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Bioscreening of Australian olive mill waste extracts: Biophenol content, antioxidant, antimicrobial and molluscicidal activities

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Abstract

The biophenolic fraction was characterized in olive mill waste (OMW) obtained as a by-product from processing of Mission and Frantoio olive fruit. OMW produced from the Mission fruit contained higher total phenol content. Individual biophenols with the exception of verbascoside and an hydroxytyrosol-secoiridoid were also present at higher concentrations in the OMW produced from Mission cultivar. Antioxidant activities were measured in aqueous (DPPH) and emulsion (BCBT) systems. The Frantoio extract was more active than the Mission extract in the DPPH assay – EC_{50} values were 28.3 $\pm$ 1.7 ppm and 34.7 $\pm$ 1.7 ppm, respectively. Activities were reversed in the BCBT, with the Mission extract (EC_{50} 60.6 $\pm$ 2.3 ppm) more potent than the Frantoio extract (EC_{50} 79.9 $\pm$ 2.0 ppm), and this may be related to the more lipophilic nature of the Mission extract. Both extracts showed broad spectrum antibacterial activity against *Staph*. *aureus*, *B*. *subtilis*, *E*. *coli* and *Ps*. *aeruginosa*; whereas individual biophenols (hydroxytyrosol, luteolin, oleuropein) showed more limited activity. Molluscicidal activity was measured against *Isidorella newcombi* and LD_{50} values were 424 ppm and 541 ppm for Mission and Frantoio extracts, respectively. The results suggest that OMW may be utilised as a source of bioactive compounds.

Keywords: olive mill waste; bioactivity; biophenol; antioxidant, antimicrobial; molluscicidal.

Abbreviations: BCBT = *β*-carotene bleaching test; CAE = caffeic acid equivalents; CCE = cyaniding chloride equivalents; cfu = colony forming unit; DPPH = 2,2′-diphenyl-1-picrylhydrazyl radical; EM = extractable matter; FC = Folin Ciocalteu; FOE = Frantoio olive mill waste-reconstituted crude extract; GAE = gallic acid equivalents; HPLC = High performance liquid chromatography; HPLC-DAD = HPLC-photodiode array detection; HTS = High throughput screening; LC-MS = Liquid chromatography-Mass spectrometry; MOE = Mission olive mill waste-reconstituted crude extract; OMW = Olive mill waste; QE = quercetin equivalents.

1. Introduction

The olive oil industry generates large quantities of a deleterious by-product known as olive mill waste (OMW). Being seasonal and resistant to degradation, OMW is a major problem for the development of a sustainable olive oil industry. Raw (untreated) OMW has broad spectrum toxicity (Niaounakis and Halvadakis, 2004) against bacteria (Capasso et al., 1995; Ramos-Cormenzana et al., 1996), fungi (Fodale et al., 1999), algae (Della Greca et al., 2001), plants (Capasso et al., 1992; Casa et al., 2003), insects (Capasso et al., 1994), animals (Niaounakis and Halvadakis, 2004) and human cells (Capasso et al., 1995). However, fractionated OMW extracts and isolated biophenols demonstrate selective or minimal toxicity (Capasso et al., 1995). Nevertheless, European research identifies OMW as a potential source for the recovery of antioxidant (Visioli et al., 1995; Aldini et al., 2006), anti-atherogenic (Leger et al., 2000) and anti-inflammatory biophenols (Visioli et al., 1999). The European work suggests the production of bioactive compounds such as biophenols (Fig. 1) from OMW as a viable alternative for value adding to this problematic by-product (Obied et al., 2005b).

Olive biophenols are most widely studied as antioxidants and free radical scavengers (Obied et al., 2005b) and Mediterranean OMW is rich in these compounds (Visioli et al., 1995; Visioli et al., 1999; Lesage-Meessen et al., 2001; Amro et al., 2002). Australian olive fruit extracts were studied by our group previously and their antioxidant activities were demonstrated under aqueous and lipophilic reaction conditions (McDonald et al., 2001). The current paper examines the biophenolic content of OMW and its bioactivity. It is the first reported investigation of the antioxidant activity of Australian OMW which is distinguished from most Mediterranean OMW by the nature of the waste (commonly two-phase generated pomace) and its distinctive biophenolic profile (Obied et al., 2005a).

Other bioactivities include antimicrobial and molluscicidal activities. Molluscicidal activity has been largely unexplored (Kubo and Hanke, 1985) whilst the antimicrobial activity of OMW was early recognized and linked to biophenol content (Niaounakis and Halvadakis, 2004). Most studies of antimicrobial activity have focused on ecological and environmental consequences (Moreno, Quevedo-Sarmiento, and Ramos-Cormenzana, 1989) or agronomic applications (Capasso et al., 1995). The activity of pure biophenols and biophenols recovered from OMW has been tested against human pathogens (Bisignano et al., 1999; Aziz et al., 1998). As part of a comprehensive study of the nature (Obied et al., 2005a) and functionality of OMW, we report here the antioxidant, antimicrobial and molluscicidal activities of OMW and biophenols. The current study is the first to report antimicrobial action of OMW extracts and biophenols against pathogenic bacteria and fungi.

2. Materials and Methods

2.1. Reagents and standards

Reagents used without further purification: Folin-Ciocalteu reagent, 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), β-carotene (Type I, synthetic), linoleic acid, and Tween 20 (Sigma-Aldrich, Steinheim, Germany); HPLC grade methanol and n-hexane (Mallinckrodt, Paris, USA), acetonitrile anhydrous (Unichrome, Sydney, Australia); glacial acetic acid, hydrochloric acid (32%), calcium chloride, sodium carbonate, sodium bicarbonate, sodium chloride, magnesium chloride, magnesium sulfate hydrate and sodium molybdate dihydrate (Univar, Sydney, Australia); chloroform and copper sulfate pentahydrate (Chem-Supply, Adelaide, Australia); and absolute ethanol (Biolab, Melbourne, Australia).

Water used in all analytical work was purified by a Modulab Analytical model water system (Continental Water Systems Corporation, Australia). De-ionized water, RO Pure LP Barnstead (Dubuque, Iowa USA), was used for molluscicidal bioassays.

Phenolic standards used without further purification were: catechol, gallic acid, caffeic acid, *p*-coumaric acid, rutin, luteolin, and quercetin dihydrate from Sigma-Aldrich (Steinheim, Germany); tyrosol from Aldrich (Milwaukee, WI, USA); hydroxytyrosol from Cayman (Michigan, USA); oleuropein, verbascoside and cyanidin chloride from Extrasynthese (Genay, France); *p*-hydroxybenzoic acid from Fluka (Switzerland). Most standards were dissolved in 80% methanol to prepare stock solutions of 1 mg/mL. The exceptions were: quercetin dihydrate and luteolin, which were dissolved in absolute methanol; rutin, which was dissolved in hot 80% methanol; and cyanidin chloride, which required 80% methanol containing 1% HCl for stabilization.

2.2 Olive Mill Waste

OMW from two cultivars was used in this study. Frantoio fruit, 90% black skin coloration, was processed at Riverina Olive Grove, Wagga Wagga, Australia in May 2003 using a commercial two-phase olive oil mill (Pieralisi, Italy) and a malaxation time and temperature of 1 h and 20 ± 1 ºC, respectively. OMW was also obtained from Mission fruit that was processed using a laboratory-scale olive mill. The fresh waste from either process was stored under liquid nitrogen without delay and then freeze dried in a Dynavac FD12 freeze dryer (Sydney, Australia). The freeze dried OMW was stored in screw capped amber-coloured glass containers at -20 °C. The weight of the freeze dried OMW powder was taken as the dry weight.

2.3. Extraction of Biophenols

Freeze dried OMW (10 g) was extracted with aqueous methanol (80% v/v; pH 2, HCl; 40 mL) for 30 min at ambient temperature (20 ± 2 °C). After filtration, the raffinate was re-extracted with 15 mL of the same extraction solvent and filtered over the first filtrate. The combined filtrate was defatted twice with *n*-hexane (2 x 30 mL). The defatted crude extract (CE) was concentrated in a rotary evaporator for 1 h (at < 35 °C). The concentrated crude extract was taken to near dryness under a current of nitrogen at ambient temperature for 2 h. The syrupy residue was reconstituted in 10 mL 50% ethanol to give the reconstituted crude extract (RCE). The RCE was filtered through GF/F filter paper followed by a 0.45 µm plastic non-sterile filter (Advantec MFS, Japan). RCE was stored at –20 °C and aliquots were used for different assays. Extractable matter (EM) of the RCE was determined as previously described (Obied et al., 2005a).

2.4. Spectrophotometric measurements

Spectrophotometric measurements were performed with a Cary 50 UV/visible spectrophotometer, using Cary WinUV "version 3" software (Varian, Australia). An aliquot of the RCE (20 µL) was diluted to 10 mL with water and this diluted extract was used for all spectrophotometric analyses as described previously (Obied et al., 2005a) by reference to a six-point regression curve for each phenolic determination. Total phenols were determined using Folin Ciocalteu reagent and results expressed as gallic acid equivalents (GAE). Results for *o*-diphenols measured colorimetrically at 370 nm are expressed as caffeic acid equivalents (CAE). Total biophenols were also measured directly at 280 nm using gallic acid as standard whilst 320 nm was used to determine hydroxycinnamic acid derivatives using caffeic acid as standard; 360 nm to estimate flavonols using quercetin as standard, and 520 nm for anthocyanins using cyanidin chloride as standard. All results are expressed as mg of the relevant standard per g dry weight of freeze-dried material.

2.5. High Performance Liquid Chromatography

HPLC-DAD was performed with a Varian 9021 solvent delivery system equipped with a Varian 9065 Polychrom UV diode array detector (190-367 nm). Software used for data processing was Star Polychrom version 5.2. Separation was performed by gradient elution on a Luna C-18(2) column, 5 µm particle size; (150 mm x 4.6 mm) (Phenomenex, Australia) attached to a SecurityGuard guard cartridge (Phenomenex, Australia). Analysis conditions were as described previously (Obied et al., 2005a).

Liquid chromatography-Mass Spectrometry (LC-MS) of the OMW extracts was performed on a Micromass Quattro micro tandem quadrupole mass spectrometer (Waters, Manchester, UK). LC separation was provided by a Waters liquid chromatograph (Waters, Milford, USA), consisting of a 2695 Separation Module and 2487 dual wavelength UV detector operated at 240 and 280 nm. An SGE Wakosil C18 column (150 mm × 2 mm; 5µm) was used. Aqueous formic acid (1%) and methanol + acetonitrile + formic acid (89.5+9.5+1 v/v) served as solvents A and B, respectively. A seven-step linear gradient analysis for a total run time of 75 min was used as follows: Starting from 90% solvent A and 10% solvent B increasing to 30% solvent B over 10 min, then isocratic for 5 min, increased to 40% solvent B over 10 min, to 50% over 15 min and to 100% solvent B over 10 min, back to 10% solvent B over 5 min and finally isocratic for 10 min. An injection volume of 10 µL and a constant flow of 0.2 mL/min was used for each analysis. The entire flow from the LC was directed into the mass spectrometer. Data were acquired by the Masslynx data system for both the MS and UV data. The mass spectral data were acquired for four alternative scans; Scan 1: Positive ion mode, cone voltage 35 V; Scan 2: Positive ion mode, voltage 70 V; Scan 3: Negative ion mode,

cone voltage 30 V; Scan 4: Negative ion mode, cone voltage 70 V. All scans were performed in the range 80 to 1500 *m/z* in 1 sec.

2.6. Free radical scavenging activity

DPPH radical scavenging activity of OMW extracts was evaluated according to Kulisic et al. (Kulisic et al., 2004) with minor modification as follows. Aliquots of RCE ranging from 5 µL to 120 µL were diluted to a volume of 10 mL. The diluted extract (200 µL) was added to an aqueous methanolic solution (82%) of DPPH radical (32 mg/L; 3 mL) in plastic macro-cuvettes (1 cm). The macro-cuvettes were covered, well shaken, and kept in the dark for 1 h and the absorbance was then measured at 517 nm. The percentage scavenging of DPPH radical was calculated according to the formula:

$$\% \text{ scavenging} = 100 \times (A_{control} - A_{sample})/A_{control}.$$

Caffeic acid (0.8-6.3 ppm) was used as a positive control for comparison.

The molar concentration of DPPH radical was calculated using the literature value of 12509 $M^{-1}cm^{-1}$ for molar absorptivity (Fukumoto et al., 2000). TEAC (Trolox equivalent antioxidant capacity) was determined as the amount of Trolox equivalent to the amount of test substance that resulted in 50% scavenging of DPPH radical.

2.7. Antioxidant activity using β-Carotene Bleaching Test (BCBT)

Although BCBT is widely used to determine the antioxidant activity in an emulsified system, our initial results demonstrated poor reproducibility. The following procedure was optimized from a systematic study for high reproducibility of results under our laboratory conditions based on the

methodology of Kumazawa et al. (Kumazawa et al., 2004), Matthäus (Matthäus, 2002), Kulisic (Kulisic et al., 2004) and von Gadow (von Gadow et al., 1997). Linoleic acid (50 µL) was added to Tween 20 (400 mg) and *β*-carotene solution (3 mL; 0.1 mg/mL in chloroform) in a round bottom flask. The chloroform was evaporated completely by heating at 37 ºC under vacuum for 10 min. Aerated water (100 mL) was added in portions with vigorous shaking. Blending with an Ultra-Turrax at 19,000 rpm for 2 min in an ice-bath under nitrogen stream was followed by sonication for 2 min in an ice bath. An aliquot (3 mL) of the emulsion was added without delay to 50 µL of different concentrations of the antioxidant solution in 50% ethanol in 10 mL-screw capped Pyrex glass tubes and vortexed. Absorbance at 470 nm was measured immediately and time was assigned as T_0. The absorbance was re-measured after 1 h incubation at 40 ºC (T_{60}). Aqueous ethanol (50%; 50 µL) was used as a control whilst caffeic acid (20-329 ppm) was used as a positive control for comparison. An emulsion containing no *β*-carotene was prepared daily and used for blanking the absorbance. The percentage inhibition of *β*-carotene bleaching was calculated using the following formula:

% Inhibition = $[(A_{60} - C_{60}) / (C_0 - C_{60})] \times 100$ where A_{60} is the absorbance of the test compound or extract at $T_{60,}$ C_{60} absorbance of the control at $T_{60,}$ and C_0 is the absorbance of the control at T_0.

2.8. Antimicrobial activity

Antimicrobial activity was tested against a panel of microorganisms: *Staphylococcus aureus* and *Bacillus subtilis*, both Gram positive bacteria; *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa*, both Gram negative bacteria; and two fungi: *Candida albicans*, a yeast; and *Aspergillus niger*, a mould. All cultures with the exception of *E. coli* were originally obtained from the University of NSW culture collection. The disc diffusion method, known as the Kirby-Bauer method, was used to determine the antimicrobial activities of OMW extracts and biophenols (oleuropein, hydroxytyrosol, caffeic acid, verbascoside and luteolin). For antibacterial activity the method of Bisignano et al.

(Bisignano et al., 1999) was applied with variation of the concentrations of bacteria to 10^5-10^6 cfu/mL as used in the majority of current standard methods. OMW extracts were reconstituted in 25% aqueous ethanol to obtain concentrations of 5, 10 and 20% w/v. Hydroxytyrosol, oleuropein, and verbascoside were dissolved in 25% aqueous ethanol, while caffeic acid was dissolved in 50% ethanol and luteolin could only be dissolved in absolute ethanol. The concentrations were made up so as to test 100, 200, and 300 µg/disc of each phenolic compound. Mueller Hinton Agar (Oxoid, UK) was used for bacteria and Mueller Hinton Agar with 2% glucose (Lab Supply, Australia) plus 0.5µg/mL methylene blue was used for fungi as per NCCLS methodology M44-P in which glucose is used to improve growth of *C. albicans* and *A. niger*, while the methylene blue enhances the zone edge definition. Agar plates (22 mL/plate) were prepared, allowed to set, and surface dried at 37 °C for 30 min. Bacterial cultures were diluted in Nutrient broth (Oxoid, UK) to give a count of approximately 10^6cfu/mL. *Candida* was treated as mentioned for bacteria. Cultures of *A. niger* were grown on Malt extract agar slopes (Oxoid, UK) for 7 days at 35 °C. Spores were then removed with 2 × 5mL aliquots of sterile saline to which one drop of Tween 80 had been added. Serial dilutions of the spore suspension were performed in sterile saline (from 10^{-1} to 10^{-7} cfu/mL, then 250µL plated onto Sabouraud's Dextrose Agar (Oxoid, UK) to determine the concentration of the spore suspension. The plates were then incubated for 24 h at 25 °C. The volume of the inoculum was 100µL.

Blank sensitivity discs, 6mm, (Oxoid, UK) were allowed to warm to room temperature for 1 h then impregnated with 25 µL of each standard, extract or controls then left to dry in a sterile Petri dish for 90 min. Negative controls for standards and extracts were 25, 50 and 100% ethanol. Positive controls were tetracycline discs 30 µg (Oxoid, UK) for antibacterial activity, and amphotericin B (10 µg) and ketoconozole tablets (10 µg) (Dutec Diagnostics, Sydney, Australia) were used for antifungal activity. All plates were incubated at 37 °C for 16-18 h for bacteria, at 35 °C for 24 h for fungi. The

diameter of the inhibition zone was measured in mm (including disc) with callipers, three replicates were performed and the assays were duplicated.

2.9. Molluscicidal activity

Collection and breeding of snails: Fresh water snails *Isidorella newcombi* were collected from irrigation supply channels on a rice field near Yanco, NSW on 20th February 2004. The snails were transferred in thermally isolated plastic tanks using irrigation water. On arrival at the laboratory the snails were washed with de-ionized water and placed in aerated aquaria filled with 1X Martin's rearing solution (MRS), an artificial fresh water for acclimatization (Martin et al., 1980). Snails were fed on lettuce leaves. Mature snails (shell height ≥5 mm) were transferred after 3 d to breeding tanks. The breeding tanks were aerated glass aquaria filled with 1X modified Martin's rearing solution (MMRS) (Schodde, 1997). The MMRS was replaced every five days. Acclimatization, breeding and bioassays were performed in a temperature controlled room maintained at 24 ± 1 °C. After one week the immature snails were used for the molluscicidal bioassay.

Bioassay: The immature snails (7 to 10 days of age "less than 1 mm shell height") were used for screening for molluscicidal activity according to Stevens' method (Stevens, 2003b). The bioassay was performed in 50 mL glass beakers containing a final volume of 30 mL MRS and covered with a plastic film. A 1 cm^2 hole was made in the plastic film to permit aeration. To each beaker a group of 10 snails was randomly transferred with aid of 5 mL MRS. Different volumes of RCE were volumetrically diluted to 25 mL using MRS. Seven different concentrations were applied for each cultivar. Doses used were determined with a pre-screen to get partial % mortality (10 - 98%). Each bioassay was performed on 7 replicates over three days resulting in 70 snails exposed to each concentration. Negative controls were performed for each bioassay using MRS alone. Positive controls using copper sulfate 12 ppm (100% mortality) were also performed daily. Snails were exposed for 24 h and then

rinsed with de-ionized water. Mortality was assessed under a stereo-microscope after 24 h recovery in 30 mL MRS. Snails were considered dead if there was no response when a dissecting needle was applied to the edge of the mantle.

2.10. Statistical Analysis

All experiments were performed at least in triplicate. Data are expressed as means ± SD. Student t-test was used to test significant differences. For antioxidant activity, concentrations that resulted in 50% scavenging (EC_{50}) were calculated from the dose-response curves fitted to data points using Microsoft Excel software (Savelev et al., 2003). Statistical analysis of the mortality data was performed by Probit5 software package (NSW Agricultural Institute), where mortalities were corrected using Abbott's formula. The LC_{50} and LC_{90} values and their 95% confidence intervals were estimated by probit analysis (Schodde, 1997).

3. Results

3.1. Biophenol Content

Gross characterization of the biophenol content of OMW extracts is provided in Table 1. MOE had higher amounts of extractable matter and total phenols, as measured by both Folin Ciocalteu assay and direct spectrophotometric measurement at 280 nm, than FOE. The levels of individual biophenol classes in the extracts were indistinguishable with the exception of pigments, presumably anthocyanic, absorbing at 520 nm.

HPLC provided separation of individual biophenols in the extracts as illustrated in Fig. 2 for detection at 278 nm where both qualitative and quantitative differences between MOE and FOE are observed. Identification of biophenols was performed by comparing retention times and UV spectra of standards in HPLC-DAD and confirmed by relevant molecular mass data from LC-MS. Where an authentic standard was not available, as in the case of hydroxytyrosol glucoside, relative retention time and molecular mass from LC-MS provided a tentative assignment of the structure. As the main aim of this study was to screen OMW extracts for biological activities, a detailed characterization of individual compounds was not attempted and only the major peaks appearing at 278 nm were identified to assist understanding of the relation between the chemical composition and the observed bioactivities.

3.2. Antioxidant Activity

Both FOE and MOE showed concentration dependent DPPH radical scavenging activity with a linear relationship at concentrations less than 50 ppm (FOE, r^2 = 0.995; MOE, r^2 =0.994; Trolox, r^2 =1.00). The EC_{50} values of FOE and MOE were 28.3 ± 1.7 ppm and 34.7 ± 1.6 ppm, respectively while for caffeic acid and Trolox EC_{50} values were 1.73 ± 0.07 ppm and 4.2 ± 0.1 ppm, respectively. Expressing H-donating activity in terms of the amount of substrate scavenged per gram DPPH provides a more reasonable comparison as different starting concentrations of DPPH radical are used in different studies (Roginsky et al., 2005). On this basis, at concentrations equal to their EC_{50}, 650 mg FOE, 797 mg MOE, 39 mg caffeic acid and 96 mg Trolox reacted with one gram DPPH radical. For comparison, the TEAC values for FOE and MOE were 0.148 and 0.121, respectively. Reaction kinetics for radical scavenging (Fig. 3) show that MOE had a faster onset of action, but that FOE had a slightly higher activity by the end of the reaction.

Both FOE and MOE protected linoleic acid and subsequently minimized the decolourization of *β*-carotene in the BCBT (Fig. 4). MOE had an EC_{50} of 60.6 ± 2.3 ppm while FOE was less potent with an EC_{50} of 79.9 ± 2.0 ppm. The EC_{50} for caffeic acid was 50.4 ± 4.3 ppm.

3.3. Antimicrobial Activity

No antibacterial or antifungal activity was observed for the negative controls (25, 50, &100% ethanol) whilst the zone of inhibition for positive controls was as follows: 18.7 ± 0.5 mm and 20.7 ± 1.5 mm for amphotericin B (10 µg) against *Candida* and *Aspergillus*, respectively; 23.5 ± 4.7 mm and 15.1 ± 0.6 mm for ketoconozole (15 µg) against *Candida* and *Aspergillus*, respectively. No antifungal activity was found for OMW extracts or any of the biophenols tested and although luteolin did not show antifungal activity, its diffusion area turned green on *Candida* growth plates.

At lower concentrations, the extracts exhibited differential antibacterial action, but at 5 mg/disc both the OMW extracts were active against all the challenge bacteria (Table 2). Antibacterial activity results for individual biophenols were variable. None of the tested biophenols were active against *E. coli*. Verbascoside and caffeic acid, up to 300 µg, were totally ineffective against all the challenge microorganisms. Oleuropein was only active against *B. subtilis* with some observed resistance. Hydroxytyrosol had good activity against *Staph. aureus* and showed mild activity against *Pseudomonas aeruginosa* only at 300 µg. Luteolin had mild antibacterial activity against both the Gram positive *Staph. aureus* and *B. subtilis*, yet surprisingly the activity decreased slightly, but consistently, upon increasing the concentration.

3.4. Molluscicidal Activity

LD_{50} values are mostly determined for adult animals. However, the water-leaving behavior, the large volume bioassay vessels, and the need for aeration make adulticidal bioassays inappropriate for high throughput screening (HTS) of molluscicidal activity. Stevens (Stevens, 2003a) developed an HTS technique using immature snails which was adopted in the current study, recognizing that immature snails are likely to be more susceptible. A typical sigmoid dose-response curve was obtained for both OMW extracts (Fig. 4). MOE was more potent than FOE and this was reflected in their LD_{50}'s of 424 ppm and 541 ppm, respectively (Table 3). While the MOE dose-response curve exhibited the typical gradual increase in molluscicidal activity upon increasing the concentration, the FOE curve had an abrupt increase followed by a drop before reaching the steep portion of the curve. At high concentrations a nearly instantaneous toxic action of MOE was detected by visual observation of snails during the first few hours of bioassay suggesting a rapid onset of action. Stereo-microscopic examination revealed more severe disintegration of snails after 24 h exposure to MOE compared with snails subjected to FOE.

Although utilization of Stevens' technique (Stevens, 2003a) was largely for the lack of avoidance behavior (water-leaving behavior) exhibited by immature snails, our study produced a different outcome. The percentage of escape for MOE and FOE, i.e. the proportion of the snails that climbed out of the molluscicidal bioassay solution, was significantly high, between 4-14% (Fig. 6). The expelled snails were not included in the mortality statistics. Whilst the water-leaving behavior was negligible and similar for both controls (1.4%), different doses of FOE and MOE showed different water-leaving behavior.

4. Discussion

We have previously used sodium metabisulfite and formic acid in the extracting solvent to maximize recovery of biophenols (Obied et al., 2005a). However, their reducing properties are unsuitable for biological screening and hence the extraction solvent contained hydrochloric acid, which was also suitable for protecting biophenols and is more compatible with screening assays. Quantification and identification of biophenols in plant extracts is the first step towards evaluation of antioxidant activity (Becker et al., 2004) and can also provide insight into other biological activities. Both gross spectroscopic (Table 1) and chromatographic techniques were applied to assess the biophenol content of the extracts.

OMW samples were chosen arbitrarily as the purpose of this study was not to assess differences due to agronomic parameters. Thus, differences in phenolic composition between the two extracts may be ascribed to any or all of varietal, seasonal, processing and farming practice differences. For example, the very high pigment content of MOE was most probably due to the late harvesting of Mission fruit but varietal differences can not be ignored. For all other gross features presented in Table 1, FOE and MOE were comparable since the increase in the amount of total phenols was not proportional to the large increase in the amount of extractable matter.

HPLC with detection by DAD and ESI-MS provides valuable information on biophenolic composition. Qualitative and quantitative differences are obvious between the profiles of FOE and MOE (Fig. 2). MOE had the higher total phenol content consistent with its greater abundance of individual phenols with the exception of verbascoside (**5**) and the hydroxytyrosol-secoiridoid (**9**), which were higher in FOE. The reduced levels of (**5**) and (**9**) in MOE can be attributed to hydrolysis of both compounds to form hydroxytyrosol, hydroxytyrosol glucoside and caffeic acid. Although there was no significant difference in the flavonol content (Table 1), the amount of flavonoids recovered from MOE was

notably larger than FOE (Fig.1). Moreover, quercetin (**17**), luteolin-glucoside III (**15**) and apigenin-glycoside (**11**) which could not be detected in FOE were identified in MOE chromatograms. The increased recovery of the polymeric humps, **P1**, **P2**, and **P3,** in the case of MOE was consistent with the high readings at 520 nm.

Antioxidant activity

Antioxidant capacity assays can be classified in various ways; for example, as an electron-transfer or hydrogen atom transfer process (Huang et al., 2005; Roginsky et al., 2005). Antioxidant screening of plant extracts requires multidimensional evaluation of antioxidant activity (Frankel et al., 2000) combined with simple, fast and inexpensive assays. Thus, DPPH radical scavenging (electron-transfer, homogenous system) and BCBT (hydrogen-transfer, heterogenous system) were chosen for the current study. Although the DPPH assay was considered as a hydrogen atom transfer reaction, it has been found recently that the rate-limiting step is an electron-transfer reaction from the phenoxide anion to the DPPH radical in protic solvents e.g. methanol and ethanol (Foti & Ruberto, 2001).

EC_{50} is inversely proportional to antioxidant activity and hence FOE was more active than MOE in trapping DPPH radicals. However, the use of EC_{50} alone to depict antioxidant activity without examining reaction kinetics can be misleading (Roginsky et al., 2005). Study of the kinetics in the present work supports this observation. MOE reacted faster than FOE with the DPPH radical and reached a steady state after 20 min while FOE required 30 min to attain equilibrium. For the first 30 min, MOE was a more efficient scavenger, but after 30 min both extracts had similar efficiencies. The reaction followed a general multiplicative model equation: $\ln [DPPH^{\bullet}] = b \ln t + \ln a$, (Sánchez-Moreno et al., 1998) ($R^2 = 0.9792$ for FOE and $R^2 = 0.9227$ for MOE). The biphasic behaviour of the

phenolic reaction with the DPPH radical (Villano et al., 2006) was evident; in less than 1 min, 60-70 % of the DPPH radicals were scavenged, the reaction proceeding slowly to a plateau after 20 min. The initial fast reaction is due to the original biophenols, and the slower subsequent decay is due to their degradation products (Villano et al., 2006).

Microemulsion models provide a means to measure not only the antioxidant capacity but also the accessibility (partitioning) of the antioxidant to substrate. Oxidation happens within the micelles and hence antioxidants of hydrophobic nature are expected to inhibit the chain oxidation more efficiently. BCBT is one of the early model systems (Miller, 1971) that is still frequently applied in screening for antioxidant activity, mainly due to commercial availability of its components at an economic price. In contrast to the DPPH assay, MOE was a more efficient chain-breaking antioxidant than FOE in BCBT. This suggests the more hydrophobic nature of the MOE antioxidants, which can be ascribed to the higher polymeric pigment content and/or the hydrolysis of some compounds e.g. verbascoside (**5**). Interestingly, the EC_{50} value (50.4 ± 4.3ppm) for caffeic acid in BCBT was 30 times the EC_{50} value (1.73 ± 0.07 ppm) in the DPPH assay. On the other hand, the corresponding ratios for FOE and MOE were ca. three and ca. two, respectively. Unless there are some very potent chain-breaking antioxidants that caused much of the observed activity in BCBT, this is an example of the broad spectrum antioxidant activity of a plant extract versus that of a pure compound.

Comparison of bioactivity data is hindered by the absence of standardised method(s) for measuring antioxidant activity, as well as different conditions, measurement techniques, data analysis or presentation. For example, DPPH radical scavenging was reported for Italian OMW (Visioli et al., 2002), French OMW (Lesage-Meessen et al., 2001), and Jordanian olive mill cake (Amro et al., 2002). The antioxidant activity of a hydroalcoholic extract of two-phase OMW was reported with an EC_{50} of

25.31 ± 3.21 ppm for the Italian variety Coratina (Aldini et al., 2006). This value is similar to our reported activity for FOE (12% higher) and MOE (37% higher). However, caffeic acid under their conditions had an EC_{50} of 2.82 ± 0.07 ppm which is higher than our value of 1.73 ± 0.07 ppm. Under our conditions and using caffeic acid as a standard, the EC_{50} for Coratina is 15.5 ppm which is 200% more active.

There are no reported data on the activity of OMW and olive biophenols using BCBT. Both OMW extracts were effective free radical scavengers and chain-breaking antioxidants that acted as primary antioxidants. However, further cleanup and fractionation is required to identify and extract the potent antioxidant(s) from the less active matrix components.

Antimicrobial activity

Neither extract exhibited activity against tested fungi. This is unexceptional since antifungal activity requires considerable lipophilicity while OMW biophenols are essentially hydrophilic, the more lipophilic constituents are partitioned into the olive oil during processing. In the case of luteolin, the green colour developed on the *Candida* growth plate at the end of the incubation period was investigated. Luteolin has a yellow colour in acidic and neutral solutions with an increase in intensity as pH increases but without development of a green colour. However, both oxidation with Folin Ciocalteu reagent and concentrated H_2SO_4 as well as chelation with iron(III) and copper(II) produced a green coloured product.

Most plant extracts show activity against Gram positive bacteria but activity against the Gram negative bacteria (and fungi) is a critical measure of success. In contrast to the antifungal results,

both OMW extracts showed broad spectrum antibacterial activity at high concentrations which is common in plant extracts due to the wide array of phytochemicals that provides synergistic and additive action. FOE had higher antibacterial activity against the Gram positive bacteria whereas MOE was more active against the Gram negative bacteria. The different activities against Gram negative and Gram positive bacteria may be rationalized by considering differences in cell wall composition. Gram negative bacteria have a lipopolysaccharide component in their outer membrane that makes them more resistant to antibacterial compounds. MOE biophenols were more hydrophobic than FOE as evident from the BCBT results, which may improve their activity against Gram negative bacteria.

No activity was detected for the pure biophenols tested against *E. coli* and only very high concentrations of extracts and hydroxytyrosol were active against *Ps. aeruginosa* one of the very stubborn Gram negative organisms. Fig. 2 shows that hydroxytyrosol was one of the major constituents of both extracts with a higher content in MOE, and this was consistent with the higher activity of MOE against *Ps. aeruginosa*. There is no standard method or evaluation criteria for screening antimicrobial activity in plant extracts (Hadacek et al., 2000; Nostro et al., 2000). Differences in bacterial strains, antimicrobial assay, growth media, size of the inoculum and concentration of the challenge microorganism make comparisons of antimicrobial data of plant extracts from different sources very difficult. Various antimicrobial activities have been noted for caffeic acid (Baranowski et al., 1980; Baranowski et al., 1982; Paster et al., 1988; Aziz et al., 1998) and verbascoside (Pardo et al., 1993; Didry et al., 1999) although the results are controversial and both compounds exhibited absolutely no antimicrobial activity in the current study. Nevertheless, our results for pure compounds are generally consistent with previous reports considering differences in assay procedure and the relative purity of test substances (Aziz et al., 1998; Bisignano et al., 1999; Capasso et al., 1995; Tranter et al., 1993). Luteolin was interesting in that activity was

restricted to Gram positive bacteria but more surprisingly activity decreased with the increase in the concentration (100-300 μg).

Molluscicidal activity

Isidorella newcombi is one of the most important invertebrate pests of irrigated rice crops in NSW (Stevens et al., 1996). The LD_{50} values for FOE and MOE against *Isidorella newcombi* were 541 ppm and 424 ppm, respectively. Although LD_{50} is widely accepted as a general measurement of chemical toxicity, it is not a biological constant and it is a measure of lethality that ignores other adverse effects. The examination of the dose-response curves (Fig. 5) in addition to LD_{50} and LD_{90} can provide a better assessment of molluscicidal activity of crude extracts. At concentrations lower than LD_{50}, MOE was two to three fold more active than FOE. The molluscicidal activity of MOE showed 10% mortality at 92 ppm; while for FOE 319 ppm produced 10% mortality. The difference in activity decreased gradually upon increasing the dose; at LD_{50} MOE was 1.5 times more active, whilst at LD_{90} MOE was only 1.2 times more active than FOE. The dose-response for MOE was typical, while the curve for FOE shows apparent biphasic behaviour. Although not statistically significant at $p < 0.05$, this type of behaviour is known in experimental toxicology as hormesis (low dose stimulation and high dose inhibition) (van der Woude et al., 2005).

The water-leaving behaviour (avoidance behaviour) of palnorbids including Isidorella (Schodde, 1997) and the repellent action of phenolic compounds (Jurberg et al., 1995) are documented phenomena. Exit Index and Escape Index were introduced to measure the repellency of molluscicides in vector molluscs of schistosomiasis (Jurberg et al., 1995), but these indices represent the percentage of snails that have exited the bioassay solution and remain alive for 24 h relative to the number of live snails in the control group. MOE demonstrated an ideal model for water-leaving

behavior where the percentage of escape increased with the increase in sub-lethal doses till a maximum (14.3%) just below the LD_{50} that gradually decreased with the increase of the dose above the LD_{50} till a zero value above LD_{90}. FOE showed a similar water-leaving behavior with an abrupt increase of the escape percentage just below the LD_{90}. Water-leaving behaviour is known to increase the survival of some palnorbids after exposure to molluscicidal treatments that results in rapid re-colonization and failure of snail control campaigns (Sarquis et al., 1997). However, the real impact of this phenomenon in the case of immature snails is not clear, as immature Isidorella snails, in the current study, were highly non-resistant to desiccation, where all escaped snails died shortly after climbing out.

Conclusion

OMW extracts in the present study had free radical scavenging activity, chain terminator activity, antibacterial and molluscicidal activity. Recognizing that the crude extracts contain less than 10% biophenols, further clean up and fractionation should produce more potent activities. However, the potent antioxidant activity at low concentrations of the crude extract suggests further investment in antioxidant activity.

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References

Aldini, G., Piccoli, A., Beretta, G., Morazzoni, P., Riva, A., Marinello, C. and Facino, R.M. (2006) Antioxidant activity of polyphenols from solid olive residues of Cv. *Coratina*. Fitoterapia 77, 121-128.

Amro, B., Aburjai, T. and Al-Khalil, S. (2002) Antioxidative and radical scavenging effects of olive cake extract. Fitoterapia 73, 456-461.

Aziz, N.H., Farag, S.E., Mousa, L.A.A. and Abo-Zaid, M.A. (1998) Comparative antibacterial and antifungal effects of some phenolic compounds. Microbios 93, 43-54.

Baranowski, J.D., Davidson, P.M., Nagel, C.W. and Branen, A.L. (1980) Inhibition of *Saccharomyces cerevisiae* by the naturally occuring hydroxycinnamates. Journal of Food Science 45, 592-594.

Baranowski, J.D. and Nagel, C.W. (1982) Inhibition of *Pseudomonas fluorescens* by hydroxycimnnamic acids and their alkyl esters. Journal of Food Science 47, 1587-1589.

Becker, E.M., Nissen, L.R. and Skibsted, L.H. (2004) Antioxidant evaluation protocols: Food quality or health effects. European Food Research and Technology 219, 561-571.

Bisignano, G., Tomaino, A., Lo Cascio, R., Crisafi, G., Uccella, N. and Saija, A. (1999) On the *in-vitro* antimicrobial activity of oleuropein and hydroxytyrosol. Journal of Pharmacy and Pharmacology 51, 971-974.

Capasso, R., Cristinzio, G., Evidente, A. and Scognamiglio F. (1992) Isolation, spectoscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. Phytochemistry 31, 4125-4128.

Capasso, R., Evidente, A., Schivo, L., Orru, G., Marcialis, M.A. and Cristinzio, G. (1995) Antibacterial polyphenols from olive oil mill waste waters. Journal of Applied Bacteriology 79, 393-398.

Capasso, R., Evidente, A., Trembaly, E., Sala, A., Santoro, C., Cristinzio, G. and Scognamiglio, F. (1994) Direct and mediated effects on *Bactrocera oleae* (Gmelin) (Diptera: Tephritidae) on natural

polyphenols and some of the related synthetic compounds: structure-activity relationships. Journal of Chemical Ecology 20, 1189-1199.

Casa, R., D'Annibale, A., Pieruccetti, F., Stazi, S.R., Sermanni, G.G. and Lo Cascio, B. (2003) Reduction of the phenolic components in olive-mill wastewater by an enzymatic treatment and its impact on Durum Wheat (*Triticum durum* Desf.) germinability. Chemosphere 50, 959-966.

Della Greca, M., Monaco, P., Pinto, G., Pollio, A., Previtera, L. and Temussi, F. (2001) Phytotoxicity of low-molecular weight phenols from olive-mill wastewaters. Bulletin of Environmental Contamination and Toxicology 67, 352-359.

Didry, N., Seidel, V., Dubreuil, L., Tillequin, F. and Bailleul, F. (1999) Isolation and antibacterial activity of phenylpropanoid derivatives from *Ballota nigra*. Journal of Ethnopharmacology 67, 197-202.

Fodale, A.S., Mule, R. and Bati, B. (1999) The antifungal activity of olive oil waste water on isolates of *Verticillium dahliae* Kleb. *in vitro*. Acta Horticulturae 474, 753-756.

Foti, M. C. and Ruberto, G., 2004. Kinetic solvent effects on phenolic antioxidants determined by spectrophotometric measurements. Journal of Agricultural and Food Chemistry 49, 342-348.

Frankel, E.N. and Meyer, A.S. (2000) The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. Journal of the Science of Food and Agriculture 80, 1925-1941.

Fukumoto, L.R. and Mazza, G. (2000) Assessing antioxidant and prooxidant activities of phenolic compounds. Journal of Agricultural and Food Chemistry 48, 3597-3604.

Hadacek, F. and Greger, H. (2000) Testing of antifungal natural products: Methodologies, comparability of results and assay choice. Phytochemical Analysis 11, 137-147.

Huang, D.J., Ou, B.X. and Prior, R.L. (2005) The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry 53, 1841-1856.

Jurberg, P., Sarquis, O., Santos J.A.A. and Ferreira, R.C.R. (1995) Effect of niclosamide (Bayluscide WP 70 R), *Anacardium occidentale* hexane extract and *Euphorbia splendens* latex on behaviour of *Biomphalraia glabrata* (Say, 1818), under laboratory conditions. Memórias do Instituto Oswaldo Cruz 90 , 191-194.

Kubo, I. and Hanke, F.J. (1985) Chemically based resistance in plants. In: Chemically mediated interactions between plants and other organisms, Vol. 19New York, USA, pp. 171-194.

Kulisic, T., Radonic, A., Katalinic, V. and Milos, M. (2004) Use of different methods for testing antioxidative activity of oregano essential oil. Food Chemistry 85, 633-640.

Kumazawa, S., Hamasaka, T. and Nakayama, T. (2004) Antioxidant activity of Propolis of various geographic origins. Food Chemistry 84, 329-339.

Leger, C.L., Kadiri-Hassani, N. and Descomps, B. (2000) Decreased superoxide anion production in cultured human promonocyte cells (THP-1) due to polyphenol mixtures from olive oil processing wastewaters. Journal of Agricultural and Food Chemistry 48, 5061-5067.

Lesage-Meessen, L., Navarro, D., Maunier, S., Sigoillot, J.C., Lorquin, J., Delattre, M., Simon, J.L., Asther, M. and Labat, M. (2001) Simple phenolic content in olive oil residues as a function of extraction systems. Food Chemistry 75, 501-507.

Martin, J., Kuvangkadilok, C., Peart, D.H. and Lee, B.T.O. (1980) Multiple sex determining regions in a group of related *Chironomus* species (Diptera: Chironomidae). Heredity 44, 367-382.

Matthäus, B. (2002) Antioxidant activity of extracts obtained from residues of different oilseeds. Journal of Agricultural and Food Chemistry 50, 3444-3452.

McDonald, S., Prenzler, P.D., Antolovich, M. and Robards, K. (2001) Phenolic content and antioxidant activity of olive extracts. Food Chemistry 73, 73-84.

Miller, H.E. (1971) A simplified method for the evaluation of antioxidants. Journal of the American

Oil Chemist's Society 45, 91.

Moreno, E., Quevedo-Sarmiento, J. and Ramos-Cormenzana, A. (1989) Antibacterial activity of wastewaters from olive oil mills. In: P.N. Cheremisoff, (Ed), Encyclopedia of Environmental Control Technology, Vol. 4, Gulf Publishing Co., Houston, pp. 731-757.

Niaounakis, M. and Halvadakis, C.P. (2004), *Olive-Mill Waste Management* (First edition), Typothito-George Dardanos, Athens.

Nostro, A., Germano, M.P., D'angelo, V., Marino, A. and Cannatelli, M.A. (2000) Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Letters in Applied Microbiology 30, 379-384.

Obied, H.K., Allen, M.S., Bedgood, D.R., Prenzler, P.D. and Robards, K. (2005a) Investigation of Australian olive mill waste for recovery of biophenols. Journal of Agricultural and Food Chemistry 53, 9911-9920.

Obied, H.K., Allen, M.S., Bedgood, D.R., Prenzler, P.D., Robards, K. and Stockmann, R. (2005b) Bioactivity and analysis of biophenols recovered from olive mill waste. Journal of Agricultural and Food Chemistry 53, 823-837.

Pardo, F., Perich, F., Villarroel, L. and Torres, R. (1993) Isolation of verbascoside, an antimicrobial constituent of *Buddleja-Globosa* leaves. Journal of Ethnopharmacology 39, 221-222.

Paster, N., Juven, B.J. and Harshemesh, H. (1988) Antimicrobial activity and inhibition of aflatoxin B1 formation by olive plant tissue constituents. Journal of Applied Bacteriology 64, 293-297.

Ramos-Cormenzana, A., Juarez-Jimenez, B. and Garcia-Pareja, M.P. (1996) Antimicrobial activity of olive mill waste-waters (Alpechin) and biotransformed olive oil mill wastewater. International Biodeterioration & Biodegradation 38, 283-290.

Roginsky, V. and Lissi, E.A. (2005) Review of methods to determine chain-breaking antioxidant activity in food. Food Chemistry 92, 235-254.

Sarquis, O., Pieri, O.S. and Santos, J.A.A. (1997) Effects of Bayluscide WP70R on the servival and water-leaving behaviour of *Biomphalaria straminea*, snail host of schistosomiasis in North-east Brazil. Memórias do Instituto Oswaldo Cruz 92, 619-623.

Savelev, S., Okello, E., Perry, N.S.L., Wilkins, R.M. and Perry, E.K. (2003) Synergistic and antagonistic interactions of anticholinesterase terpenoids in *Salvia lavandulaefolia* essential oil. Pharmacology Biochemistry and Behavior 75, 661-668.

Schodde, A.L. (1997), *Toxicity of two agricultural pesticides to the fresh water snail Isidorella newcombi* Honours Thesis, Charles Sturt University, Wagga Wagga, NSW.

Stevens, M.M. (2003a) Toxicity of bait pellets, methiocarb, and endothal to the snail *Isidorella newcombi,* RIRDC Publication, *Improving bloodworm, earthworm, and snail control in rice*, Rural Industries Research and Development Corporation , Barton, ACT, Australia.

Stevens, M.M. (2003b) Development of chlorothalonil for control of the rice snail *Isidorella newcombi,* RIRDC Publication, *Improving bloodworm, earthworm and snail control in rice*, Rural Industries Research and Development Corporation, Barton, ACT, Australia.

Stevens, M.M., Faulder, R.J. and Coombes, N.E. (1996) Microcosm assessment of potential molluscicides for control of the rice snail *Isidorella newcombi sens. lat.* (Gastropoda:Basommatophora: Planorbibae). Australian Journal of Agricultural Research 47, 673-680.

Sánchez-Moreno, C., Larrauri, J.A. and Saura-Calixto, F. (1998) A procedure to measure the antiradical efficiency of polyphenols. Journal of the Science of Food and Agriculture 76, 270-276.

Tranter, H.S., Tassou, S.C. and Nychas, G.J. (1993) The effect of the olive phenolic compound, oleuropein, on growth and enterotoxin B production by *Staphylococcus aureus*. Journal of Applied Bacteriology 74, 253-259.

van der Woude, H., Alink, G.M. and Rietjens, I.M.C.M. (2005) The definition of hormesis and its

implications for *in vitro* to *in vivo* extrapolation and risk assessment. Critical Reviews in Toxicology 35, 603-607.

Villano, D., Fernandez-Pachon, M.S., Moya, M.L., Troncoso, A.M. and Garcia-Parrilla, M.C. (2006), 'Radical scavenging ability of polyphenolic compounds towards DPPH free radical', *Talanta,* In Press, doi:10.1016/j.talanta.2006.03.050.

Visioli, F., Poli, A. and Galli, C. (2002) Antioxidant and other biological activities of phenols from olives and olive oil. Medicinal Research Reviews 22, 65-75.

Visioli, F., Romani, A., Mulinacci, N., Zarini, S., Conte, D., Vincieri, F.F. and Galli, C. (1999) Antioxidant and other biological activities of olive mill waste waters. Journal of Agricultural and Food Chemistry 47, 3397-3401.

Visioli, F., Vinceri, F.F. and Galli, C. (1995) Waste waters from olive oil production are rich in natural antioxidants. Experientia 51, 32-34.

von Gadow, A., Joubert, E. and Hansmann, C.F. (1997) Comparison of the antioxidant activity of Aspalathin with that of other plant phenols of Rooibos Tea. Journal of Agricultural and Food Chemistry 45, 632-638.

CAPTIONS

Table 1. Data for extractable matter, total phenols, and different biophenolic classes recovered from Mission olive mill waste-reconstituted crude extract (MOE) and Frantoio olive mill waste-reconstituted crude extract (FOE).

Table 2. Antimicrobial activity of biophenols and OMW extracts.

Table 3. Summary of results from probit analysis on MOE and FOE molluscicidal activity on *Isidorella newcombi* immature snails.

Fig. 1. The major biophenols identified in Australian OMW extracts.

Fig. 2. RP-HPLC of the phenolic profile of FOE and MOE at 278 nm; **1**) Hydroxytyrosol glucoside, **2**) Hydroxytyrosol, **3**) Tyrosol, **4**) Caffeic acid, **5**) Verbascoside, **6**) Luteolin glycoside I, **7**) Rutin, **8**) Verbascoside isomer, **9**) hydroxytyrosol secoiridoid molecular mass 382, **10**) secoiridoid molecular mass 378, **11**) Apigenin glycoside, **12**) Luteolin glycoside II, **13**) Oleuropein, **14**) Oleuropein isomer, **15**) Luteolin-glucoside III, **16**) Unknown molecular mass 366, **17**) Quercetin and **18**) Luteolin, **P1**) , **P2**) & **P3**) polymeric substances. Peaks 2, 3, 4, 5, 7, 8, 13, 17, and 18 were identified by use of standards, and comparing retention data, UV and mass spectra. The remaining peaks were tentatively identified by comparison of UV-spectra, MS data, and relative retention time on RP-HPLC with literature data.

Fig. 3. Kinetics of DPPH radical scavenging activity of FOE and MOE.

Fig. 4. Dose-response curve of antioxidant activity of OMW extracts in BCBT.

Fig. 5. Dose-response curves for molluscicidal activity of OMW extracts: A. Dose represented as ppm (mg EM per litre of assay solvent) B. Dose represented as ppm GAE (mg GAE per litre of assay solvent). Standard deviation < 20%.

Fig. 6. Water leaving behaviour of snails exposed to FOE and MOE.

* Table 1.

	Concentration (mg/g dry weight)	
	FOE	MOE
extractable matter (dried extract)	191.1 ± 14.0a	272.4 ± 7.6b
total phenols (measured as Folin Ciocalteu reactive substances)[a]	17.7 ± 0.9a	21.1 ± 0.9b
total phenols (measured at 280 nm)[a]	9.9 ± 0.64a	14.3 ± 0.45b
hydroxycinnamic acids (320 nm)[b]	3.18 ± 0.15a	3.20 ± 0.14a
flavonols (360 nm)[c]	3.50 ± 0.08a	3.47 ± 0.21a
pigments (520 nm)[d]	0.28 ± 0.01a	2.74 ± 0.10b
o-diphenols[b]	8.85 ± 0.37a	8.61 ± 0.28a

Expressed as: [a] GAE, [b] CAE, [c] QE, and [d] CCE

Different letters in the same row indicate significantly different mean ± standard deviation of triplicates ($p > 0.05$).

Table 2.

Organism	*Staph. aureus*	*B. subtilis*	*E. coli*	*Ps. Aeruginosa*
Test substance (amount/disc)	Zone of inhibition (mm)[a]			
FOE (1.25 mg)	0	7.2[b] ±0.3	0	0
FOE (2.50 mg)	8.1 ± 0.3	8.3[b] ± 0.3	0	6.7[b] ± 0.1
FOE (5.00 mg)	8.9 ± 0.6	9.9[b] ± 0.6	6.7 ± 0.1	7.4 ± 0.4
MOE (1.25 mg)	6.8 ± 0.1	6.8 ± 0.1	0	0
MOE (2.50 mg)	7.8 ± 0.4	8.2 ± 0.4	6.8 ± 0.1	6.7 ± 0.1
MOE (5.00 mg)	9.2 ± 0.9	9.8 ± 1.1	8.2 ± 0.4	7.6 ± 0.1
oleuropein (100 µg)	0	10.6[b] ± 0.6	0	0
oleuropein (200 µg)	0	16.4[b] ± 0.5	0	0
oleuropein (300 µg)	0	18.5[b] ± 0.6	0	0
hydroxytyrosol (100 µg)	9.5[b] ± 0.3	0	0	0
hydroxytyrosol (200 µg)	17.5 ± 0.5	0	0	0
hydroxytyrosol (300 µg)	20.0 ± 2.0	0	0	7.0 ± 0.2
luteolin	7.8[b] ± 0.1	7.9[b]± 0.1	0	0

(100 µg)				
luteolin	7.5[b] ± 0.2	7.5[b]± 0.1	0	0
(200 µg)				
Luteolin	6.9[b] ± 0.1	7.1[b]± 0.1	0	0
(300 µg)				
tetracyclin	29.3 ± 1.9	20.1 ± 2.1	24.7 ± 1.1	14.7 ± 0.3
(30.0 µg)				

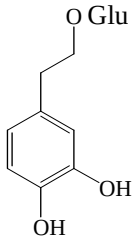
[a] Diameter of zone of inhibition (mm) including diameter of 6 mm disc; results quoted as the average of a minimum of six readings ± standard deviation; 0 mm indicates no visible zone of inhibition

[b] incomplete inhibition

Table 3.

Statistics	FOE	MOE
LD_{50} (ppm, extractable matter)	541	424
LD_{50} confidence interval	522-561	393-457
LD_{90} (ppm, extractable matter)	712	745
LD_{90} confidence interval	669-757	651-853
Slope	9.89	5.23
standard error slope	1.83	1.25
Chi squared	29.36	44.91
LD_{50} (ppm, GAE)	50	33
LD_{50} confidence interval	48-52	30-35
LD_{90} (ppm, GAE)	66	58
LD_{90} confidence interval	62-70	50-66
Slope	10.81	5.21
standard error slope	2.42	1.24
Chi squared	41.87	44.75

Fig. 1.



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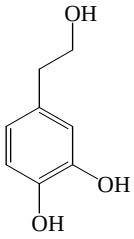

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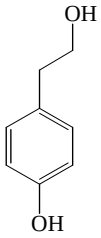
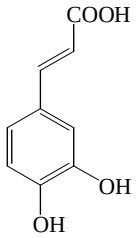
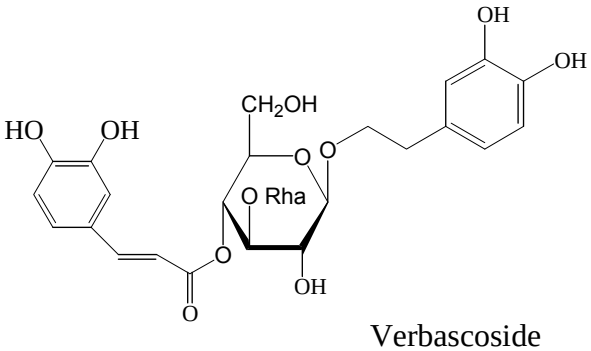


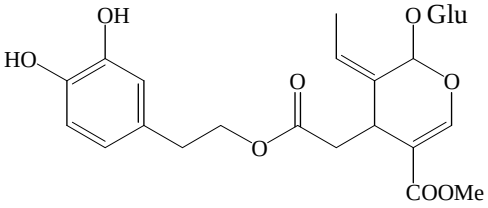
Tyrosol



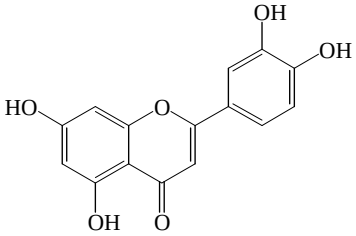
Caffeic acid



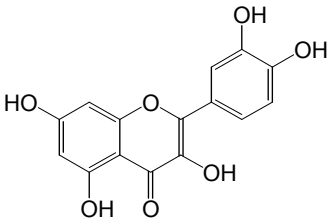
Verbascoside



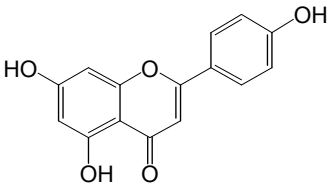
Oleuropein



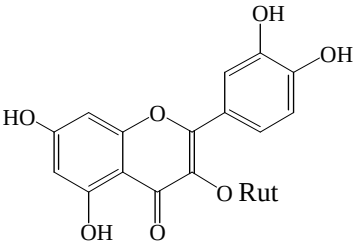
Luteolin



Quercetin



Apigenin



Rutin

Glu = Glucose

Rut = Rutinose

Rha = Rhamnose

Fig. 2

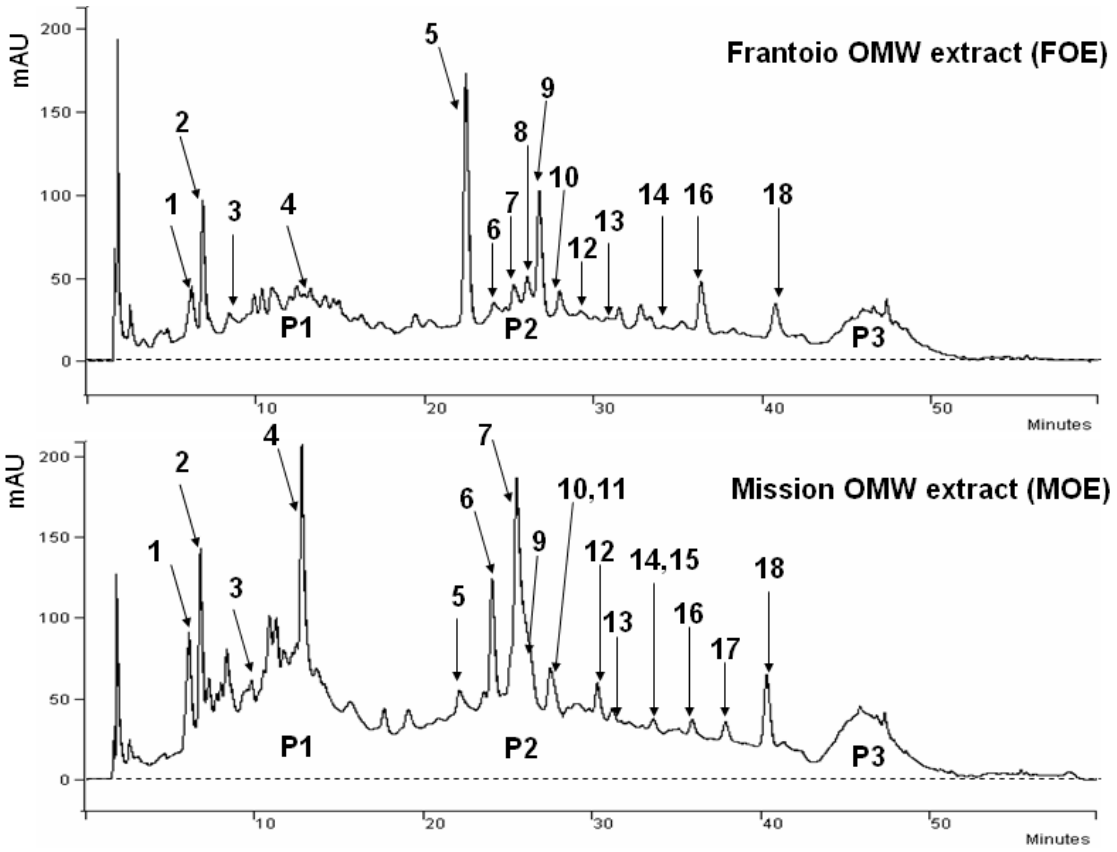

Frantoio OMW extract (FOE)
mAU
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
12
13
14
16
18
P1
P2
P3
10
20
30
40
50
Minutes
Mission OMW extract (MOE)
mAU
200
150
100
50
0
1
2
3
4
5
6
7
9
10,11
12
13
14,15
16
17
18
P1
P2
P3
10
20
30
40
50
Minutes

Fig. 3.

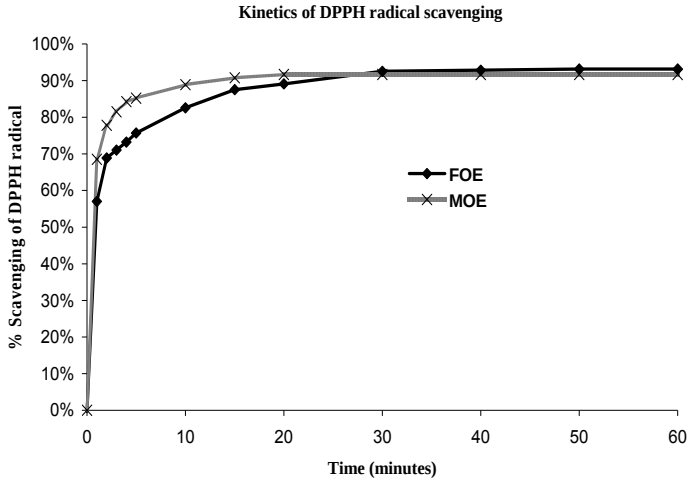

Kinetics of DPPH radical scavenging
% Scavenging of DPPH radical
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
10
20
30
40
50
60
Time (minutes)
FOE
MOE

Fig. 4.

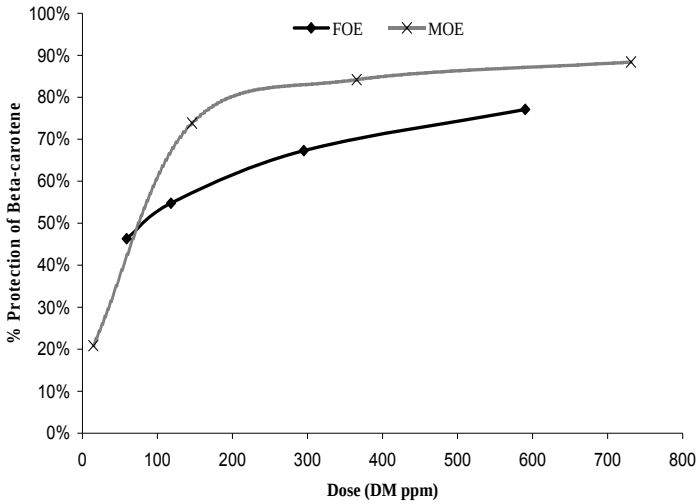
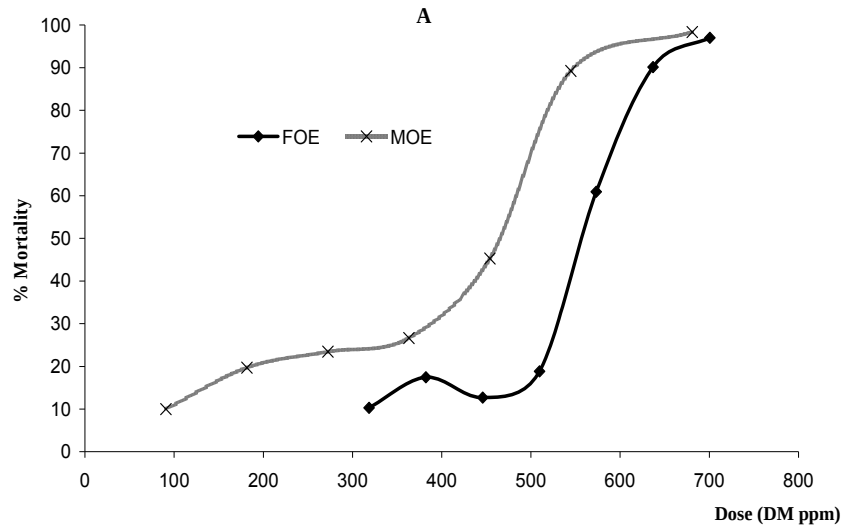

FOE
MOE
% Protection of Beta-carotene
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
100
200
300
400
500
600
700
800
Dose (DM ppm)

Fig. 5.


A
FOE
MOE
% Mortality
Dose (DM ppm)
0
10
20
30
40
50
60
70
80
90
100
0
100
200
300
400
500
600
700
800

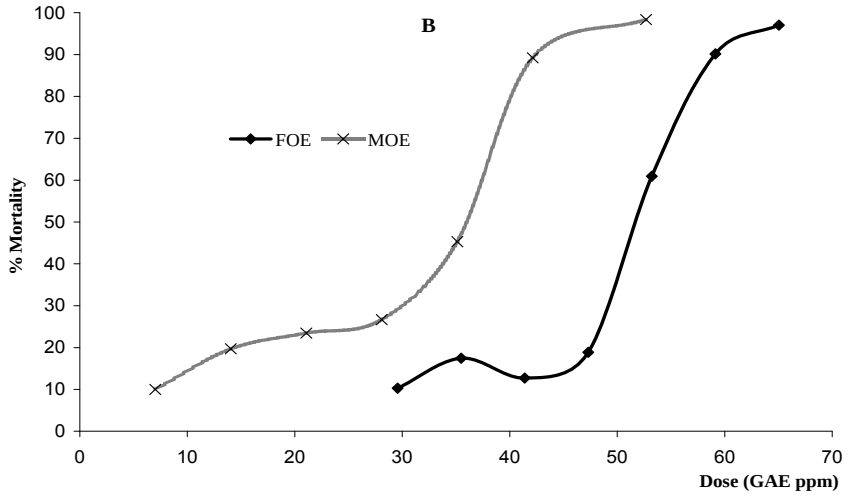
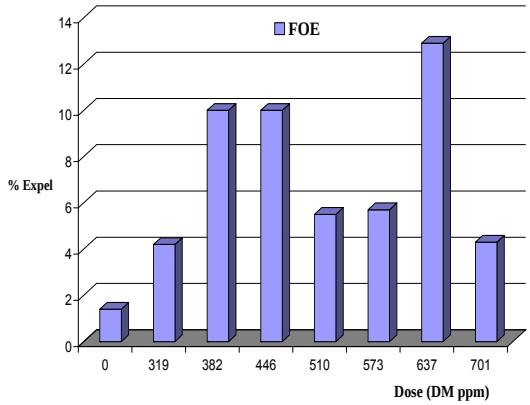
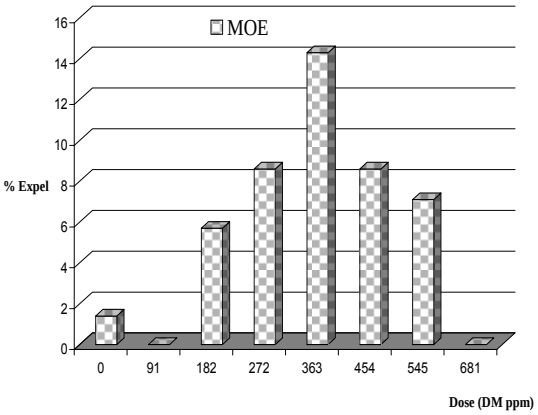

B
FOE
MOE
% Mortality
Dose (GAE ppm)
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
70

Fig. 6.


FOE
% Expel
14
12
10
8
6
4
2
0
0
319
382
446
510
573
637
701
Dose (DM ppm)


MOE
% Expel
16
14
12
10
8
6
4
2
0
0
91
182
272
363
454
545
681
Dose (DM ppm)