

Fractions of the Red skin extract obtained by chromatographic separation of the crude flavonoid fraction were tested for antimicrobial activity. Fractions 3, 4, 5 and 6 showed activity against *Campylobacter* and *Clostridium* species and initial work by Liquid Chromatography-Mass Spectrometry (LCMS) was directed at these fractions. Earlier work by HPLC had shown that the two common flavonoids, quercetin and myrecitin, were not present in these fractions but present in Fraction 8 and both this fraction and pure quercetin and rutin did not show antimicrobial activity. The

presence of these two flavonoids in Fraction 8 was confirmed by LCMS analysis. Fractions 3, 4, 5 and 6 were subjected to analysis by LCMS for molecular weight identification of the major peaks present in these fractions. The preliminary results showed that Fraction 3, although based on a single peak in the large scale chromatography on Toyopearl HW-40F, comprised many compounds. Two compounds comprised 23.6% and 18.9% of the total peak area with all other peaks being <10% of the total peak area. Mass analysis of these two peaks showed that the compound eluting at 6.50 min had a molecular weight (MW) of 410 and the compound eluting at 8.85 min a MW of 242. This fraction showed activity against *Clostridium perfingens* only at a MIC concentration of 0.5 mg/ml.

Fraction 4 showed by UV detection to have a major compound eluting at 32.31 min comprising 33.3% of the total peak area. This compound was shown by mass analysis to have a MW of 226. The other peaks present individually were <10% of the total peak area. This fraction showed activity against all *Campylobacter* spp. tested needing only a MIC of 0.25 mg/mL. Fraction 4 also inhibited most of the strains (6/7) of *Clostridium perfringens* at the 0.5 mg/ml concentration.

Fraction 5 comprise of a single compound eluting at 12.68 min and accounting for 78.6% of the total peak area. This compound was shown by mass analysis to have a MW of 198.

LCMS analysis of Fractions 6 and 7, which also showed limited activity at *Campylobacter* spp. only at a MIC of 0.5 mg/ml, showed that these fractions comprised eluting traces of the major compound present in Fraction 5. This is not unexpected, as the peak for Fraction 5 was the major peak obtained for fractions off the Toyopearl column prior to changing solvents.

The initial identity of the major peak present in Fraction 5 as ethyl gallate was based on published work performed using LCMS analysis of similar ethyl acetate extracts of red wine but without the class separation step to remove phenolic acids. Acid hydrolysis of a sub-sample of Fraction 5 and analysis by LCMS showed the loss of the peak eluting at 12.66 min with the appearance of a peak eluting at 2.13 min which corresponds by both retention time and (M-H) ion (m/z 169) to gallic acid. Final confirmation of this peak as ethyl gallate will be done by running the authentic compound when it is available. A literature search showed that gallate esters have been demonstrated to have antimicrobial activity. Chaubal *et al* (2005) showed that both gallic acid and methyl gallate were active against a range of bacteria with a MIC of >1000 mg/mL and 12.5 mg/mL respectively. It has also been shown by Adesina *et al* (2000) that ethyl gallate extracted from the leaves of *Acalypha* spp. showed antimicrobial activity against *E. coli* and *S. aureus* with MIC values of 0.4 and 0.1 mg/mL, respectively. The tentatively identified ethyl gallate present in Fraction 5 has, in this work, been shown to have an activity with a MIC of 0.25 mg/L against *Campylobacter* spp. and *Clostridium perfringens* isolates.

Implications

During this study using *in vtiro* techniques, phenolic compounds extracted from grape residues were found to inhibit *Campylobacter* and *Clostridium perfringens* microroganisms. These compound were also effective against *Salmonella* and *E.coli* bacteria. *Clostridium perfringens* is a bacterium responsible for necrotic enteritis (NE), considered a global poultry disease. This bacterium produces toxins in the chicken's intestine, resulting in high mortalities in flocks, and in its subclinical form, NE is financially damaging due to reduced growth performance in chickens. Currently, the main method to control the incidence of NE is by the use of antibiotics, but this practice is coming under increasingly critical scrutiny and has been curtailed in Western Europe due to concerns about the development of antibiotic-resistance in bacteria that are potential human pathogens. There is also increasing public concern about antibiotic residues in animal products, which has resulted in the search for natural alternatives to sustain efficient chicken meat production without reliance on antibiotics.

The discovery of the proposed natural phenolic compound during this pilot study may revolutionise the control of the major food safety pathogens in which, currently, there is no 100% effective control/prevention strategy.

The outcomes of this research may include:

- a novel consumer friendly method for preventing/controlling the colonisation of chickens by *Campylobacter*, the most important cause of food-borne gastroenteritis in humans;
- improved on-farm control programs based on the outcomes of this project and future research which will result in safer chicken meat and improve consumer confidence and, hence, improve the market penetration of chicken meat;
- the emergence of Australia as a leader in the development and commercialisation of biotechnology and utilisation of life science technologies for the betterment of society;
- improved systems to ensure the safety of food products through the chain by allowing an effective prevention/control program for unwanted bacteria in broiler chickens;
- a significant step forward in our ability to manage and control a major biohazard – *C. jejuni* and C*lostridium perfringens*, respectively the most important food-borne gastro-enteritis agent in humans and necrotis enteritis agent in poultry;
- the potential to improve the profitability of the poultry industry by adding value to poultry products (without antibiotics);
- enabling the poultry industry to enhance the clean image of Australia's food industries, adding considerable value and market appeal to these industries.

Recommendations

- For continuity, future *in vitro* works regarding antimicrobial evaluation of plant material fractions should follow all the laboratory methods established at ARI during this study;
- It is recommended that future research continue with the identification of the compounds that have shown major antimicrobial activity (*i.e.*, Fractions 3, 4 and 5 from Red skin grape material);
- Fraction 3, which showed activity against *Clostridium perfingens* and is mainly made with two compounds (23.6% and 18.9% of the total peak area), would need further work to accomplish components identification. It is recommended that from the original material (Red skin extract), to collect Fraction 3 needing further purification and fractionation using a superior resolution column to clearly separate main compounds to assist to LCMS identification;
- Fractions 4 and 5 were dominated by single compounds and it is desirable to complete their identification and perform further antimicrobial testing to establish if the activity is solely associated with single compounds or is the result of the mixture of other compounds present in the fractions;
- If the identified compounds are commercially available, then reference compounds need to be purchased for further antimicrobioal testing to ensure the identity of the compound found in the active fractions;
- If the identified compound obtained during this study is the active compound, then a search of global databases is needed to evaluate potential intellectual property on the use of these compounds;
- It is necessary to investigate how the intellectual property, of individual or group of the obtained compounds that show microbial activity in this study, could be protected;

- It is highly recommended to continue the evaluation of the obtained compounds using animals (Phase II proposal) to evaluate their efficacy in controlling undesirable bacteria without affecting feed efficiency;
- It is recommended to expand the potential use of the obtained antimicrobial fractions. For example, there is a possibility for their use as antimicrobial sprays by the food industry to increase food life during refrigeration/storage.

CONTENTS

Introduction

Concerns over the possible emergence of antibiotic-resistant microbials in the food chain, arising from the use of antibiotics in the intensive livestock industries, has led to a general consensus to reduce their use. In several European countries, in which similar types of antibiotics have been used to treat both animals and humans, partial or total bans of antibiotic use in feed have been imposed. Therefore, European poultry producers are faced with the challenge to produce poultry products without antibiotics with consequent losses in feed efficiency, performance and increased production costs.

Reducing reliance on the use of antibiotics in meat chicken production requires the introduction of alternatives to antibiotics, which will form part of nutritional management regimes. One of these alternatives may be naturally-occurring antimicrobial compounds found in certain plants.

Condensed tannins (CTs) are polymeric phenolic substances capable of tanning leather or precipitating protein from solution, a property known as astringency. Found in plant tissues, CTs with high molecular weight (500 to 3,000) have the capacity to form complexes with carbohydrates and proteins and have been shown to inhibit microbial action. The binding capacity and its force depend upon their chemical structure (cyanidin, delphinidin or pelargonidin sub-units), which, in turn, depends upon plant material origin. For example, the chemical structures and binding capacities of CTs from the grain legume, faba beans, are different from the CTs isolated from the shrub legume, *Leucaena leucocephala*. Due to this difference in chemical structure, some CTs may have the capacity to eliminate large populations of microflora in sheep fed *Acacia saligna* (Krebs *et al*, 2003).

There are other related substances in plant material with smaller molecular weights known as phenolics. Significant amounts of phenolic compounds obtained from olive fruit and pomace have been shown to have significant biological properties such as antimicrobial, hypoglycaemic, hypolopidemic, hypocholesterolic, antioxidant and free radical scavenging actions (Fleming *et al*, 1973). A recent Brazilian *in vitro* and *in vivo* study (Esquenazi *et al*., 2002) has demonstrated how a B-type procyanidins extract from *Cocos nucifera* can selectively eliminate *Staphylococcus aureus* and a herpes type 1 virus.

Therefore, the main objective of this pilot study was to evaluate *in vitro* small molecular weight phenolics and CTs extracted from grapes and olive plant material, and to determine their efficacy as antimicrobial agents.

Objectives

1. Determine the antimicrobial activity of isolated, fractionated and purified compounds from grape marc to be supplied by TARAC Technologies Pty Ltd.

2. Isolate, fractionate, purify and characterise proanthocyanidins and related phenolics from olive pomace.

3. Identify and characterise specific polyphenolic fractions from grapes and olive pomace able to inhibit microorganisms such as *Clostridium* spp., *Campylobacter* spp. and *Salmonella* spp. under *in vitro* conditions.

Chapter 1: Initial grape seed extracts provided by Tarac technologies

TARAC Industrial Process Method to Extract Grape Seed Tannins

During the wine vintage period, white grape marc is collected and the seeds are removed. The white grape seeds are gently dried then stored until required. The seeds are then loaded into an extraction vessel and the aqueous ethanol extraction liquor is circulated through the seeds. The ethanol is removed from the extraction liquor and the liquor concentrated. The extract liquor concentrate undergoes fermentation of reducing sugars. The extract liquor is then chilled and held in a tank before passing through a centrifuge to remove fine material. The extract is further concentrated and then filtered before spray drying to a fine powder.

TARAC Industrial Process Method to Extract Red Grape Marc Pigment

During the wine vintage period, grape marc is delivered from the wineries directly to Tarac. The selection process for fresh red grape marc utilises a sample inspection by the laboratory to determine if marc is within specification for pigment extraction. The seeds are removed from the red grape marc which is then conveyed into two parallel counter current extractors (CCE's) for extraction with an aqueous ethanol extraction solvent. The marc exiting the CCE's are fed into a screw press to remove the liquid from the skins. The extract liquor passes through a decanting centrifuge which continuously removes coarse solids and then a continuous centrifuge removing fine material. The extract liquor is concentrated approximately 5 times under vacuum at a low temperature. The extract liquor concentrate is stored in tanks and undergoes fermentation of reducing sugars. Following this, the concentrate is retained in the tanks until it is filtered before further concentration to the desired specification.

TARAC Method to Determine Polymeric Procyanidins (tannins) In Grape Seed Extracts

Polymeric procyanidins (tannins) in grape seed extract were determined qualitatively by injection onto a High Performance Liquid Chromatograph (HPLC) with reverse phase C18 column and UV detection at 280nm. Individual components (gallic acid, catechin, epicatechin and epicatechin gallate) were determined by comparison with standard solutions (Peng *et al*, 2000).

Tarac method for chemical analysis of fractionated grape seed

Grape Seed Extract was separated into three fractions. The aim was to produce fractions containing predominantly monomeric, oligomeric and polymeric grape seed tannins. Additionally, a further sample of precipitate in the 'racking' of the final solution prior to spray drying was analysed. The chemical analysis of the whole seed extract and these fractions was undertaken for their chemical characterisation, which included:

- Antioxidant Capacity using the Ferric Reducing Ability of Plasma (FRAP assay) LM 410;
- Total phenolics by Folin – Ciocalteau Assay, LM 400;
- Determination of Polymeric Procyanidins (Tannins) in Grape Seed Extract by HPLC, LM 611;
- Subunit composition analysis by phlorglucinol analysis.

Due to intellectual property restriction, the description of the method used by TARAC could not be disclosed in this report, but it may be obtained by directly contacting Marina Shulz (TARAC Chemistry Officer).

First set of samples from grape material generated by tarac technologies

TARAC technologies generated several fractions samples which were forwarded to the Poultry Research and Development Centre (Rider Perez, 14 November 2005). Samples were divided into two portions to be chemically evaluated at the University of Queensland (Graham Kerven) and for antibacterial activity at the Animal Research Institute (Pat Blackall). Samples are classified as follows:

Table 1. Fraction samples obtained from Tarac

Fractions obtained	Water solubility	Sulphur in sample?	Storage conditions
Grape seed extract (GSE) N05010 (5.7g)	soluble	no	store at room temperature, away from moisture and sunlight.
Monomeric fraction GSE batch N03010. (0.5g)	soluble	no	store at room temperature, away from moisture and sunlight.
Oligomeric fraction GSE batch N03010. (0.94g)	soluble	no	store at room temperature, away from moisture and sunlight.
Polymeric fraction GSE batch N03010. (1.29g)	Not soluble – dissolve in methanol – may be able to dilute with water after dissolved	no	store at room temperature, away from moisture and sunlight.
GSE 178-2005 f (2.6g)	soluble	no	store at room temperature, away from moisture and sunlight.
GSE 178-2005 XAD (2.2g)	soluble	no	store at room temperature, away from moisture and sunlight.
White skin extract 136-2005 (2.5g)	soluble	no	store at room temperature, away from moisture and sunlight.
Red skin extract (aqueous liquid) batch I05101 (50mL)	soluble	Yes Free SO_2 = 54 Total SO_2 = 352	When not in use refrigerate to approx 4^oC

Chapter 2: *In vitro* studies on initial Tarac grape seed extracts

Preliminary evaluation to establish *in vitro* methodology

Introduction

A review of literature shows that two methodologies have been used to examine the antibacterial activity of plant phenolic compounds – disc diffusion and agar-based minimal inhibitory concentration (MIC). Plant phenolics have also been dissolved in water, propylene glycol and absolute methanol.

The aim of this experiment was to gain experience in the performance of antimicrobial assays, in order to evaluate the method of the testing (disc diffusion v's MIC) and to evaluate the effect of the solvent on these methods.

Materials and Methods

Bacteria

Campylobacter jejuni ATCC 33560, the strain recommended by the National Committee Clinical Laboratory Standards (NCCLS, 2002), was used in this work. The formal reference strain for *Campylobacter coli* ATCC 33559 was also used.

Preparation of stock tetracycline solution

A stock solution of tetracycline was prepared, as recommended by Anhalt and Washington (1985). Distilled water was used as both the initial solvent and the diluent. All preparation of the antibiotic was done in plastic-ware. A total weight (accurately recorded on an analytical balance) of pure powder was obtained. The volume of distilled water that was added to the dry powder was calculated using the following formula:

$$\text{volume (ml)} = \frac{\text{weight (mg)} \times \text{potency (ug/mg)}}{\text{concentration (ug/ml)}}$$

Where:

weight (in mg) is the weight of antibiotic powder;
potency (in μg/mg) is marked by the manufacturer on the bottle;
concentration (μg/ml) is the desired concentration of the stock solution.

The stock solution was then prepared by addition of the required volume of distilled water. A standard stock concentration of 1,280 μg/ml was used in this study (NCCLS, 2002). The stock solution was held at –70°C for no more than six months, as recommended (NCCLS, 2002).

The intention is to prepare dilutions of propylene glycol and methanol and then test these dilutions in both a disc diffusion method and an MIC method.

Performance of tetracycline MIC assay

The antibiotic containing agar was prepared as recommended (NCCLS, 2002). In brief, a volume of the stock solution of tetracycline was removed from storage and thawed. The stock solution was diluted in a two-fold series in distilled water to dilutions of 10 to 320 μg/ml. Mueller-Hinton agar (Oxoid CM337) was prepared as per manufacturer's instructions. The molten Mueller-Hinton agar was cooled to 50°C, supplemented with 5% defibrinated sheep blood and the relevant dilution of

antibiotic (added as a 10% volume) and the agar poured as a 30 ml volume per 90 mm petri dish. All plates were dried and used on the day of preparation. This meant that a series of plates with a final concentration of 1 to 32 μg/ml of tetracycline had been produced.

The bacterias *Campylobacter jejuni* ATCC 33560 and *C. coli* ATCC 33559 were subcultured on 5% sheep blood agar (SBA) and incubated for 48 hours in a modified atmosphere (CampyGen Gas Pak - Oxoid CN35). A sterile saline suspension was prepared and adjusted to an optical density equivalent to a 0.5 MacFarland nephelometer tube.

The adjusted bacterial suspension was loaded into the master plate of a Mast Multipoint Inoculator (SCAN 100). The tetracycline containing agar plates (which were pre-dried) were then inoculated. The plates were then left at room temperature until the moisture in the inoculum spots had dried into the agar. The plates were then incubated at 37^{o}C in a modified atmosphere (CampyGen Gas Pak - Oxoid CN35). In each run, two SBA plates were inoculated – one as the first plate and the other as the last plate.

The SBA plates were checked for purity. Only cultures which showed pure growth on both SBA plates were accepted. The MIC of tetracycline for the reference strains for tetracycline was determined as the lowest concentration of tetracycline that completely inhibited colony formation. This was done at 24 and 48 hours post inoculation.

Evaluation of the inhibitory effects of methanol and propylene glycol

Using sterile distilled water, dilutions of both methanol and propylene glycol were prepared. An aliquot (20 μl) of the relevant solvent dilution was added to sterile paper discs. As well, agar plates containing dilutions of the two solvents were prepared, as described above for tetracycline.

The paper discs were used in a standardised disc diffusion assay (NCCLS, 2002). The reference strains were grown on SBA at 37ºC for 48 hrs in a modified atmosphere (CampyGen Gas Pak - Oxoid CN35). A sterile saline suspension was prepared and adjusted to an optical density equivalent to a 0.5 MacFarland nephelometer tube. The suspension was swabbed across the surface of pre-dried Muller Hinton agar (with 5% defibrinated sheep blood) plates. The paper discs containing the solvent were then placed onto the inoculated plates – no more than three discs per plate. A purity check, performed by inoculation onto sheep blood agar, was performed for all suspensions. The inoculated MHA and purity check plates were incubated at 37ºC in the modified atmosphere (CampyGen Gas Pak - Oxoid CN35). Following incubation, the purity plates were examined to confirm the purity of the inocula. The diameter of inhibition zones around the discs was measured to the nearest whole millimetre using a Vernier calliper. This was done at 24 and 48 hours post inoculation. The MIC methodology described above was used to determine the MIC for methanol and propylene glycol for both reference strains.

Results and Discussion

Tetracycline MIC study

At 24 hours post inoculation, the MIC for tetracycline was 2 μg/ml for *C. coli* ATCC 33559 and 4 μg/ml for *C. jejuni* ATCC 33560. At 48 hours post inoculation, the MIC for tetracycline for both *C. jejuni* and *C. coli* was 4 μg/ml.

Inhibitory Effects of Solvents

The results of this work are presented in Table 2.

Table 2. Results of disc diffusion and MIC testing of possible solvents

		Size of zone (mm) around disc containing indicated dilution				Growth on MIC plate at indicated dilution			
		C. jejuni ATCC 33560		*C. coli* ATCC 33559		*C. jejuni* ATCC 33560		*C. coli* ATCC 33559	
Solvent	**Dilution**	**24 hr**	**48 hr**	**24 hr**	**48 hr**	**24 hr**	**48 hr**	**24 hr**	**48 hr**
Methanol	Neat	?[1]	<6[2]	?	<6	-	-	?	-
	1/10	?	<6	?	<6	?	+	?	+
	1/100	?	<6	?	<6	+	+	?	+
	1/400	?	<6	?	<6	+	+	?	+
Glycol[3]	Neat	?	8.2	?	8.2	-	-	?	-
	1/10	?	<6	?	<6	+	+	?	+
	1/100	?	<6	?	<6	+	+	?	+
	1/400	?	<6	?	<6	+	+	?	+

[1] ? = weak growth – difficult to read; [2] <6 = growth right to disc which has a diameter of 6 mm
[3] Glycol = Propylene glycol

Antimicrobial activity can be detected in many different ways. When working with pure antibiotics, there are a set of formally validated methodologies that are widely accepted around the world. The methodologies are fully described by the NCCLS documentation (NCCLS, 2002). In this documentation, two basic methods are described – a disc diffusion method and a Minimal Inhibitory Concentration (MIC) method. For *Campylobacter* isolates, the NCCLS methodology allows only an agar-based MIC method (NCCLS, 2002).

The NCCLS (2002) MIC method uses *C. jejuni* ATCC 33560 as the formal quality control strain. Using the full NCCLS methodology should result in *C. jejuni* ATCC 33560 showing an MIC of 1-4 μg/ml (NCCLS 2002). In the first tetracycline MIC study, we demonstrated an MIC for tetracycline of 2 μg/ml for *C. jejuni* ATCC 33560, whether read at 24 or 48 hours post inoculation. This result indicates that our experimental methodologies and techniques for MIC-based methods are valid and our results are accurate and precise.

The second experiment was performed to determine if the two available solvents (methanol and propylene glycol) had any inhibitory effect when tested as blanks in either an MIC or a disc diffusion method. The results indicated that both solvents did cause inhibition when tested neat but that the inhibitory effect was not detectable once the solvent was diluted 1 in 10.

In the solvent study, the 24 hour results were difficult to read due to the weak growth of the reference strains. This indicates that readings at 24 and 48 hours post inoculation should be performed when testing the plant compounds to ensure that no results are mis-read due to weak growth.

In vitro antimicrobial activity of grape extracts against poultry *Campylobacter* and *Clostridium* perfringens (first evaluation)

Introduction

In the previous section, the basic methodologies for the evaluation of the antimicrobial activity of grape extracts against bacteria have been established and validated. In this work, we have examined the antimicrobial activity of a range of grape extracts supplied by Tarac (Table 1) as well as pure reference chemical compounds (supplied by UQ) against a collection of poultry *Campylobacter jejuni* and *Clostridium perfringens.*

Bacteria

Campylobacter jejuni ATCC 33560, the strain recommended by the National Committee Clinical Laboratory Standards (NCCLS, 2002), was used in this work, along with the formal reference strain for *Campylobacter coli* ATCC 33559. We used the following *C. jejuni* isolates – C338, C627, C838, C858 and C1209, as supplied by Jillian Templeton (DPI&F) Queensland. The isolates have been obtained in prior RIRDC research projects (Miflin 2001; Templeton and Miflin, 2005) and represent different genotypes based on *fla*A restriction fragment length polymorphisms. The isolates were selected to represent the five most common genotypes seen in broilers in south-east Queensland. All *Clostridium perfringens* isolates used in this work are part of the DPI&F Queensland collection belonging to Dr Pat Blackall, Animal Research Institute.

Extracts, chemicals and antibiotic

A total of seven grape extracts were provided by Tarac and labelled as 136-2005, 178-2005F, 178-2005AXA, oligomeric GSE; polymeric acid; N05010 and monomeric GSE. Pure powders of caffeic acid, p-courmeric acid, syringic acid and vanillic acid were provided by Dr G. Kerven (UQ). Pure tetracycline powder was also used.

Preparation of stock solutions

Stock solutions of the various compounds listed above were prepared as recommended by Anhalt and Washington (1985). Distilled water was used as both the initial solvent and the diluent for 136-2005, 178-2005F, 178-2005AXA and oligomeric GSE; N05010, monomeric GSE and tetracycline. For the caffeic acid, p-courmeric acid, syringic acid, vanillic acid polymeric acid, the initial solvent was a 50:50 mix of methanol and distilled water. All compounds using the 50:50 methanol/water mix also required a round of sonication to ensure full solubilisation. A total weight (accurately recorded on an analytical balance) of pure powder was obtained. The volume of distilled water or methanol/distilled water that was added to the dry powder was calculated using the following formula:

$$\text{volume (ml)} = \frac{\text{weight (mg)} \times \text{potency (ug/mg)}}{\text{concentration (ug/ml)}}$$

Where:

weight (in mg) is the weight of antibiotic powder;
potency (in μg/mg) is marked by the manufacturer on the bottle;
concentration (μg/ml) is the desired concentration of stock solution.

The stock solution was then prepared by addition of the required volume of distilled water. A standard stock concentration of 1,280 μg/ml of tetracycline and 10 mg/ml of all other compounds was used in this study.

Performance of MIC assay

The assay was performed as described in the preceding chapter. The plates were prepared such that tetracycline was tested at final concentrations of 1, 2, 4 and 8 μg/ml. All other compounds were tested at final concentrations of 0.25, 0.5 and 1 mg/ml. The results are shown in Table 3.

Table 3. Results of MIC tests on grape extracts, pure compounds and tetracycline

Compound	Concentration (mg/ml)	*C. jejuni* ATCC 33560	*C. coli* ATCC 33559	Field *C. jejuni* (4 isolates)	*Cl. Perfringens* (7 isolates)
136-2005	0.25	+	+	+	+
	0.5	+	+	+	+
	1	+	+	+	+
178-2005F	0.25	+	+	+	+
	0.5	+	+	+	+
	1	+	+	+	+
178-2005AXA	0.25	+	+	+	+
	0.5	+	+	+	+
	1	+	+	+	V (6/7 +)
oligomeric GSE	0.25	+	+	+	+
	0.5	+	+	+	+
	1	+	+	+	V (6/7 +)
caffeic acid	0.25	-	-	-	+
	0.5	-	-	-	+
	1	-	-	-	+
p-courmeric acid	0.25	+	+	+	+
	0.5	-	-	-	+
	1	-	-	-	+
syringic acid	0.25	+	+	+	+
	0.5	-	-	-	+
	1	-	-	-	+
vanillic acid	0.25	+	+	+	+
	0.5	-	-	-	+
	1	-	-	-	+
polymeric acid	0.25	+	+	+	+
	0.5	+	+	+	+
	1	-	+	V (1/4+)	V (6/7 +)
N05010	0.25	+	+	+	+
	0.5	+	+	+	+
	1	+	+	+	+
Monomeric GSE	0.25	+	+	+	+
	0.5	+	+	+	+
	1	+	+	+	+
Skin extract	1/10[a]	-	-	-	-
	1/20	-	-	-	-
	1/40	-	-	-	+
	1/80	+	-	+	+
Tetracycline	0.25 µg/ml	ND	ND	ND	V (6/7 +)
	0.5 µg/ml	ND	ND	ND	V (5/7 +)
	1 µg/ml	+	+	V (1/4 +)	V (5/7 +)
	2 µg/ml	+	+	V (1/4 +)	V (5/7 +)
	4 µg/ml	-	-	V (1/4 +)	ND
	8 µg/ml	-	-	V (1/4 +)	ND

[a=] Skin extract was used as dilutions of the liquid material provided by Tarac

While readings were performed at 24 and 48 hours incubation, Table 3 shows the 48 hour reading only as there was no difference in the reading. Strain 1209 gave poor growth on the control plates (SBA) and has been excluded from the analysis.

Most of the grape extracts showed no inhibitory activity at the dilutions tested in this work. The extract termed polymeric acid did show inhibitory activity – *C. jejuni* ATCC 33560 and three of the four field isolates were inhibited at a concentration of 1 mg/ml of this extract. Polymeric acid was also affecting C*lostridium perfringens. C. coli* ATCC 33559 and one of the field isolates of *C. jejuni* were not inhibited by this concentration of this extract. Skin extract inhibited all type of microorganisms, although sulphur dioxide present in the skin extract solution could have affected results.

Of the four pure reference compounds tested, three (p-courmeric acid, syringic acid and vanillic acid) inhibited the two reference strains and the four field isolates at 1 and 0.5 mg/ml. Caffeic acid inhibited the two reference strains and the four field isolates at all three concentrations tested (1, 05 and 0.25 mg/ml). The *Campylobacter jejuni* ATCC 33560 strain gave the correct result for tetracycline of 2 μg/ml – confirming the acceptability of the assays.

Chapter 3: Liquid chromatography mass spectrometry evaluation of initial Tarac grape seed extracts

Introduction

A range of Grape seed extracts supplied by Tarac Technologies (Table 1) were concurrently evaluated for anti-microbial activity and were received for analysis by liquid chromatography mass spectrometry (LCMS) at the University of Queensland lab. The samples were dissolved in methanol/water mixtures and run on an LCMS system using a PDA and mass spectrometer detector. Details of the methods are included.

LCMS methodology

The samples were dissolved in methanol/deionised water mixtures to provide a solution concentration of 5 mg/mL. Where necessary, the solutions were filtered through a 0.45 µm nylon filter after wasting the first portion to minimise adsorption loss on the filter membrane. The samples were then run on the LCMS system using an established mobile phase and gradient used for phenolic acid/ flavonoid work. Samples were run with the PDA set for 280 nm (phenolic acids) and 340 nm (flavonoids). The mass spectrometer trace was post-processed for (M-H)- ions associated with the UV peaks to provide molecular weight identity and any characteristic fragment ions. It had been established that a Cone voltage of 34 V would result in significant fragmentation of glycosidic flavonoids and phenolic acids to yield the aglycone ion. In addition to the LCMS analysis, all samples were subjected to the butanol/HCl assay for condensed tannins. The results for this were normalised to the polymeric fraction after the LCMS UV traces showed that the sample was predominately proanthocyanidins (condensed tannins).

Results

The polymeric fraction chromatogram showed only small peaks for lower molecular weight catechins and broad late eluting peaks that are presumed to be proanthocyanidins. Under the mass spectrometer ionisation conditions, these did not ionise efficiently due to suppression by the formic acid. Other work has shown that it is necessary to add ammonia post-column to raise the pH and ionise the larger MW compounds. Multiple charging is then a problem in identifying the (M-H)- ions.

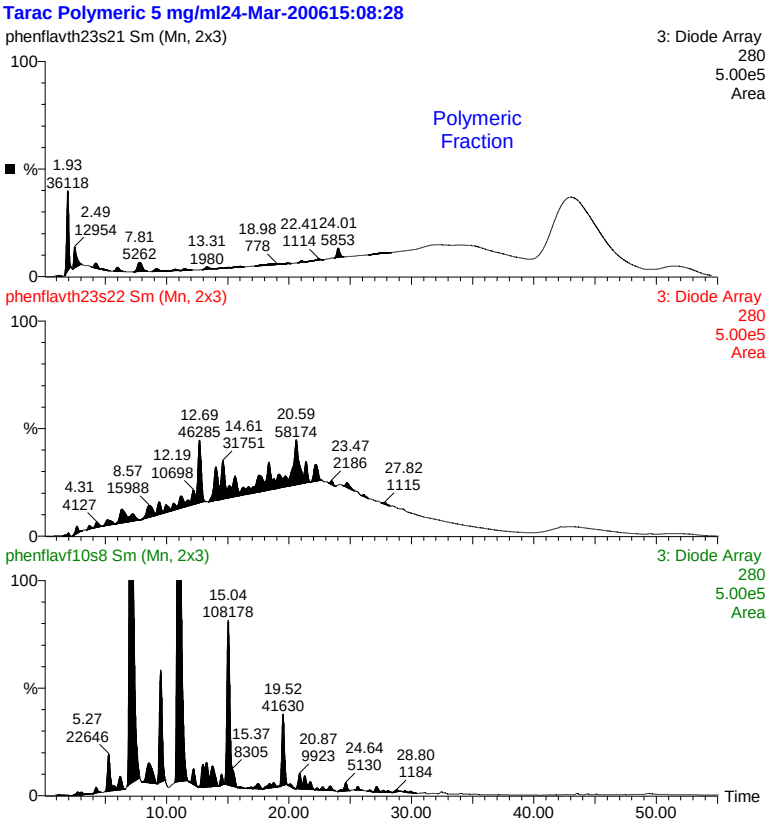

Figure 1. Chromatograms for Tarac purified fractions of Grape Seed Extract. UV traces @ 280 nm for phenolics. Broad peaks eluting after 25 min in Oligomeric and Polymeric fractions probably higher DP Proanthocyanidins

Table 1. Peak list table for major $(M-H)^-$ ions in Monomeric fraction

RT (min)	Area	Area %	$(M-H)^-$ Ions	MW	Name
4.253	2892.293	0.24			
5.27	19013.795	1.61	577+483+425	578	Procyanidin B
6.22	8341.034	0.71	577+425+137	578	Procyanidin B
7.07	417520.375	35.35	289	290	Catechin
8.553	15801.338	1.34			
8.687	10154.014	0.86			
9.52	65001.82	5.50	577+425	578	Procyanidin B
10.953	395247.938	33.47	289	290	Epicatechin
12.203	9397.237	0.80			
12.97	13795.327	1.17			
13.27	17879.609	1.51			
13.753	15190.751	1.29			
13.92	4510.811	0.38			
14.487	5612.913	0.48			
15.037	91254.477	7.73	729+577+425	730	
17.47	2341.753	0.20			
18.203	1020.642	0.09			
18.403	2736.633	0.23			
18.753	2577.027	0.22			
19.52	40174.531	3.40	441	442	Epicatechin gallate
20.087	1674.065	0.14			
20.87	9739.468	0.82	729+441	730	Epicatechin gallate glycoside
21.287	8772.987	0.74	463+449	464	
21.737	5024.93	0.43			
22.737	1839.676	0.16			
23.353	3022.57	0.26			
24.637	4276.054	0.36	439+209	440	
25.62	1771.683	0.15			
27.153	3309.587	0.28			
28.803	1132.134	0.10			

Table 2. Normalised values for Butanol/HCl estimation of Condensed Tannin content

Sample ID	mg sample	A 550 nm	CT (Normalised)
Monomeric fraction	3.98	0.468	33.9
Oligomeric fraction	4.13	1.233	86.1
Polymeric fraction	5.52	1.915	100
N05010	5.68	1.14	57.9
136-2005	7.37	0.732	28.6
178-2005f	5.2	0.913	50.6
178-2005 XAD	4.26	1.091	73.8

Note: Samples dissolved in methanol reacted with BuOH/HCl reagent @ 95°C 15 min, measured at 550 nm. Values normalised on mg/mg to Polymeric fraction.

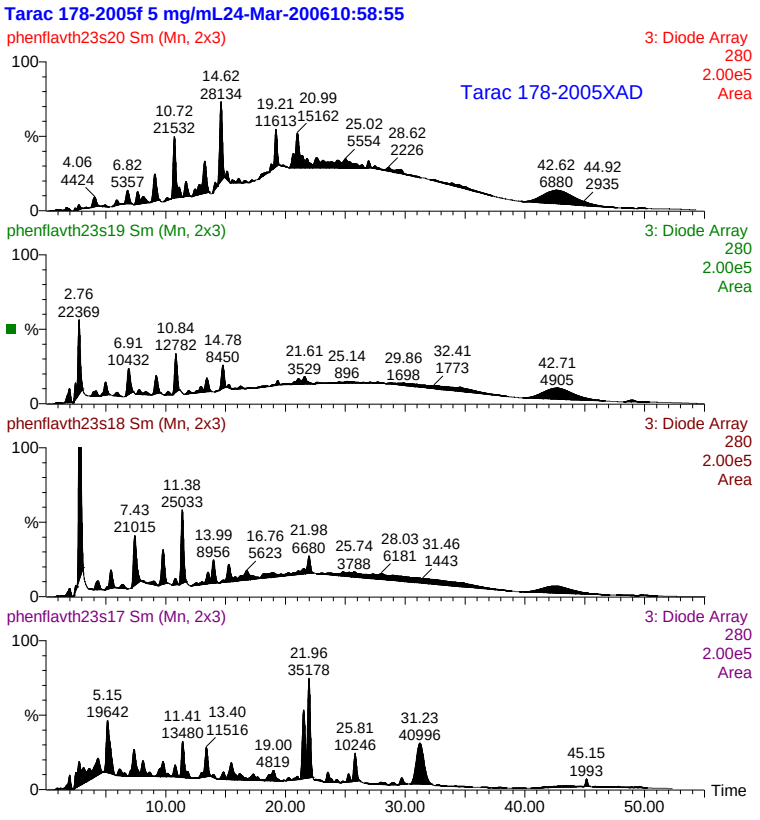

Figure 2. Chromatograms of Tarac Grape Seed Extracts (Freeze dried). UV traces @ 280 nm for phenolics.

Table 3. $(M-H)^-$ ions associated with selected UV peaks in Tarac extracts

Sample ID	RT 280 nm	RT 340 nm	$(M-H)^-$ ions	Name
178-2005XAD	19.21		441+289+169	Epicatechin gallate
		21.14	523+463+449	
		21.66	729+577+477+301	
178-2005f				
N05010	2.83		169	Gallic acid
	5.43		577+425+289	Procyanidin B
	7.45		289	Catechin
	9.78		577+425+289	Procyanidin B
	11.38		289	Epicatechin
	15.28		729+577+289	Procyanidin ?
		21.56	463	Flavonoid
		21.98	477+301	Flavonoid
		25.71	433+285	Flavonoid
136-2005	13.40		366	Phenolic acid
	21.53		463+449	Flavonoid
	21.96		477+301	Flavonoid
	25.81		433	Phenolic acid
	31.23		285	Luteolin or Kaemphferol

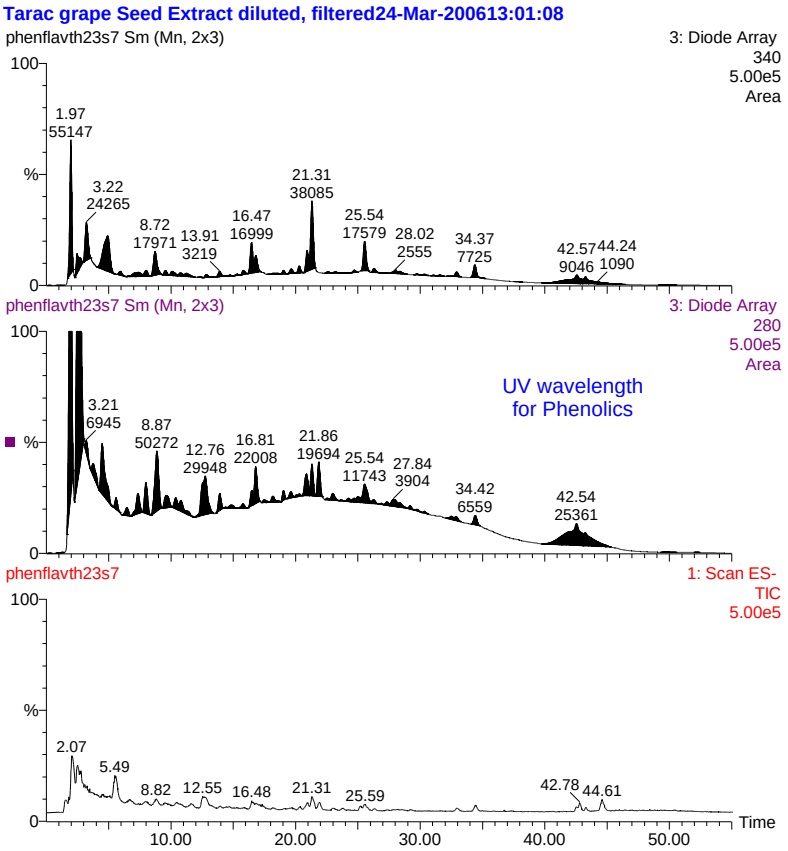

Figure. 3 Chromatograms for crude Grape Seed Extract @ 280 nm (phenolics) and 340 nm (flavonoids).

Table 4. Peak list for Crude Grape seed extract (UV 340 nm trace)

RT (min)	Area	Area %	$(M-H)^-$ ions	MW	Name
2.69	4912.708	1.97			
3.223	24264.627	9.75	616+275	617	
4.807	23225.883	9.33			Weak ESI- ionisation
4.907	25359.639	10.19			Weak ESI- ionisation
5.923	2479.101	1.00			
7.207	1788.311	0.72			
7.373	3335.042	1.34			
8.007	4114.676	1.65			
8.723	17970.91	7.22	577	578	Procyanidin B?
9.557	2588.776	1.04			
10.09	2139.584	0.86			
10.207	1487.909	0.60			
10.773	2462.897	0.99			
11.24	3410.926	1.37			
12.84	1649.353	0.66			
13.907	3218.517	1.29			
15.823	2423.444	0.97			
16.473	16999.457	6.83	509+479	510	
16.807	10518.116	4.23	559+515+353	560	Chlorogenic acid glycoside?
19.023	1669.485	0.67			
19.657	2870.854	1.15			
20.29	3762.125	1.51			
20.923	10897.241	4.38	463+347	464	
21.307	38085.453	15.31	477+301	478	Ellagic acid glycoside?
24.723	1244.792	0.50			
25.54	17579.23	7.06	507+477+227	508	
26.29	2594.096	1.04			
28.023	3047.328	1.22			
28.373	1991.18	0.80			
32.923	3009.056	1.21			
34.373	7725.21	3.10	301	302	Ellagic acid?

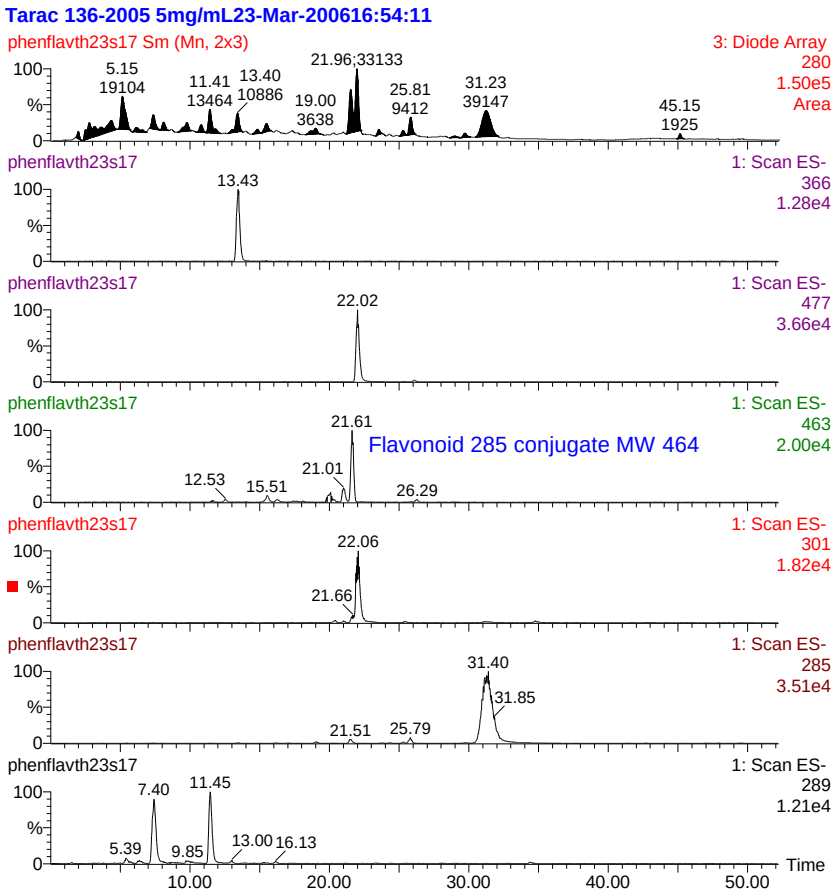

Figure 4. Extracted ion traces for sample 136-2005. (M-H)- ions extracted from TIC.

Discussion

The chromatograms (Fig. 1) and BuOH/HCl assay (Table 2) show that the polymeric and oligomeric are dominated by the presence of proanthocyanidins in fractions. Catechins identified as Catechin, Epichatin, Epicatechin gallate and Epigallocatechin gallate (Table 1), however, dominate the monomeric fraction. Broad, unresolved peaks present in the chromatograms were presumed to be proanthocyanidins but poor ionisation prevented their MW identity to be established. Direct injection of the fractions into the mass spectrometer ESI interface, after being made alkaline with ammonium hydroxide, which had been shown by Lazarus *et al* (1999) to favour their ionisation in negative mode, did show ions at m/z values in the range expected from other work, but the absence of compound separation by chromatography prevented any clear identity to be established.

The broad, hump-shaped peaks present in the chromatograms (Fig. 2) for the 2005 extracts showed a relative size in agreement with the normalised CT values (Table 2) obtained by BuOH/HCl assay, indicating these broad peaks are also probably associated with proanthocyanidins. Table 3 lists selected UV peaks for the chromatograms for the 2005 extracts, where clearly identified (M-H)- ions permitted association of TIC peaks with UV peaks. Where a general class name of phenolics or flavonoids has been assigned, this is on the basis of a strong adsorption band at 340 nm normally associated with flavonoids but not phenolic acids. Initial work to identify the UV peaks in sample 136-2005 is shown in Fig. 4, where marker ions have been extracted from the TIC trace and used in conjunction with the UV spectral information to provide probable identifications of the UV peaks.

Following the initial study by Pat Blackall which showed no anti-microbial for the Tarac samples, but similar activity for a range of phenolic acids (Caffeic, p-coumaric, syringic and vanillic acids) to that shown by Aziz *et al* (1998), all of the TIC traces for the Tarac samples were then subjected to

extracted ion analysis for the marker ions of these phenolic acids. The results (Table 5) show that the marker ions (M-H)-) for these phenolic acids are present in a number of the samples. However, in no case were the marker ions associated with large UV peaks at 280 nm, the wavelength used to quantify phenolic acids.

Table 5. Qualitative estimates for the presence of phenolic acids in Tarac samples

Sample ID	p-Coumaric acid	Caffeic acid	Syringic acid	Vanillic acid
Monomeric fraction	+	-	+	-
Oligomeric fraction	-	-	-	-
Polymeric fraction	-	-	-	+
136-2005	+	-	-	-
N05010	+	+	+	-
178-2005f	-	+	-	-
178-2005 XAD	+	+	+	-

This suggests that while the phenolic acids that have been shown in this work to have anti-microbial activity present, their concentrations are either too low to be effective or their effects are being masked by the presence of higher concentrations of other compounds.

Conclusion

The analysis of grape seed extracts by LCMS provides a useful tool to aid in the identification of unknown peaks. In the Electrospray negative ion mode, both the phenolic acids and flavonoids ionise relatively efficiently without significant suppression at 0.1% formic acid or the formation of adduct ions. Operating the ion source at a Cone voltage of 34 V was shown to fragment the parent ions into the associated aglycones where the original compounds are present as sugar conjugates. Using the extracted ion method, known marker ions can be easily identified in the TIC ion trace.

For the higher molecular weight proanthocyanidins, the present chromatography gives poor separation and the presence of 0.1% formic acid suppresses their ionisation. The post-column addition of ammonium hydroxide is necessary to raise the pH for effective ionisation. However, multiple charged ions can then present a problem in assigning the m/z values to (M-H)- parent ions.

Summary of in vitro work and characterisation of phenolics (first evaluation)

During May 2006, a meeting was held between Tarac, UQ, ARI, PRDC and CRC representatives to evaluate the initial work performed during 2005. A summary of the main aspects are presented as follows:

In vitro evaluation (ARI, Pat Blackall)

- *In vitro* methodologies for the evaluation of antimicrobial activity of grape extracts against bacteria were established and validated.
- Reference phenolic acids (caffeic acid, p-courmeric acid, syringic acid supplied by UQ, Graham Kerven) were useful as they showed activity against gram negative bacteria.
- Only polymeric acid at 1 mg/ml exhibited activity for some *C. jejuni* and was affecting *Clostridium perfringens*. It was therefore recommended to increase the concentration of polymeric acid fraction in the study using 10, 5, 2.5, and 1.25.
- Skin extract inhibited both types of microorganisms, but sulphur dioxide present could have affected results. Thus, the need to evaluate skin extract free of sulphur dioxide (TARAC needs to produce and provide new skin extract without sulphur) .

Characterision of phenolic fractions (UQ, Graham Kerven)

- The initial samples provided by Tarac exhibited large amounts of proanthocyanidins (condensed tannins) which are mainly in the form of polymers and oligomers.
- It was also shown that Cathechins, Epicatechin gallate and others dominate the monomeric fractions.
- Examples were given in which LCMS analysis provided molecular weight of peaks that assisted in the identification of some of the compound present.
- It was indicated there was a need for further purification steps on relevant fractions to improve resolution.
- It was indicated that to the SO_2-free extract polymeric fraction would be further purified and fractionated using ethyl acetate (EA). This will allow us to check effectiveness on microbial inhibition by fractionating it into two fractions - the EA extractable phenolics and the non-EA extractable (including condensed tannins) to produce at least three fractions to test: the original extract, the EA fraction and the no-EA extractables.

TARAC summary of major aspect (Marina Shulz)

- The need to provide a new batch of skin extract without SO_2 for antimicrobial testing.
- Provision of polymeric acid fraction for further evaluation at higher concentrations.
- Discussions were made regarding delivery of olive pomace material from Adelaide University.

Chapter 4: Fractionation, preparation and identification of additional Tarac grape seed extracts and olive pomice

Introduction

Following the meeting held on May 9th 2007, it was agreed that Tarac would supply additional quantities of the Polymeric fraction that had shown some anti-microbial activity in the first tests, as well as a Red skin extract free of sulphur dioxide (SO_2) to eliminate this as the compound responsible for some anti-microbial activity demonstrated in the first tests.

The proposal was for the University of Queensland to fractionate the polymeric fraction to remove the small quantity of phenolics present in the material from the bulk of the condensed tannins for further testing and to fractionate the SO_2 free Red skin extract into various fractions for testing.

Methodology

Polymeric Fraction

The polymeric fraction was dissolved in 40% methanol at a concentration of 50 mg/mL, filtered through a 0.45 µm nylon filter and loaded onto a Sephadex LH-20 column (2.6 x 20 cm) in 5 mL batches and eluted for 80 min with 40% methanol to elute the phenolics followed by 70% acetone for 40 min to elute the retained condensed Tannins (CT). The two fractions were then Rotary Evaporated and finally vacuum evaporated to provide:-

i) phenolic and
ii) CT fractions.

Red Skin Extract

The red skin extract was adjusted to 70% with acetone, sonicated for 30 min, filtered through a GF/A filter and Rotary Evaporated to remove the acetone and concentrate the water solution by a factor of x2. The extract was then fractionated following the method of Kader *et al* (1995). The extract was acidified to pH<2 with HCl, extracted five times with Ethyl acetate and the extracts combined. The combined extract was then back extracted with 5% $NaHCO_3$ five times to remove the phenolics. The ethyl acetate fraction was vacuum evaporated to yield the flavonoid fraction. The $NaHCO_3$ fraction was then re-acidified with HCl to a pH <3 and again extracted four times with ethyl acetate (50 mL). The combined ethyl acetate was vacuum evaporated to yield the phenolic fraction.

The results of the fractionation steps showed that the polymeric fraction comprised 93.7% condensed tannins with the remainder as phenolics. The red skin extract had a flavonoid content of 2.8 mg/mL and a phenolics content of 8 mg/mL.

Evaluation on fractionated Red Skin Flavonoids

The crude flavonoid fraction obtained by the procedures previously outlined was then subjected to Size Exclusion Chromatography on Toyopearl HW-WF to further fractionate the classes of compounds present. This process provided two groups of compounds classified as purified flavonoids and condensed tannins based on previous work (Perez-Maldonado and Norton, 1996) using this fractionation process for plant extracts.

Methodology

1. The crude flavonoid fraction was dissolved in 40% methanol at a concentration of 100 mg/mL, filtered through a 0.45 µm nylon filter and loaded onto a Toyopearl HW-40F column (2.6 x 20 cm) in 5 mL batches and eluted for 60 min with 40% methanol to elute the flavonoids followed by 70% acetone for 40 min to elute the retained condensed Tannins (CT). The two fractions were then Rotary Evaporated and finally vacuum evaporated to provide the i) purified falvonoids and ii) CT fractions.
2. A portion of the purified fraction was then subjected to acid hydrolysis to cleave the glycosidic bonds and provide the free aglycones for analysis.
3. The fractions were then analysed by HPLC after a range of common red grape compounds were run to obtain their retention times.

Results

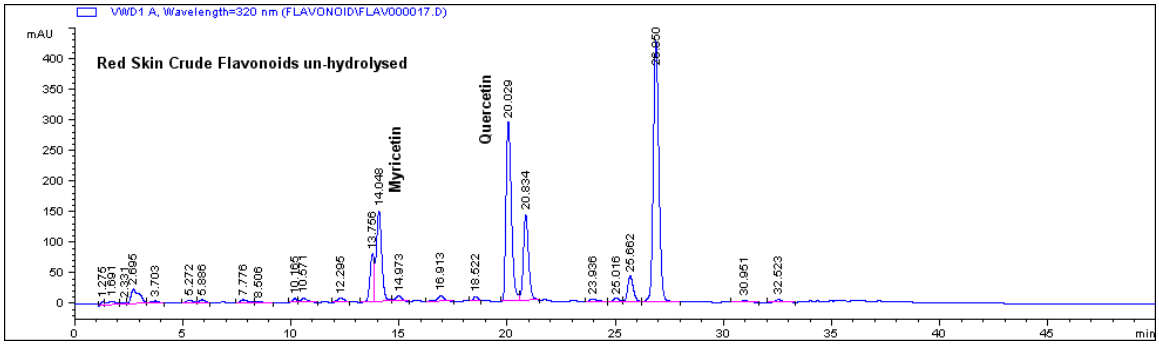

Figure 1. Chromatogram of Red Skin Crude Flavonoids un-hydrolysed

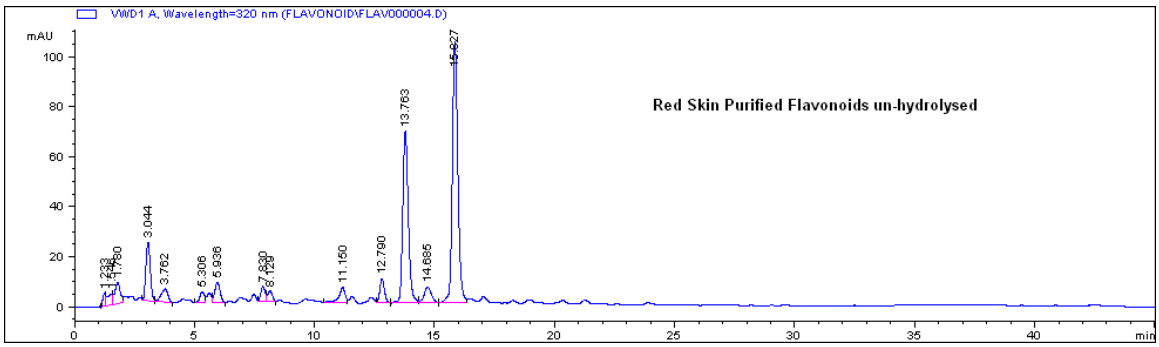

Figure 2. Chromatogram of Red Skin Purified Flavonoids un-hydrolysed

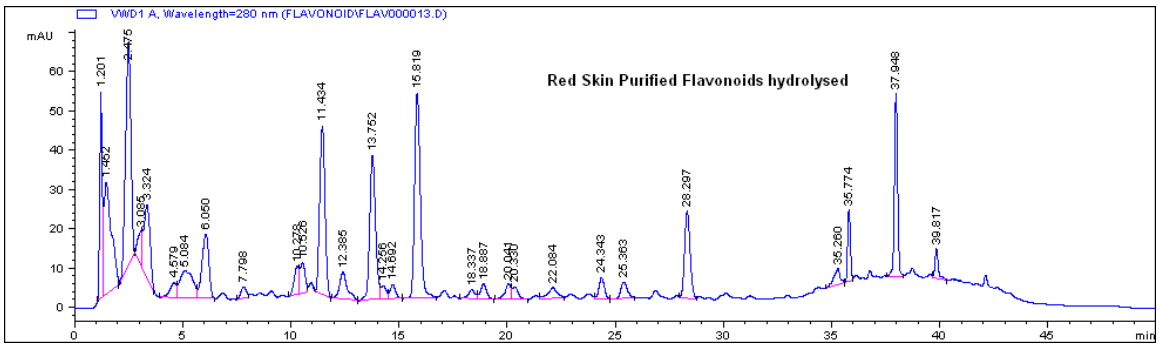

Figure 3. Chromatogram of further fractionated Red Skin Purified Flavonoids after acid hydrolysis

HPLC analysis of the flavonoid fraction obtained by solvent extraction showed the presence of quercetin and myricetin as the free aglycones in the crude fraction which had not been subjected to further separation (Fig. 1). Fractionation of this by SEC on Toyopearl HW-40F and HPLC analysis of the methanol eluent where the flavonoids were expected to be present however did not show the presence of quercetin or myricetin (Fig. 2). Acid hydrolysis of the methanol fraction to break the glycosidic bonds and release the free aglycones did show the presence of a small peak that was identified as quercetin (Fig. 3). The results of the HPLC analysis are summarized in Table 1.

Table 1. Presence of compounds identified to be present in various red skin flavonoid fractions

Compound	RT (min)	Crude flavonoids	Unhydrolysed Purified flavonoids	Hydrolysed purified flavonoids
Catechin	2.55	-	-	-
Epicatechin	4.07	-	-	-
Rutin	10.48	-	-	-
Myricetin	14.04	++	-	-
Resveratrol	15.02	-	-	-
Quercetin	20.02	++	-	trace
Unknown peaks				
RT (min)	2.48	-		+
	3.04	-	+	Trace
	6.05	-		+
	11.43	-		+
	13.75	++	++	++
	15.82	Trace	++	++
	20.83	+		-
	25.66	+		-
	26.85	+++		Trace
	28.3	-		+
	35.77	-		+
	37.95	-		+

HPLC analysis of the crude flavonoid fraction obtained by solvent extraction with ethyl acetate, and previously tested to show antimicrobial activity, indicated the presence of the two common flavonoids quercetin and myricetin (McDonald et al 1998) as well as other unidentified peaks. Fractionation of this by SEC using an established procedure that separates the flavonoids from the condensed tannins di not however show the presence of these two flavonoids in the expected methanol eluent fraction despite many large peaks being present in the chromatogram. Acid hydrolysis of this did reveal a small peak for quercetin indicating that it was present as a glycosidic form not as the free form present in the original extract.

The acetone eluent fraction expected to represent the condensed tannins did show a positive response with the butanol/HCl colorimetric test but not a strong colour. This suggests that these flavonoids were strongly retained and not eluted until the mobile phase was changed to acetone/water. A subsequent test on the Toyopearl column with quercetin as a marker compound showed that it was retained at the head of the column and did not elute until the solvent was changed to acetone/water at 60 min and then eluted at 81 min during the period when the brown colored condensed tannins also eluted. Myricetin would be expected to behave in a similar manner.

Fractions obtained from grape extracts

For anti-microbial testing the following fractions were produced:

1) Polymeric fraction as supplied
2) Condensed tannin fraction from polymeric fraction
3) Phenolic fraction from polymeric fraction
4) Red skin extract as supplied (nil SO_2)
5) Water fraction of Red skin extract after acetone solvation and filtering and extraction with ethyl acetate
6) First ethyl acetate extract fraction (phenolics + flavonoids)
7) Phenolic fraction
8) Flavonoid fraction

Olive Pomace Fractions

The olive pomace fractions were prepared by The University of Adelaide (Dr Graham Jones). The material arrived at Poultry Research and Development Centre in September 2006.

Four samples (1 g each) of phenolic extracts from olive-derived materials were received for antimicrobial testing. The material was described as follow:

1) OLI-2322-1B - Mixture of total phenolics from olive leaf (approx 1g) . This material is 100% phenolic material in aqueous methanol at a concentration of 25% w/v. The methanol concentration is nominally 60% v/v.

2) OL2-2322-1B - purified oleuropein (approx 1g). This material is soluble in water but more so in aqueous methanol or aqueous ethanol (add alcohol and dilute with water to the required alcohol concentration).

3) OW1-2322-1B - Mixture of total phenolics from olive mill pomace (approx 1g). This material is 100% phenolic material in aqueous methanol at a concentration of 25% w/v. The methanol concentration is nominally 60% v/v.

4) OW2-2322-1B - Mixture of total phenolics from olive mill black water. This material is 100% phenolic material in aqueous methanol at a concentration of 25% w/v. The methanol concentration is nominally 60% v/v.

Chapter 5: In vitro antimicrobial activities in additional grape seed extracts and olive pomace fractions against poultry *Campylobacter* and *Clostridium perfringens*

The grape extracts compounds provided by the University of Queensland (Graham Kerven) and the University of Adelaide (Graham Jones) have been tested against the same set of *Campylobacter jejuni/coli* strains and *Clostridium perfringens* isolates used in the initial screening.

Methods

The same methodology was used in the current work as in the previous work. Essentially, the approach is to prepare the test samples at a standard concentration. The samples are then diluted and agar plates poured with these dilutions incorporated into the agar. Standardised overnight cultures of the test organisms are spotted onto the agar plates and the plates incubated. Various controls, including tetracycline containing plates, solvent containing plates as well as blank plates (*i.e.*, agar with no additional test compound) are used. The agar we use is the standard agar used for antimicrobial testing. The *Campylobacter* work was performed in the week beginning the 4th December 2006 and the *Clostridium* work in the week beginning the 11th December 2006.

Campylobacter work

A. Based on advice received, the compounds were all prepared as a 10 mg/ml stock solution using 70% methanol.

B. For Tarac Red Skin, Flavonoid Fraction and Tarac Red Skin, Phenolic Fraction, the weight provided on the label had to be used as the material could be weighed.

C. For Tarac Red Skin, Polymeric Phenolics, there was no labelled weight. Hence, this compound was simply dissolved in the minimum amount of 70% methanol that would allow both the *Campylobacter* and *Clostridium* work to be performed.

D. All compounds were tested at a 1/10, 1/20 and 1/40 dilution (final concentration in the agar plate) meaning that the concentrations tested were 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml.

E. Control tests using tetracycline (final concentrations of 1, 2, 4, and 8 μg/ml) were included.

F. A further control of a blank solution that contained only the solvent (70% methanol) was used. In these methanol controls, the final plates contained 7%, 3.5% and 1.75% methanol (in the 1/10, 1/20 and 1/40 dilution series).

Clostridium work

A. The following compounds were re-used from the *Campylobacter* work (ie the same stock solution of 10 mg/ml in 70% methanol) – Tarac Red Skin, Flavonoid Fraction; Tarac Red Skin, Phenolic Fraction; Tarac Red Skin, Polymeric phenolics.

B. As the *Campylobacter* results were available by the time of this work, the compounds were prepared with either no methanol or as little methanol as possible.

C. The following compounds had no methanol used Tarac Red Skin, Bulk; Tarac Red Skin, Water Fraction; Univ Adelaide OL1-2322-1B; Univ Adelaide OW1-2322-1B and Univ Adelaide OW2-2322-1B.

D. The remaining compounds had 28% methanol in the stock solution:- Tarac Red Skin, Polymeric Fraction and Univ Adelaide OL2-2322-1B.

E. Tarac Red Skin, Flavonoid and Phenolics stock had 24% methanol.

Results

The detailed results are shown in Table 1.

Table 1. Results of MIC tests on grape extracts, olive extracts, methanol and tetracycline

Compound	Concentration (mg/ml)	*C. jejuni* ATCC 33560	*C. coli* ATCC 33559	*C. jejuni* (5 isolates)	*Cl. perfringens* (7 isolates)
Tarac Red Skin Bulk	0.25 0.5 1	+ + -	+ + -	+ V (3/5 +) -	+ + +
Tarac Red Skin Water Fraction	0.25 0.5 1	+ + -	+ + -	+ V (3/5 +) -	+ + +
Tarac Red Skin Flavonoid and	0.25 0.5	+ -	+ -	V (3/5 +) -	+ +
Phenolic	1	-	-	-	+
Tarac Red Skin Flavonoid	0.25 0.5 1	- - -	- - -	- - -	V (5/7 +) - -
Tarac Red Skin Phenolic	0.25 0.5 1	+ + -	+ + -	+ V (2/5 +) -	+ + +
Tarac Red Skin Polymeric	0.25 0.5 1	+ + -	+ + -	+ V (3/5 +) -	+ + V (6/7 +)
Tarac Red Skin Condensed Tannins	0.25 0.5 1	+ + -	+ + -	+ V (3/5 +) -	+ + V (6/7 +)
Tarac Red Skin Polymeric Phenolics	0.25?? 0.5?? 1??	+ + -	+ + -	V (3/5 +) V (3/5 +) -	+ + +
Univ Adelaide OL1-2322-1B	0.25 0.5 1	+ + -	+ + -	V (3/5 +) V (2/5 +) -	+ + +
Univ Adelaide OL2-2322-1B	0.25 0.5 1	+ - -	+ - -	+ - -	+ + +
Univ Adelaide OW1-2322-1B	0.25 0.5 1	+ + -	+ + -	+ V (3/5 +) -	+ + +
Univ Adelaide OW2-2322-1B	0.25 0.5 1	+ + -	+ + -	+ V (2/5 +) -	+ + +
Tetracycline	0.25 µg/ml 0.5 µg/ml 1 µg/ml 2 µg/ml 4 µg/ml 8 µg/ml	ND ND + + - -	ND ND + + - -	ND ND V (2/5+) V (2/5+) V (2/5+) V (2/5+)	V (5/7 +) V (5/7 +) V (5/7 +) V (5/7 +) ND ND
Methanol 70%	1/40 1/20 1/10	+ + -	+ + -	+ + -	+ + +

+ indicates growth; - = no growth

Comments - *Campylobacter*

For the *Campylobacter* work, the methanol blank plates showed inhibition of all strains in the 1/10 dilution. This means that all inhibitions detected in the test compounds at 1 mg/ml are due to the inhibitory effect of the solvent. Hence, only the 0.5 and 0.25 mg/ml plates (where the matching solvent plates showed no effect from the methanol) can be regarded as showing specific effects due to the compound test. The tetracycline results were exactly as expected and thus validated the overall testing technology.

The Tarac Red Skin, Flavonoid fraction inhibited all strains of *Campylobacter* at 0.5 and 0.25 mg/ml concentration. The next most effective compound was the Tarac Red Skin, Flavonoid and Phenolics fraction – with most, but not all strains being inhibited at the highest dilution (0.25 mg/ml) tested.

The most active of the olive extracts was the OL1-2322-1B fraction – although the level of activity was less than the best performed of the red skin extracts.

Comments - *Clostridium*

For the *Clostridium* work, the methanol blank plates showed no inhibition of any strains at any dilution. This means that all inhibitions detected in the test compounds are due to the specific inhibitory effect of the test compound. The tetracycline results were exactly as expected and thus validated the overall testing technology.

The only compound to show any major activity was the Tarac Red Skin, Flavonoid fraction which inhibited all strains of *Clostridium perfringens* at 1.0 and 0.5 mg/ml concentrations and 2/7 strains at the 0.25 mg/ml concentration.

Future *in vitro* work

Based on the above results, it would be logical to obtain more of the Tarac Red Skin, Flavonoid fraction and complete the dilution work (*i.e*., test greater dilutions until an end point is reached - minimum inhibitory concentration - with all strains of both *Campylobacter* and *Clostridium perfringens*). We no longer have sufficient material to do any further testing with the remaining amount of this fraction.

Chapter 6: *In vitro* antimicrobial activity of Tarac red skin flavonoid fractions against poultry *Campylobacter* and *Clostridium perfringens*

Introduction

Previous work has demonstrated that the only compound that has shown any major activity on all bacteria tested was the Tarac Red Skin, Flavonoid fraction, which inhibited all strains of *Campylobacter* (at concentrations 0.25, 0.5 and 1 mg/ml) and *Clostridium perfringens* (at 1.0 and 0.5 mg/ml concentrations and 2/7 strains at the 0.25 mg/ml).

It was suggested that further testing using red skin flavonoid fraction at lower dilutions was needed, using greater dilutions, until an end point is reached with both *Campylobacter* and *Clostridium perfringens* isolates used in the initial screening. As well, a collection of *Salmonella enterica* isolates (each a different serovar from a different broiler farm) would be tested against this promising flavonoid fraction.

Preparation of red skin extract to produce phenolic acid and flavonoid fractions (University of Queensland)

Initial sample clean up

The red skin extract requires filtration to remove insoluble material adjustment with acetone to the equivalent of a standard 70% acetone extraction and vacuum concentration to remove the acetone and any sulphur dioxide (Note: pH was low enough to ensure removal of any SO_2).

Measure out 500 ml of the extract and add acetone to make a solution of 70% acetone strength. Using a combination of centrifugation (3000 rpm, 10 min, 16 °C) and filtration through GF/A filters remove all insoluble material. Now concentrate the extract on a Rotary Evaporator to a volume of approx 300 ml.

Extraction of combined Phenolics + Flavonoids

Acidify the concentrate to a pH <3 with HCl in two separate batches. Now extract each batch with 50 ml of ethyl acetate five times by transferring to a 500 ml Separating Funnel, adding the solvent, capping, shaking for 1 min and allowing for phase separation. Combine each portion of solvent and then centrifuge this with some added water in 50 ml Falcon tubes to ensure complete phase separation.

Back extraction of phenolic acids

Transfer the ethyl acetate to a clean 500 ml Separating Funnel and add 50 ml of 5% NaHCO3. Shake for 1 min and then allow for phase separation. Separate the phases into two portions, the alkali water phase contains the phenolic acids and the ethyl acetate phase the flavonoids. Repeat this step a total of five times combining the aqueous phase. Again centrifuge each phases with the addition of the opposite solvent to ensure complete phase separation.

Extraction of Phenolic acids

Transfer all of the alkali extract to a 500 ml beaker and place on the magnetic stirrer in the Fume Cupboard. Now slowly add HCl using a pH strip test to bring the pH to <3. Now transfer this in two batches to a clean 500 ml Separating Funnel and extract five times with 50 mL of ethyl acetate. Again add water and centrifuge this to ensure complete separation. Now concentrate this to a volume of <20 ml on the Rotary Evaporator. This is then transferred to a dried weighed 20 ml glass vial(s) and vacuum dried to a constant weight. Record the yield of phenolic acids. Store at 5 °C.

Concentration of Flavonoids

Rotary Evaporate the ethyl acetate solvent that contains the flavonoids after back extraction with 5% NaHCO3 and washing with water to remove traces of alkali to a volume of <20 ml. Transfer this to dried weighed 20mL glass vials and vacuum dry to a constant weight. Record the yield of flavonoids. Store at 5 °C.

Combined Phenolics/Flavonoids

Rotary evaporate an exact portion ($1/5^{th}$) of the original ethyl acetate extract to < 20ml and again finally dry in a 20 ml glass vial under vacuum. Record the yield of combined phenolics/flavonoids. Store at 5 °C.

Safety Precautions:

1. Wear plastic gloves and a face mask when adding concentrated HCl to samples.
2. Perform all organic solvent operations in the Fume Cupboard in N124.
3. Wear Safety glasses when undertaking all vacuum operations.

Fractions Yield

Combined fraction	3.95g/100mL
Phenolic fraction	1.24g/100mL
Flavonoid fraction	0.305g/100mL

Comments on methodologies for bacteria *in vitro* evaluations

The same methodology, as outlined in previous work, was used in the current work. Essentially, the approach is to prepare the test samples at a standard concentration. The samples are then diluted and agar plates poured with these dilutions incorporated into the agar. Standardised overnight cultures of the test organisms are spotted onto the agar plates and the plates incubated. Various controls, including tetracycline containing plates, solvent containing plates as well as blank plates (*i.e.*, agar with no additional test compound) are used. The agar we use is the standard agar used for antimicrobial testing. The formal description of the methodologies is provided in the Clinical Laboratory Standards Institute documentation (NCCLS 2002).

Details of dilution range

- Based on advice received (Uni of Qld), the compounds were all prepared as a 10 mg/ml stock solution. The initial dilution was in 3 ml of methanol with the remaining volume consisting of sterile distilled water.
- All compounds were tested at a 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640 dilution (final concentration in the agar plate) for the *Campylobacter* cultures. This means that the concentrations tested were 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.03125 mg/ml and 0.01562 mg/ml. For the *Clostridium* and *Salmonella* isolates, a smaller dilution range was tested - 1/20, 1/40, 1/80 and 1/160 (final concentration in the agar plate). This means that for the

Clostridium and *Salmonella* isolates, the concentrations tested were 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml.

- Control tests using tetracycline (final concentrations of 1, 2, 4 and 8 μg/ml) were included.
- A further control of a blank solution that contained 15% methanol (the final concentration in the stock solutions) was used. In these methanol controls, the final plates contained 1.5% and 0.75% methanol (in a 1/10 and 1/20 dilution series).

In vitro *work results*

The detailed results of the Tarac red skin fractions against *Campylobacter* and *Clostridium perfringens* strains are shown in Table 1. The results for the *Salmonella* strains are shown in Table 2.

Table 1. Results of MIC tests on grape extracts methanol and tetracycline – *Campylobacter* and *Clostridium perfringens*

Compound	Concentration (mg/ml)	C. jejuni ATCC 33560	*C. coli* ATCC 33559	C. jejuni (5 isolates)	Cl. perfringens (7 isolates)
Phenolics and	0.01562	+	+	+	ND
Flavonoids	0.03125	+	+	+	ND
	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	+	+	+	+
	0.5	+	+	+	+
Flavonoids	0.01562	+	+	+	ND
	0.03125	+	+	+	ND
	0.0625	+	+	+	+
	0.125	+	+	V (4/5+)	+
	0.25	-	-	-	+
	0.5	-	-	-	V (1/7+)
Phenolics	0.01562	+	+	+	ND
	0.03125	+	+	+	ND
	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	+	+	+	+
	0.5	+	+	+	+
Anthocyanine	0.01562	+	+	+	ND
	0.03125	+	+	+	ND
	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	+	+	+	+
	0.5	+	+	V (4/5+)	+
Tetracycline	0.25 μg/ml	ND	ND	ND	V (4/7 +)
	0.5 μg/ml	ND	ND	ND	V (5/7 +)
	1 μg/ml	-	+	V (2/5+)	V (5/7 +)
	2 μg/ml	-	+	V (2/5+)	V (5/7 +)
	4 μg/ml	-	-	V (2/5+)	ND
	8 μg/ml	-	-	V (2/5+)	ND
Methanol 15%	1/20	+	+	+	+
	1/10	+	+	+	+

+ indicates growth; - = no growth; ND = Not Done

Table 2. Results of MIC tests on grape extracts methanol and tetracycline – Salmonella

Compound	Concentration (mg/ml)	*E. coli* ATCC 25922	Salmonella (6 isolates)
Phenolics and	0.01562	ND	ND
Flavonoids	0.03125	ND	ND
	0.0625	+	+
	0.125	+	+
	0.25	+	+
	0.5	+	+
Flavonoids	0.01562	ND	ND
	0.03125	ND	ND
	0.0625	+	+
	0.125	+	+
	0.25	+	+
	0.5	-	V (2/6+)
Phenolics	0.01562	ND	ND
	0.03125	ND	ND
	0.0625	+	+
	0.125	+	+
	0.25	+	+
	0.5	+	+
Anthocyanine	0.01562	ND	ND
	0.03125	ND	ND
	0.0625	+	+
	0.125	+	+
	0.25	+	+
	0.5	+	+
Tetracycline	0.25 μg/ml	+	+
	0.5 μg/ml	+	+
	1 μg/ml	+	V (6/7 +)
	2 μg/ml	-	-
	4 μg/ml	ND	ND
	8 μg/ml	ND	ND
Methanol 15%	1/20	+	+
	1/10	+	+

+ indicates growth; - = no growth ; ND = Not Done

Comments - *Campylobacter*

There was no inhibition detected in the solvent (methanol) blank. The tetracycline results for the *C. coli* strain were exactly as expected. The tetracycline results for the five *C. jejuni* field isolates were exactly as expected. The reference strain of *C. jejuni* (33560) was inhibited at all dilutions of tetracycline tested – in the past this strain has shown an ability to growth at the two lowest dilutions tested (1 and 2 μg/ml). The formal methodology standard (NCCLS 2002) followed in this study uses this strain of *C. jejuni* (33560) as a Quality Control strain and indicates that the minimal inhibitory concentration for tetracycline can vary from 1 to 4 μg/ml. In the current trial, this QC strain (ATCC 33560) showed an MIC of $\leq$1 μg/ml. Overall, the tetracycline results have validated the testing technology.

The Flavonoid fraction inhibited all strains of *Campylobacter* at 0.25 mg/ml concentration. No other tested compound showed any activity except that the anthocyanine fraction inhibited one *Campylobacter* at 0.5 mg/ml.

Comments - *Clostridium*

The methanol blank plates showed no inhibition of any strains at any dilution. The tetracycline results were exactly as expected and thus validated the overall testing technology.

The Flavonoid fraction inhibited most strains (6/7) of *Clostridium perfringens* at the 0.5 mg/ml concentration. No other tested compound showed any activity.

Comments - Salmonella

The methanol blank plates showed no inhibition of any strains at any dilution. The tetracycline results for the *E. coli* QC strain was 2 μg/ml, this is within the range permitted for this strain (NCCLS 2002). Overall, the tetracycline results have validated the testing technology.

The Flavonoid fraction inhibited most strains (4/6) of Salmonella at the 0.5 mg/ml concentration. No other tested compound showed any activity.

Overall Comments

These results are consistent with the previous results recorded for the *Campylobacter* and *Clostridium* strains in our earlier reports. The flavonoid fraction was the only fraction to demonstrate marked anti-bacterial activity. The flavonoid fraction inhibited all strains of *Campylobacter* at the 0.25 mg/ml concentration. The flavonoid fraction also inhibited most strains (6/7) of *Clostridium perfringens* and most strains (4/6) of *Salmonela* and *E coli* at the 0.5 mg/ml concentration. These results are consistent with the previous result recorded for *Campylobacter* and *Clostridium* strains. This current round of testing has established the formal minimal inhibitory concentration (MIC) of this compound for all strains used in the study. The flavonoid fraction has been shown to be active against *Campylobacter* with typical MIC of 0.25 mg/ml as well as *Clostridium perfringens* and *Salmonella* and *E. coli* with most strains showing an MIC of 0.5 mg/ml.

Future work

To complement the work undertaken in this study, further work is required to:-

- Try to identify the antimicrobial compounds in the flavonoid fraction of Tarac red skin extract by using high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LCMS) analysis.
- From the obtained flavonoid fraction (made of several fractions), search for a more active fraction by further fractionation using LH-20 or equivalent gels to obtain individual flavonoids fractions which need to be further testing for antimicrobial activity.

Chapter 7: Macro scale fractionation of red skin extract flavonoid fraction and final *in vitro* antimicrobial evaluation of flavonoid fractions (fourth evaluation)

In order to provide sufficient material for each fraction corresponding to the peaks eluting off the Toyopearl HW-WF column, a fresh bulk of red skin extract was supplied by Tarac Technologies for processing. This material was solvent extracted and separated into two groups of phenolic acids and crude flavonoids following the method of Kader *et al* (1996), concentrated and run in a series of batches on the Toyopearl column. The eluate was collected on a Fraction Collector and then divided into a group of eight fractions, concentrated and finally dried under vacuum. The dried material was then submitted for antimicrobial testing.

Methodology

1. Initial sample clean up

The red skin extract was filtered through a 20 µm nylon mesh filter to remove insoluble material and adjusted to a pH of 3 prior to extraction. The acidified extract was then extracted in batches of 500 ml lots with 200 ml of ethyl acetate five times. After each extraction the combined solutions (water and ethyl acetate were separated by centrifugation at 3000 rpm for 5 min. The ethyl acetate phase was separated from the water phase each time, combined and finally concentrated by Rotary Evaporation to <100 ml.

2. Back extraction of phenolic acids

The concentrated ethyl acetate extract was transferred to a clean 500 ml Separating Funnel and 50 ml of 5% $NaHCO_3$ added, the funnel capped and shaken for 1 min and then allow to phase separate using centrifugation if necessary. The alkaline water phase was disregarded and the ethyl acetate phase now containing the crude flavonoids retained. After collection of all of the ethyl acetate from the back extraction to remove phenolic acids, this was back washed with deionised water to remove traces of alkaline solvent. The ethyl acetate fraction was then again concentrated by Rotary Evaporation to remove all solvent.

3. Fractionation by Size Exclusion Chromatography

The dried concentrate of crude flavonoids was re-dissolved in 40% methanol and filtered serially through a GF/A filter membrane followed by a 47 mm nylon 0.45 µm membrane filter. The solution strength was adjusted to be >100 mg/ml. A 2.6 cm glass column was then packed with Toyopearl HW-40F resin suspended in 40% methanol to a depth of 30 cm, washed with 40% methanol mobile phase at a flow arte of 5 ml/min. The eluate was connected to a UV Detector set at 350 nm and finally a Fraction Collector. Mobile phase run until the baseline of the detector was flat. 2 ml aliquots of concentrated crude flavonoids were loaded onto the column by a Sample loop. The Integrator was started and also the Fraction Collector set to collect fractions at 2 min intervals. After pumping 40 % methanol mobile phase for 70 min, the mobile phase was switched to 70% acetone and fraction collection continued until 110 min. The Fraction Collector was stopped also at this time and the eluate switched to waste. The mobile phase was then switched back to 40% methanol and solvent pumped for 45 min before loading the next batch of concentrate.

4. Concentration of Fractions

The chromatogram was examined for its major peaks and fraction marks made at appropriate valley points. A total of eight (8) fractions were defined. The defined fractions from each run were then combined, concentrated initially by Rotary Evaporation and finally transferred to weighed 20 ml glass vials and dried under vacuum to constant weight.

Results of Fraction Trial

The chromatogram for the elution pattern of the crude flavonoid extract run on the Toyopearl HW-WF column (Fig. 4) shows the early elution of higher molecular weight compounds followed by a major peak at 61.3 min. The sharp increase in detector signal at 100 min correspondes to the change in mobile phase from 40% methanol to 70% acetone eluting off the column washing out the highly retained condensed tannins and flavonoids quercetin and myricetin. The break points for the fractions are marked on the left of the chromatogram. The proportional distribution of the separated fractions is shown in Table 1.

Table 1. Results of fractionation of Crude Flavonoids extracted from 5.4 L of Red Skin Extract on Toyopearl HW-40F

Fraction	Weight Recovered (g)	Fraction concentration in Original Red skin extract (mg/l)
1	0.980	181
2	0.423	78
3	0.587	109
4	0.760	141
5	1.206	223
6	0.260	48
7	0.229	42
8	3.269	605
Total	7.714	1428

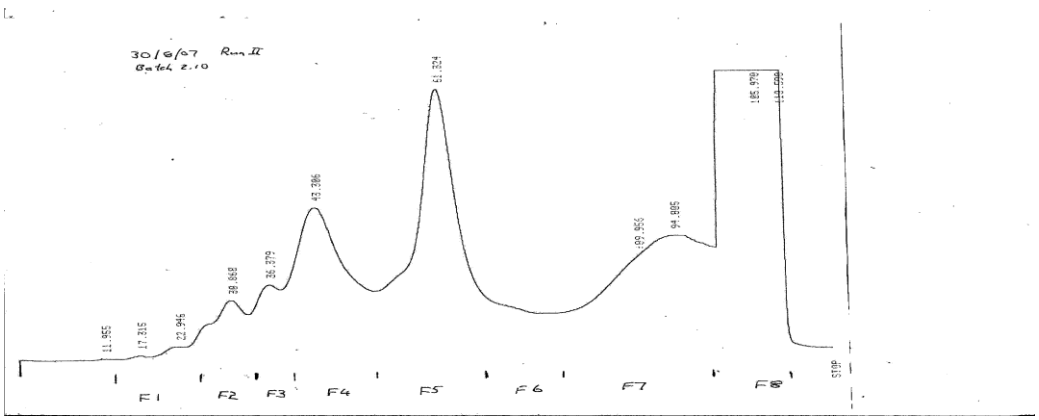

Figure 1. Chromatogram of crude flavonoids separated on a Toyopearl HW-40F column 2.6 x 30 cm column eluted with 40% methanol till 70 min then 70% acetone until 110 min at a flow rate of 5 mL/min.

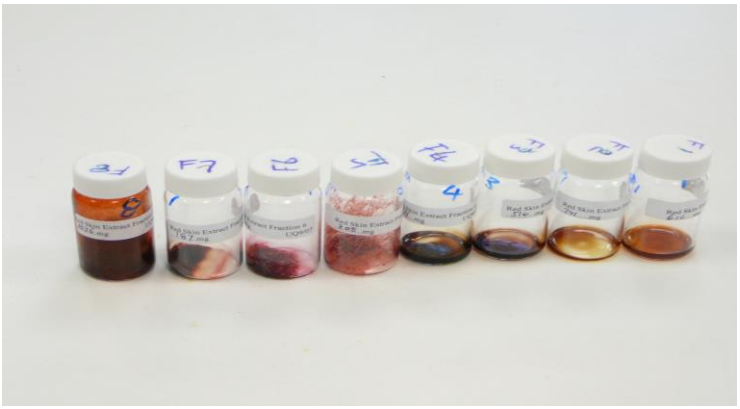

Figure 2. Rotary and vacuum evaporated flavonoids fractions

Final *In vitro* antimicrobial evaluation of flavonoid fractions (fourth evaluation)

In the latest work, extracts have been tested against the same set of *Campylobacter jejuni/coli* strains and *Clostridium perfringens* isolates used in the initial screening. In the current work the same methodology was used as described previously.

Details of dilution range

The compounds were all prepared as a 10 mg/ml stock solution in sterile distilled water containing 15% methanol. All compounds were tested at a 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640 dilutions (prepared in sterile distilled water) for the *Campylobacter* cultures. This means that the concentrations tested were 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.03125 mg/ml and 0.01562 mg/ml (final concentration in the agar plate). For the *Clostridium* isolates, a smaller dilution range was tested - 1/20, 1/40, 1/80 and 1/160. This means that for the *Clostridium* isolates, the concentrations tested were 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml (final concentration in the agar plate).

Control tests using tetracycline (final concentrations of 1, 2, 4, 8 and 16 μg/ml for the *Campylobacter* cultures and 0.25, 0.5 and 1 μg/ml for the *Clostridium* cultures) were included.

A further control of a blank solution that contained 15% methanol (the final concentration in the stock solutions) was used. In these methanol controls, the final plates contained 1.5%, 0.75% and 0.375% methanol (in a 1/10, 1/20 and 1/40 dilution series). The 1/20 dilution represented the lowest dilution used in the test series – meaning that this 1/20 dilution is the highest concentration of methanol present in the test series.

Results

The detailed results of the *Campylobacter* and *Clostridium perfringens* strains are shown in Table 1.

Table 1. Results of MIC tests on grape extracts methanol and tetracycline – *Campylobacter* and *Clostridium perfringens*

Compound	Concentration (mg/ml)	*C. jejuni ATCC 33560*	*C. coli ATCC 33559*	*C. jejuni (5 isolates)*	*Cl. perfringens (7 isolates)*
Red Skin Extract Fraction 1	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	+	+	+	+
	0.5	+	-	+	+
Red Skin Extract Fraction 2	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	+	+	+	+
	0.5	+	+	+	+
Red Skin Extract Fraction 3	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	+	+	+	+
	0.5	+	+	+	-
Red Skin Extract Fraction 4	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	-	-	-	+
	0.5	-	-	-	V (1/7 +)
Red Skin Extract Fraction 5	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	-	-	V (3/5 +)	+
	0.5	-	-	-	+
Red Skin Extract Fraction 6	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	+	+	V (4/5 +)	+
	0.5	-	-	-	+
Red Skin Extract Fraction 7	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	+	+	+	+
	0.5	+	-	V (3/5 +)	+
Red Skin Extract Fraction 8	0.0625	+	+	+	+
	0.125	+	+	+	+
	0.25	+	+	+	+
	0.5	+	+	V (4/5 +)	+
Methanol 15%	1/40	+	+	+	+
	1/20	+	+	+	+
	1/10	+	+	+	+
Tetracycline	0.25 µg/ml	ND	ND	ND	V (4/7 +)
	0.5 µg/ml	ND	ND	ND	V (4/7 +)
	1 µg/ml	-	-	V (2/5+)	V (4/7 +)
	2 µg/ml	-	-	V (2/5+)	ND
	4 µg/ml	-	-	V (2/5+)	ND
	8 µg/ml	-	-	V (2/5+)	ND
	16 µg/ml	-	-	V (2/5+)	ND

+ indicates growth; - = no growth ; ND = Not Done

Comments - *Campylobacter*

The tetracycline results for the five field isolates were exactly as expected. The reference strain of *C. jejuni* and *C. coli* were both inhibited at all dilutions of tetracycline tested – in the past these strains has shown an ability to growth somtimes grow at the two lowest dilutions tested (1 and 2 μg/ml). The formal methodology standard (NCCLS 2002) followed in this study uses this strain of *C. jejuni* as a QC strain and indicates that the minimal inhibitory concentration for tetracycline can vary from 1 to 4 μg/ml. In the current trial, this QC strain (ATCC 33560) showed an MIC of ≤1 μg/ml. Overall, the tetracycline results have validated the testing technology.

The methanol control plates showed that all *Campylobacter* isolates were capable of growth on the 1/10, 1/20 and 1/40 dilution plates. As a 1/20 dilution was the lowest dilution of the test compounds, this confirms that any inhibitory activity seen with the test compounds is due to those compounds and not to the methanol solvent.

Fractions 4, 5 and 6 showed an ability to inhibit all strains of *Campylobacter* at the 0.5 mg/ml concentration. Fraction 4 also inhibited all of the strains at the 0.25 mg/ml concentration. No other tested compound showed any activity except that Fraction 1 inhibited the *C. coli* reference strain at the 0.5 mg/ml concentration.

Comments - *Clostridium*

The tetracycline results were exactly as expected and thus validated the overall testing technology. The methanol control plates showed that all *Clostridium perfringens* isolates were capable of growth on the 1/10, 1/20 and 1/40 dilution plates. As a 1/20 dilution was the lowest dilution of the test compounds, this confirms that any inhibitory activity seen with the test compounds is due to those compounds and not to the methanol solvent.

Fraction 4 inhibited most of the strains (6/7) of *Clostridium perfringens* at the 0.5 mg/ml concentration. No other tested compound showed any activity.

Comments on the red skin material and fractions obtained

- Solvent extraction of the red skin material by ethyl acetate showed that the antimicrobial activity was in the ethyl acetate phase and not in the remaining aqueous phase.
- Fractionation of the ethyl acetate phase into phenolic acids and crude flavonoids showed that the antimicrobial activity was only in the crude flavonoid fraction and not associated with the phenolic acid fraction.
- Fractionation of the crude flavonoids by Size Exclusion Chromatography on Toyopearl HW-40F into two groups showed that the antimicrobial activity was associated with the compounds eluted initially with methanol/water and not with the retained fraction subsequently eluted with acetone/water.
- Antimicrobial testing showed that the test flavonoids quercetin and rutin did not posess antimicrobial activity.
- Subsequent testing on the Toyopearl column showed that quercetin was retained on the column along with condensed tannins and not eluted until the solvent was changed to acetone/water.
- Macro scale fraction on a Toyopearl HW-40F column into seven fractions of the methanol/water eluent showed that the antimicrobial activity was associated with the major peaks eluting at the low molecular weight time window.

- The antimicrobial activity originally demonstrated in the red skin extract is not associated with the major flavonoids, quercetin and myricetin, present in wine grape skin nor the phenolic acids or the condensed tannins but with an as yet unidentified group of compounds.

Chapter 8: Initial LCMS identification of active antimicrobial fractions

Fractions of the Red skin extract obtained by chromatographic separation of the crude flavonoid fraction were tested for antimicrobial activity. Fractions 3, 4, 5 and 6 showed activity against *Campylobacter* and *Clostridium* species and initial work by Liquid Chromatography-Mass Spectrometry (LCMS) was directed at these fractions. Earlier work by HPLC had shown that the two common flavonoids, quercetin and myrecitin were not present in these fractions but present in fraction eight and both this fraction and pure quercetin and rutin did not show antimicrobial activity. The presence of these two flavonoids in fraction eight was confirmed by LCMS analysis.

Fractions three, four, five and six were subjected to analysis by LCMS for molecular weight identification of the major peaks present in these fractions. The results of this preliminary investigation are attached.

Results

The fractions were dissolved in 40% acetonitrile at concentrations ranging 100 – 300 µg/mL and separated on a Waters X-Terra column (150 x 2.1 mm 3.5 µm). Detection was by a PDP (200 – 400 nm) for UV detection and peak integration followed by Electrospray ionisation and mass analysis on a Micromass ZMD Mass Spectrometer with mass scans in the range 100 – 700 m/z.

The UV trace at 280 nm was integrated for area per cent estimation of proportional content of the major peaks. The TIC trace was mass analysed to identify the (M-H)- ions associated with each of the major peaks. An addition acid hydrolysis was carried out on Fraction 5 to assist in the identification of its major peak.

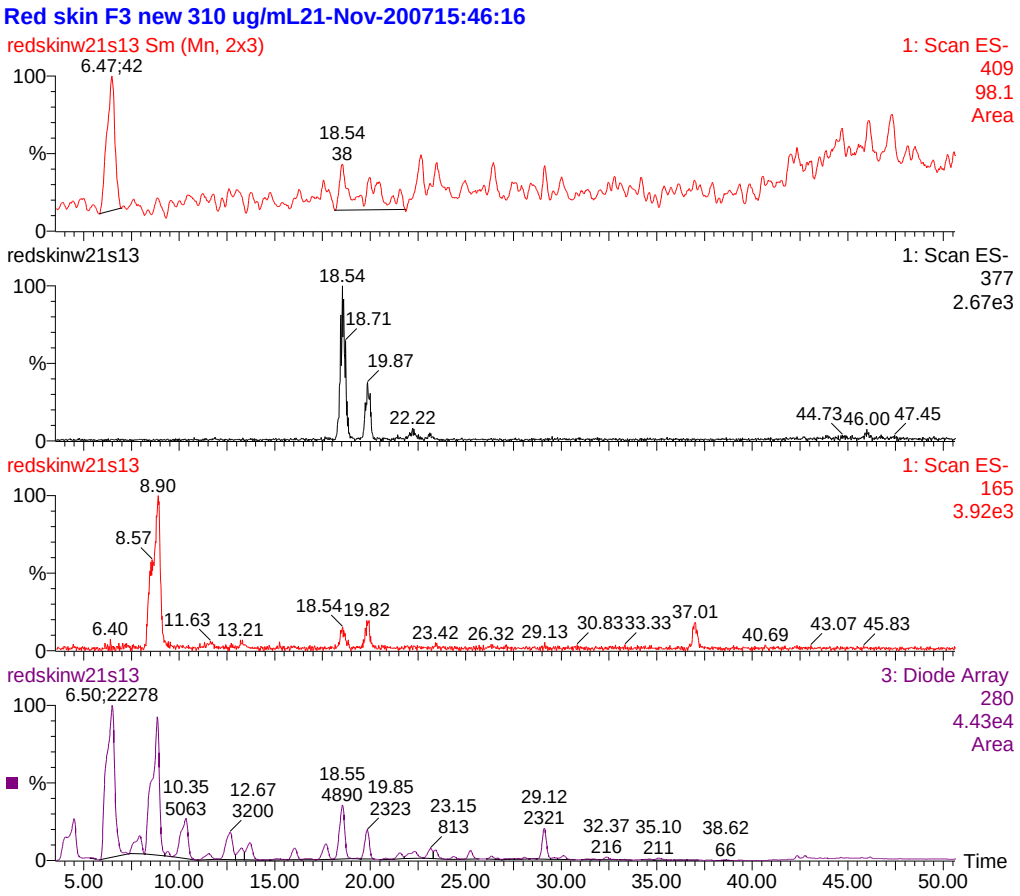

Figure 1. Chromatogram for Red skin Fraction 3 off Toyopearl HW-40F run on LCMS

The UV trace in the chromatograms for fraction 3 (Fig. 1) showed that this fraction, although based on a single peak in the large scale chromatography on Toyopearl HW-40F, comprised of many compounds. Two compounds (retention times 6.50 and 8.85 min) comprised 23.6 and 18.9% of the total peak area with all other peaks being <10% of the total peak area. Mass analysis of these two peaks showed that the compound eluting at 6.50 min had a molecular weight (MW) of 410 and the compound eluting at 8.85 min a MW of 242. This fraction showed activity against *Clostridium perfingens* only at a MIC concentration of 0.5 mg/ml.

Fraction 4 showed by UV detection (Fig. 2) to have a major compound eluting at 32.31 min comprising 33.3% of the total peak area. This compound was shown by mass analysis to have a MW of 226. Again the other peaks present individually were <10% of the total peak area. This fraction showed activity against all *Campylobacter* spp. tested needing only a MIC of 0.25 mg/mL. Fraction 4 also inhibited most of the strains (6/7) of *Clostridium perfringens* at the 0.5 mg/ml concentration.

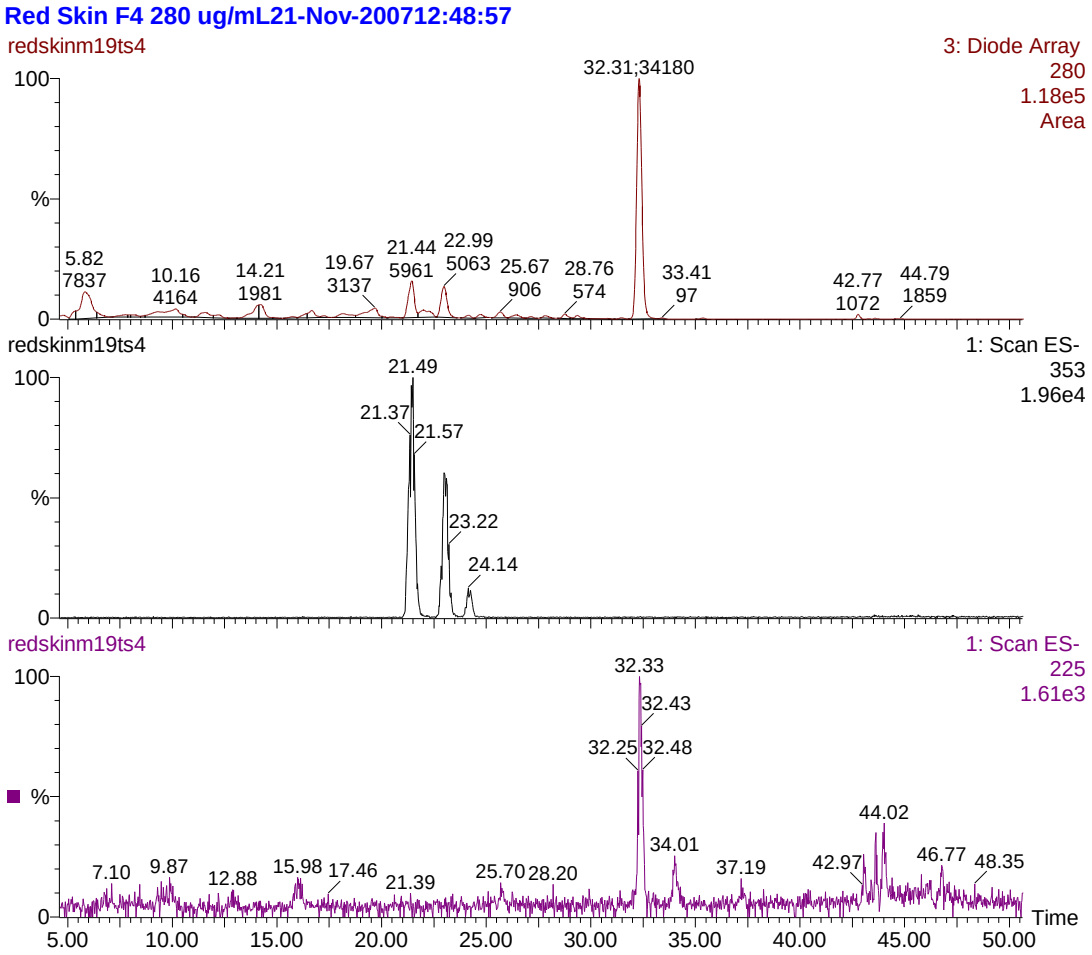

Figure 2. Chromatogram for Red skin Fraction 4 off Toyopearl HW-40F run on LCMS

Fraction 5 showed by UV detection (Figure 3) to comprise of a single compound eluting at 12.68 min and accounting for 78.6% of the total peak area. This compound was shown by mass analysis to have a MW of 198.

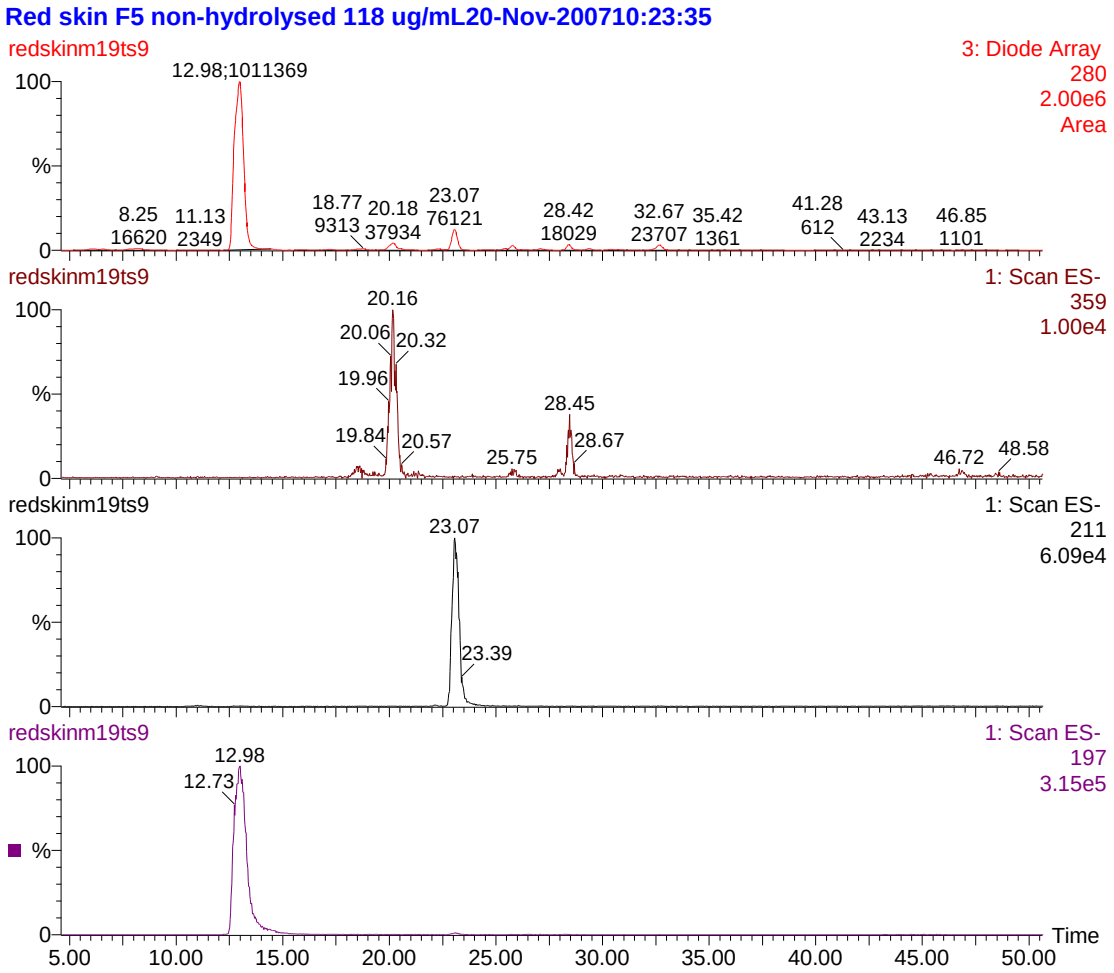

Figure 3. Chromatogram for Red skin Fraction 5 off Toyopearl HW-40F run on LCMS

LCMS analysis of fractions six and seven which also showed limited activity at *Campylobacter* spp. only at a MIC of 0.5 mg/ml showed that these fractions comprised eluting traces of the major compound present in fraction five. This is not unexpected as the peak for fraction five was the major peak obtained for fractions off the Toyopearl column prior to changing solvents.

A summary of the peak data for each fraction is shown in tables one, two, three for each of the fractions three, four, five.

Table 1. Peak data for fraction 3

Peak number	RT (min)	Area %	(M-H)- ion	Name
1	4.50	9.8	153 +123	
2	6.50	23.6	409+	Multiple fragment ions
3	8.85	18.9	241	
4	10.35	5.4	195 +180	
5	17.68	1.6	419	
6	18.55	5.2	377+329+195	
7	19.92	2.6	377+329+195	
8	23.15	1.4	621	
9	29.12	2.5	543+263+153	
10	36.94	0.1	227	Resveratrol isomer

Table 2. Peak data for fraction 4

Peak number	RT (min)	Area %	(M-H)- ion	Name
1	3.1	5.6	125	
2	5.82	9.1	109	
3	10.16	4.5	241	
4	14.21	4.3	265	
5	21.44	5.8	353	
6	22.99	4.9	353	
7	32.31	33.3	225+210	
8	37.04	0.2	227	
9	42.77	1.6	327	Resveratrol isomer

Table 3. Peak data for fraction 5

Peak number	RT (min)	Area %	(M-H)- ion	Name
1	5.93	1.2	183	
2	7.99	1.3	177	
3	12.66	78.6	197	Ethyl gallate
4	19.94	2.9	359	
5	22.84	5.9	211+196	
6	25.67	1.2	433+361	
7	28.29	1.4	341	
8	32.56	1.9	615+417	

The initial identity of the major peak present in fraction five as ethyl gallate was based on work published by Monogas *et al* (2005) further who performed LCMS analysis of similar ethyl acetate extracts of red wine but without the class separation step to remove phenolic acids. Acid hydrolysis of a sub-sample of fraction five and analysis by LCMS showed the loss of the peak eluting at 12.66 min with the appearance of a peak eluting at 2.13 min which corresponds by both retention time and (M-H)- ion (m/z 169) to gallic acid. Final confirmation of this peak as ethyl gallate will be done by running the authentic compound when it is available.

A literature search showed that gallate esters have been demonstrated to have antimicrobial activity. Chaubal *et al* (2005) showed that both gallic acid and methyl gallate were active against a range of bacteria with a MIC of >1000 mg/mL and 12.5 mg/mL respectively. It has also been shown by Adesina et al (2000) that ethyl gallate extracted from the leaves of *Acalypha* spp. showed antimicrobial activity against *E. coli* and *S. aureus* with MIC values of 0.4 and 0.1 mg/mL respectively. The tentatively identified ethyl gallate present in fraction five has in this work been shown to have an activity with a MIC of 0.25 mg/L against *Campylobacter* spp. and *Clostridium perfringens* isolates.

Conclusions

Preliminary work to date has shown that LCMS can provide molecular weight information of the various compounds present in the antimicrobial active fractions. However, this alone does not provide absolute identification of the compounds and further work is necessary to provide this information. Fractions 4 and 5 were dominated by single compounds and it is desirable to complete their identification and perform further antimicrobial testing to establish if the activity is solely associated with single compounds or is the result of the mixture of other compounds present in the fractions.

Chapter 9: Implications and recommendations

During this study using *in vitro* techniques, phenolic compounds extracted from grape residues were found to inhibit *Campylobacter* and *Clostridium perfringens* microroganisms. These compound wese also effective against *Salmonella* and *E.coli* bacteria. *Clostridium perfringens,* a bacterium responsible for necrotic enteritis (NE) has been considered a global poultry disease. This bacterium produces toxins in the chickens intestine resulting in high mortalities in flocks, and in its subclinical form, NE is financially damaging because of reduced growth performance in chickens (Mikkelsen *et al*. 2007). Currently, the main method to control the incidence of NE is by the use of antibiotics, but this practice is coming undder increasingly critical scrutiny and has been curtailed in Western Europe due to concerns about the dvelopment of antibiotic-resistant bacteria that are potential human pathogens. There is also increasing public concern about antibiotic residues in animal products, resulting in the search for natural alternatives to sustain efficient chicken meat production without reliance on antibiotics (Williams, 2001).

If the use of commercial antibiotics in the poultry industry is prohibited substantial losses would be expected due to an increased incidence of NE in poultry. It has been pointed out that the main economic losses from enteritis include :

- About 10% worse feed efficiency
- 2%-13% reduced live weight
- Up to 6% mortality
- Up to 1% increased condemnations.

The figures for economic losses in Australia due to bacterial enteritis are difficult to obtain but reported figures for other regions of the world are 3.3/4.5 euro cents/bird or more. Therefore, it can be calculated that in Australia the losses would be close to 5.7/7.8 Australian cents/bird. Hence, the potential saving to the Australian chicken meat industry, with chicken meat obtained from 474.5 million birds annually, would be close to AUD32 million annually.

The Australian poultry industry considers *Campylobacter* and *Salmonella* as major food safety pathogens detrimental to humans when ingested. For this reason, the RIRDC Chicken Meat Research Program has encouraged the development and dissemination of enhanced on-farm and processing plant food safety programs and developed through-chain strategies for control of *Campylobacter* and other food safety pathogens. *Campylobacter jejuni/coli* is the most common cause of food-borne human gastro-enteritis in Australia, with poultry being regarded as one source of the organism. Therefore, controlling these microorganisms through the use of natural antimicrobial compounds will have considerable commercial potential.

The discovery of the proposed natural phenolic compound during this pilot study may revolutionise the control of the major food safety pathogens, for which currently there is no 100% effective control/prevention strategy.

The production of the obtained phenolic compound from grapes would be economical, as the grape is one of the fruit crops most widely grown throughout the world. It has been demonstrated that grapes contain large amounts of phenolic compounds that have exhibited many favourable effects in human health with antibacterial and antioxidant properties (Baydar *et al* 2003 ; Jayaprakasha *et al* 2002).

In Australia there is interest in research and development of technology applied to transforming wine residuals such as grape marc (skins & seeds) and other winemaking residuals into commercially valuable products. For example, in 1999 Tarac Technologies constructed a modern production facility at Nuriootpa in the Barossa Valley, South Australia, which incorporates state-of-the-art extraction

technology. The company collects and processes significant quantities of winery residuals in Australia each year – including approximately:

- 100,000 tonnes of grape marc;
- 5,000 tonnes of filter cake; and
- 30 million litres of distillation wine, lees, tank washings and other winemaking residuals.

Tarac is considered by industry to be at the leading edge and this plant recovers tannins and red colour from the skins of red grape marc and extracts from the seeds of both red and white grape marc.

Other benefits

The outcomes of this research will:

- result in a novel, consumer-friendly method for preventing/controlling the colonisation of chickens by *Campylobacter*, the most important cause of food-borne gastroenteritis in humans;
- result in improved on-farm control programs based on the outcomes of this project and future research which will result in safer chicken meat, improved consumer confidence and increased market penetration of chicken meat;
- enable Australia to remain a leader in the development and commercialisation of biotechnology and utilisation of life science technologies for betterment of society;
- improve systems to ensure the safety of food products through the chain by allowing an effective prevention/control program for unwanted bacteria in broiler chickens;
- result in a significant step forward in our ability to manage and control a major biohazard – *C. jejuni* and *C. perfringens*, the most important food-borne gastro-enteritis agent in humans and necrotis enteritis agent in poultry, respectively;
- potentially improve the profitability of the poultry industry by adding value to poultry products (without antibiotics);
- enable the poultry industry to enhance the clean image of Australian food industries, adding considerable value and market appeal to these industries.

Recommendations

- For continuity, future *in vitro* works regarding antimicrobial evaluation of plant material fractions should follow all the laboratory methods that were established at Animal Research Institute during this study.
- It is recommended that future research continue with the identification of the compounds that have shown major antimicrobial activity (*i.e.*, fractions three, four and five from red skin grape material).
- Fraction three, which showed activity against *Clostridium perfingens* and is mainly made with two compounds (23.6 and 18.9% of the total peak area), would need further work to accomplish components identification. It is recommended that from the original material (red skin extract) collect fraction three that needs further purification and fractionation using a superior resolution column to clearly separate main compounds to assist LCMS identification.

- Fractions four and five were dominated by single compounds and it is desirable to complete their identification and perform further antimicrobial testing to establish if the activity is solely associated with single compounds or is the result of the mixture of other compounds present in the fractions.
- If the identified compounds are commercially available then reference compunds need to be purchased for further antimicrobial testing to ensure the identity of the compound found in the active fractions.
- If the identified compound obtained during this study is the active compound, a search of global databases around the globe is needed to evaluate potential intelectual property on the use of these compounds.
- It is necessary to investigate how the intellectual property could be protected - of individual or group of the obtained compounds that show microbial activity in this study.
- It is highly recommended to continue the evaluation of the obtained compounds using animals (Phase II proposal) to evaluate their efficacy as controlling undesirable bacteria without affecting feed efficiency.
- It is recommended to expand the potential use of the obtained antimicrobial fractions. For example, there is a possibility for their use as antimicrobial sprays in the food industry to increase food life during refrigeration/storage.

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Plain English Compendium Summary

Project Title:	Characterisation of proanthocyanidins and related phenolics for inhibition of gastrointestinal tract microorganisms (Phase I)
Project No.:	05-03
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Organisation:	Department of Primary Industries and Fisheries, Poultry Research and Development Centre
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Objectives	1) Determine the antimicrobial activity of isolated, fractionated and purified compounds from grape residues. 2) Isolate, fractionate, purify and characterise condensed tannins and related phenolics from olive pomace. 3) Identify and characterise specific polyphenolic fractions from grapes and olive pomace able to inhibit microorganisms such as *Clostridium* spp., *Campylobacter* spp. and *Salmonella* spp. under *in vitro* conditions.
Background	The general public is becoming increasingly aware of problems from the over-use and misuse of traditional antibiotics. Coupled with this view is the general consensus among the medical profession that the use of antibiotics by the poultry industry has the potential for creating "superbugs", which are microorganisms highly resistant to clinical antibiotics. As a result of this concern, antibiotics have currently been restricted, and a total ban has been in place, in Europe since January 2006. Therefore European poultry producers are faced with the challenge to produce poultry products without antibiotics with consequent losses in feed efficiency, performance and increased in production costs. There is a general acceptance that production of meat without the use of antibiotics is likely to increase within the Asia Pacific Region during the next decade. It is expected that Australia will follow this trend due to the economic benefits obtained as a leading "clean green" image country. The increasing demand for 'natural' and drug-free meat production throughout the more affluent countries of the world reinforces the need for the evaluation of plant antimicrobial compounds as alternatives to antibiotics.
Research	The proposal was created by PRDC with Tarac technologies generating all grape material and the University of Adelaide all the olive fractions. The University of Queensland did the purification, separation and identification of all grape extracts. The animal research institute performed all *in vitro* evaluations for antimicrobial activity of grape and olive extracts against poultry *Campylobacter* and *Clostridium perfringens* isolates.
Outcomes	During this study using *in vitro* techniques, phenolic compounds extracted from grape residues were found to inhibit *Campylobacter* and *Clostridium perfringens* microroganisms. These compounds wese also effective against Salmonella and *E.coli* bacteria.
Implications	• A significant step forward in our ability to manage and control a major biohazard, *Campylobacter* and *Clostridium* the most important food-borne gastro-enteritis agent in humans and necrotis enteritis agent in poultry, respectively. • The potential for improving the profitability of the poultry industry by adding value to poultry products (without antibiotics) and producing safer chicken meat. • Enabling the poultry industry to enhance the clean image of Australina food industries, adding considerable value and market appeal.
Publications	Abstract submitted to the next World Poultry Congress 2008