



Research Journal of Pharmaceutical, Biological and Chemical Sciences

Totarol Content and Cytotoxicity Varies Significantly in Different Types of Propolis

Elizabeth Jane Zammit^a, Krystle Blair Theuma^b, Stephanie Darmanin^b, Marilena Muraglia^c, Marie Therese Camilleri-Podesta^b, Joseph A. Buhagiar^d, Jean Calleja-Agius^b, Maurice Zarb Adami^a, Mark Micallef^e, Carlo Franchini^c, Pierre Schembri-Wismayer^{b*}

^a Department of Pharmacy, Faculty of Medicine and Surgery, University of Malta, Msida, Malta,

^b Department of Anatomy, Faculty of Medicine and Surgery, University of Malta, Msida, Malta,

^c Dipartimento Farmaco-Chimico, University of Bari, Bari, Italy,

^d Department of Biology, Faculty of Science, University of Malta, Msida, Malta

^e Department of Clinical Pharmacology and Therapeutics, Faculty of Medicine and Surgery, University of Malta, Msida Malta

ABSTRACT

Propolis is a complex honeybee product deposited in the beehives, where it protects the hive and its occupants from microbial infection. Propolis has several reported medical applications in view of its numerous bioactive properties. The water insoluble fraction of crude Maltese honeybee propolis was extracted in methanol. Analysis by gas chromatography – mass spectrometry (GC-MS) showed the diterpenoid totarol to be the predominant constituent in all samples. The evaporated methanol residue was dissolved in dimethyl sulphoxide (DMSO) and used for cytotoxicity testing on human cancer cell lines using standard 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assays. Results obtained show that the propolis collected from Malta has cytotoxic activity in cancer cells in vitro. However, propolis collected from different sites, only a few miles apart and at different times of the year, showed marked variations in the cytotoxicity, which correlated clearly with totarol content. This reflects the differences in the species of plants, on which the bees had foraged and indicates the importance of collection site and season of collection on the bioactivity of propolis products.

Keywords: propolis; cytotoxicity; totarol

**Corresponding Author*



INTRODUCTION

Propolis is the odorous, sticky, resinous, pale-yellow to dark-brown material which bees use to strengthen and join the hive cells, and to seal their hives from penetration by water and cold [1, 2]. It is furthermore thought to provide protection to the beehives from microbial infection [3]. Propolis is made of a complex mixture of beeswax, together with small amounts of sugars and plant exudates collected by honeybees from buds of some trees notably conifers and aromatic plants [4, 5]. The chemical composition of propolis depends on the season of collection and on the vegetation of the area and this is reflected in variations of its colour and odour [6].

Several biological properties have been reported for propolis or its constituents, including cytotoxic, antioxidant, anti-inflammatory, antiseptic, antimycotic, bacteriostatic, astringent, spasmolytic, and anaesthetic properties [7-9]. The antimicrobial activity of Egyptian propolis includes activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* [10]. Due to these supposed beneficial effects, there is a renewal of interest in the composition and biological activities of propolis.

Propolis is produced by honeybees using substances actively secreted by plants, or exuded from their wounds including lipophilic materials on leaves and leaf buds, resins, mucilages, gums and lattices [11]. The main chemical classes found in propolis, which appear to be the principal components responsible for the biological activities of propolis samples, include flavonoids, aromatic acids, diterpenic acids and phenolic compounds [2, 6, 12, 13]. The flavonoids concentrated in propolis are powerful antioxidants that can protect tissues from the DNA-damaging effects of a variety of harmful chemicals. Flavonoids can prevent cancer by scavenging oxidizing species and preventing DNA damage [6].

Furthermore the use of propolis as an immuno-stimulatory adjuvant for treatment of tumours is slowly gaining ground. Pre-clinical studies showed that co-administration of a propolis extract together with traditional chemotherapy resulted in better regression of tumours [14].

Several studies have shown that the concentrations of biologically active compounds extracted from natural products differ according to the sampling season, leading to differences in their cytotoxic effect [15, 16]. Re-number these references

The Maltese islands are situated in the middle of the Mediterranean Sea and span an area less than 50 km long all together and around 20 km wide. They possess a very rich wild flora with over one thousand flowering plants recorded. The islands were well known in the ancient world for the abundant production of honey and remained renowned for this honey production through successive centuries including the Arab and Norman colonisations, as reported by Arab geographer Al Idrisi in the 12th century AD [17].

The climate is predominantly semi-arid and the hot, dry summer months are very stressful to plant growth. The natural vegetation is therefore characterized by evergreen trees and shrubs which resist the adverse summer heat and drought, and a very large

number of herbaceous plants. The main periods of plant growth are autumn and spring, due to the optimal temperature and rainfall in these seasons [18].

The purpose of this study was therefore to analyse an extract of Maltese honeybee propolis, collected from different locations in Malta, a very small Mediterranean island, identify the major chemical constituents and investigate the *in vitro* cytotoxic activity of these extracts against human cancer cell lines.

MATERIALS AND METHODS

Propolis extraction

Crude honeybee propolis was collected from beehives, located in three areas of the Maltese Islands with different vegetation. Propolis sample 1 was collected in an area in the North of Malta, in March whilst bees were foraging on *Pinus halepensis* Mill., *Eucalyptus gomphocephala* DC., and *Cupressus sempervirens* L. Propolis sample 2 was collected in November from the same area whilst the bees were predominantly foraging on *Ceratonia siliqua* L. Propolis sample 3 was collected from an area in Central Malta in July whilst bees were foraging on *Lycopersicum esculentum* Miller, *Cucurbita pepo* L., *Prunus persica* (L.) Batsch and *Prunus armenaica* L. Propolis sample 4 was collected from another area in the North of Malta in June 2002 whilst bees were foraging largely on *Coridothymus capitatus*. The extraction method followed was according to a previously published method [6]. The crude propolis (4g) was added to water (15ml) heated to 80°C and stirred continuously. After incubating at 80°C for two hours, the water extract was filtered. The process was repeated and the combined filtrate was set aside for future analysis.

The insoluble residue was incubated in 15 ml of methanol under reflux for two hours at 80°C with continuous stirring. The warm mixture was filtered using glass wool. The undissolved residue was refluxed for a further two hours in 15mls fresh methanol and the methanol extract was again filtered through glass wool. The combined methanol extracts were concentrated to dryness using a rotary evaporator.

Gas chromatography – mass spectrometry (GC-MS)

The dried propolis methanol extract was redissolved in ethyl acetate. An Agilent 6890N-5973N MSD system (Agilent Technologies) was used to analyse the extract. A (5% Phenyl) - methylpolysiloxane (HP 5 MS) column (30 m × 0.25 mm diameter, with 0.25 µm film thickness consisting of 5% phenyl and 95% methylsilane) was used with helium as the carrier gas. Relative percentage amounts of the separated compounds were calculated automatically from peak areas of the total ion chromatogram, using the NIST98.L mass spectrometer data base to identify individual compounds.

Cell Lines Used

The human cancer cell lines HT29 (colon), MCF7 (breast), COLO 679 (melanoma), OAW42 (ovary) and K562 (leukaemia) were originally sourced from the European Collection of Animal Cell Cultures (ECACC), Wiltshire, UK. SK-MEL-28 (melanoma) was originally

sourced from the American Type Culture Collection (ATCC®), USA. Peripheral blood mononuclear cells (PBMC) were extracted from fresh donor's blood using Histopaque®-1077 (Sigma Diagnostics®) according to the manufacturer's instructions.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) Assay

A 40mg/ml stock solution was prepared by dissolving the dried methanol extract in dimethyl sulphoxide (DMSO) (Sigma – Aldrich) and stored at 4°C in the dark. This stock solution was then used to prepare different test concentrations of propolis methanol extract (10, 20, 50, 100, 200, 300 µg/ml).

Cellular susceptibility to the drug was determined using standard MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assays. Exponentially growing cells were harvested and a single cell suspension was plated in 96-well microtitre plates using the recommended seeding rates. Following 24 hours of incubation at 37°C and 5% carbon dioxide, the cells were treated with the above prepared concentrations of the propolis extracts and incubated for 24, 48 or 72 hours. A minimum of three repeats were carried out at each concentration. Following the addition of MTT (5mg/ml) and a four-hour incubation period, the purple formazan crystals formed were dissolved in DMSO. The optical density (OD) in each well was read by means of an ultra microplate reader (ELX808, Bio-Tek Instruments, Inc., U.S.A.) at a wavelength of 545nm with a reference wavelength of 650nm and recorded using KC Junior® (Bio-Tek Instruments, Inc., U.S.A.) software. Relative absorbance (as a percentage of the untreated control) was plotted against concentration of extract in (µg/ml). The median inhibitory concentration (IC₅₀) was determined from the graphs.

STATISTICAL ANALYSIS

Statistical analysis was performed using SSPS (Science Products to SYSTAT Software). Each experiment was repeated a minimum of three times and a mean value was derived in each case. The student's t test was used to detect statistical significant differences between the susceptibility of different cell types in the cytotoxicity assays.

EXPERIMENTAL RESULTS AND DISCUSSION

Propolis extracts yield and composition

The weight per weight (w/w) yield of all the propolis extracts in terms of starting crude material is shown in Table 1.

GC-MS analysis of the four different propolis extracts identified totarol, a diterpenoid as the major component of all 4 propolis samples. These results were consistent with another study carried out on Maltese propolis, where totarol was one of the most abundant compounds, and it was found in all the Maltese propolis samples [19]. Propolis 3 and 4 methanol extracts contained a greater percentage of other constituents than did propolis 1 and 2 samples, which had a higher relative percentage of totarol content (Table 2). Propolis 3 in particular, showed a large number of additional peaks, as may be expected

by the fact that the area from which it is collected is agricultural land and the bees were foraging on a larger number of plant species (Figure1 and Table 2).

The chemical composition of propolis is known to vary considerably from area to area [20 – 21]. Previous studies have shown that propolis contains predominantly phenolic compounds such as flavonoids and cinnamic acid derivatives [13]. Most European propolis have high content of black poplar resinous secretions [20], however, those in the Mediterranean region are known to differ somewhat, containing largely diterpenic acids [22]. This was confirmed by our study to be also the case with Maltese propolis.

Table 1 W/W yield of prepared extracts in terms of starting crude material

Propolis sample	Crude (wet) weight in g	Final weight in g of dry methanol extract	w/w yield
Propolis 1	4.157	1.18	0.283
Propolis 2	4.017	1.237	0.308
Propolis 3	4.005	0.799	0.200
Propolis 4	4.400	1.544	0.351

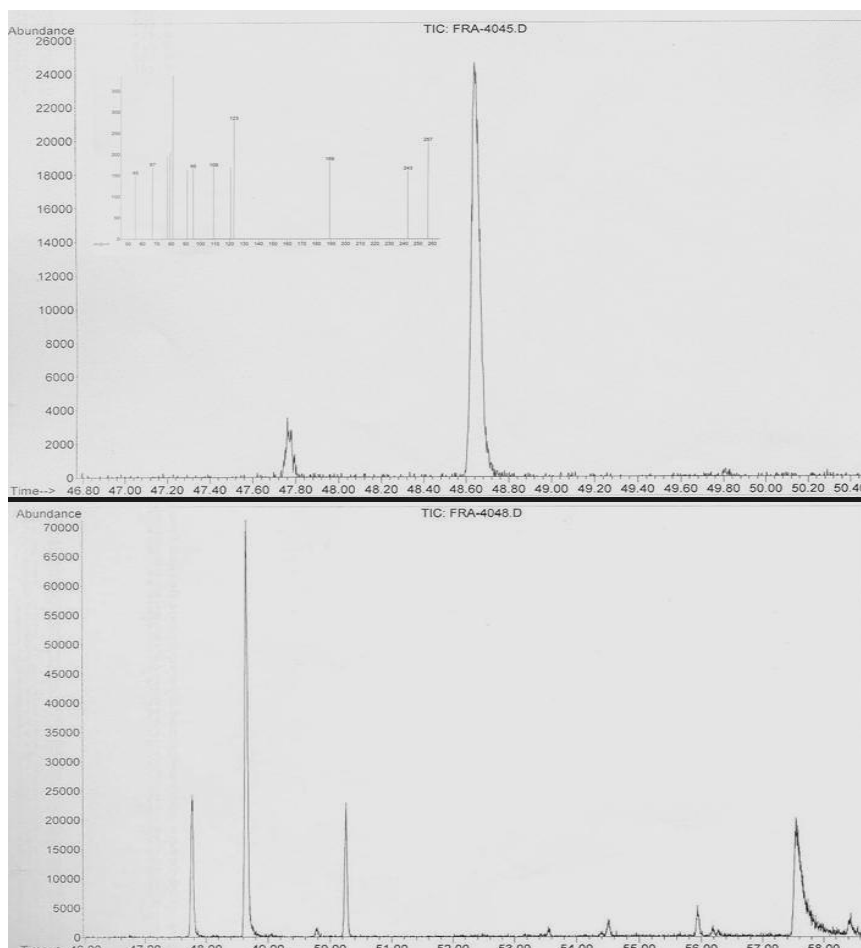
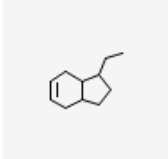
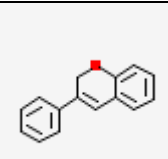
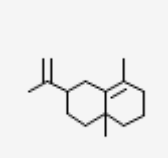
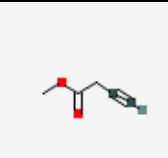


Figure 1 Top: Typical chromatogram for propolis sample 1 methanol extract analysed by using a GC-MS. The largest peak has a retention time of 48.64 minutes. **Insert:** Mass spectrum taken from this main GC peak – representing totarol. **Bottom:** Chromatogram for Propolis 3 extract showing greater number of components.

Table 2 Chemicals found in the various propolis samples using GC-MS, in order of relative concentration.

Common name/chemical structure	IUPAC name	Propolis 1		
		NIST02.L Name	Retention time (mins.)	Area under graph (%)
Totarol	4b,8,8-trimethyl-1-propan-2-yl-5,6,7,8a,9,10-hexahydrophenatren-2-ol	2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-,(4bS-trans)-	48.64	92.62
 C ₁₁ H ₁₈	1-ethyl-2,3,3a,4,7,7a-hexahydro-1H-indene	Trans-7-ethyl-bicyclo[4.3.0]non-3-ene	47.76	7.38
Propolis 2				
Totarol	4b,8,8-trimethyl-1-propan-2-yl-5,6,7,8a,9,10-hexahydrophenatren-2-ol	2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-,(4bS-trans)-	48.64	93.41
 C ₁₅ H ₁₂ O	3-phenyl-2H-chromene	3-Phenyl-2H-chromene	98.28	0.7
Propolis 3				
Totarol	4b,8,8-trimethyl-1-propan-2-yl-5,6,7,8a,9,10-hexahydrophenatren-2-ol	2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-,(4bS-trans)-	48.64	37.76
 C ₁₅ H ₂₄	1,4a-dimethyl-7-prop-1-en-2-yl-3,4,5,6,7,8-hexahydro-2H-naphthalene	2-(4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalen-2-yl)	47.77	11.39
Propolis 4				
Totarol	4b,8,8-trimethyl-1-propan-2-yl-5,6,7,8a,9,10-hexahydrophenatren-2-ol	2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-,(4bS-trans)-	48.64	64.36
 C ₅ H ₆ O ₂	methyl but-3-ynoate	Methyl-3-butynoate	49.77	35.67

Cell morphology during cytotoxicity assays

Consistent changes in cell size and morphology were observed using phase contrast microscopy (300×) upon exposure to propolis extracts, albeit at different concentrations for each extract (Figure 2). The cells displayed an irregular body and appeared shrunken at low concentrations while at higher concentrations, the cells appeared fragmented. These morphological changes are suggestive of apoptotic cell death.

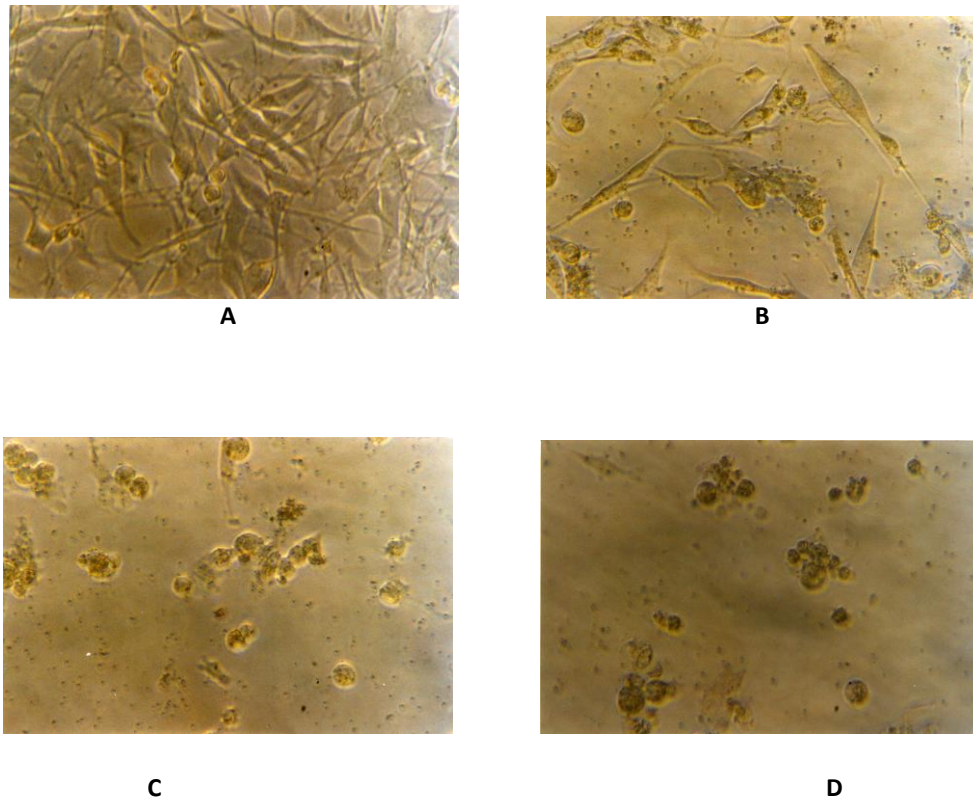


Figure 2 Morphological changes, following 48-hour exposure, of COLO 679 control cells (A), 20µg/ml (B), 50µg/ml (C) and 100µg/ml (D) propolis 1 extract.

Dose-dependent inhibition

COLO 679 and K562 were used to determine the cytotoxicity of all the propolis methanol extracts. Table 3 is a representation of the mean IC₅₀ values obtained from the dose response plots following 48 hours of exposure of K562 and COLO 679 cell lines to different propolis extracts. The same results are depicted graphically in Figure 3.

Propolis 1, being the most cytotoxic from the Maltese propolis extracts, overall, was chosen to carry out further cytotoxicity assays on HT29, MCF7, SK-MEL-28 and OAW42 cell lines as well as peripheral blood mononuclear cells (PBMCs). The cytotoxicity towards different human tumour cell lines and PBMCs, exposed to this methanol extract is indicated in Table 4.

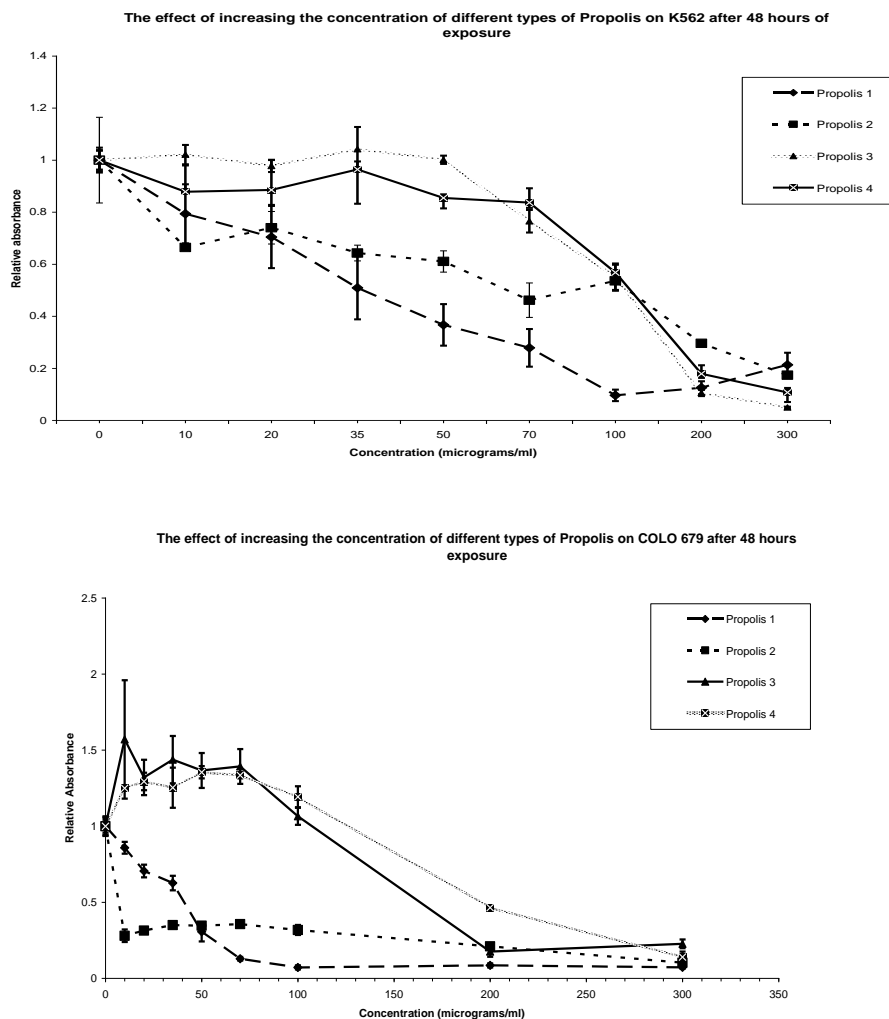


Figure 3. Top: Cytotoxicity curves based on MTT assays on K562 leukaemia cells using different Maltese propolis methanol extracts after 48 hrs exposure. **Bottom:** Cytotoxicity curves based on MTT assays on Colo 679 melanoma cells using different Maltese propolis methanol extracts after 48 hrs exposure.

Table 3: Mean IC₅₀ values obtained from the dose response plots for the drug sensitivity assays after 48hours of exposure to propolis from different areas. Means are calculated from IC₅₀ of at least three replicate experiments.

Propolis sample	K562 Mean IC ₅₀ ± SEM (µg/ml)	COLO 679 Mean IC ₅₀ ± SEM (µg/ml)
Propolis 1	39.67 ± 0.33	39.00 ± 2.08
Propolis 2	45.67 ± 19.41	7.33 ± 0.33
Propolis 3	108.33 ± 13.64	163.33 ± 3.33
Propolis 4	116.67 ± 8.82	193.33 ± 6.67

The results obtained, when using the MTT cell viability assay, show that all the methanol extracts of crude Maltese propolis had a dose-dependent cytotoxic effect on all the human cells and cell-lines tested. The dose-response plots in Figure 4, have a shape which is typical of cytotoxic drugs, showing a reduction in cell viability with increasing concentration. Through comparison of the literature, the methanol extract of Maltese propolis 1, (the most potent), appears to be more cytotoxic, prima face to K562 and PBMC than Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), an active ingredient of Brazilian

propolis. Artepillin C exhibited an IC₅₀ of 70 µg/ml on K562 leukaemia cells and an IC₅₀ of 100µg/ml on PBMC after exposure for 48 hours [23] while crude Maltese propolis 1 methanol extract had an IC₅₀ of 39.67 µg/ml on the K562 cell line and an IC₅₀ of 51.67µg/ml on PBMC after the same time period. This must obviously be confirmed through further comparison in the same laboratory.

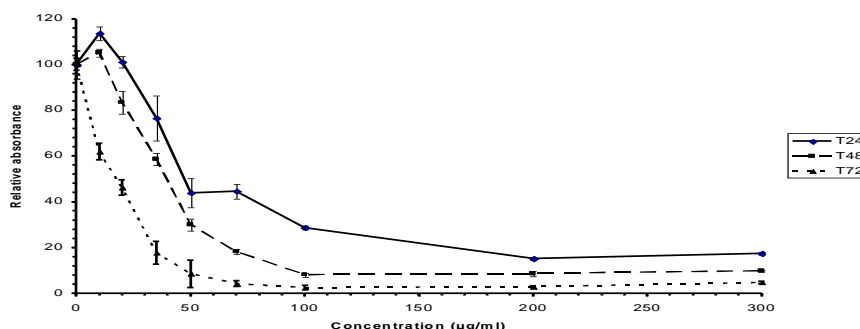


Figure 4 Dose response plots for propolis methanol extract MTT assay on COLO 679, after 24 (A), 48 (B) and 72 (C) hours of exposure to propolis 1

A somewhat biphasic response in cell viability, with an increase in relative absorbance at low doses, can be seen particularly following exposure of COLO 679 for a shorter period of 24 hours, (Figure 4). This increase in relative absorbance may be due to components of the complex extract which stimulate cell growth or metabolism at low concentrations in which cytotoxic effects are as yet undetected. A similar biphasic effect was seen in a study using Artepillin C, on leukemic cell line growth. Low concentrations appeared stimulatory while high concentrations were inhibitory to cell proliferation [23].

Time-dependent inhibition

Figure 4, shows a dose-response plot for COLO 679 exposed to propolis 1 extract for different time periods. The IC₅₀ values decreased as the drug exposure time increased and the cytotoxicity curve is displaced to the left, indicating a greater effect with a longer exposure. This is most marked at lower concentrations of the extract (10 – 100 µg/ml), which show a drastic reduction of relative absorbance when increasing the exposure time from 24 to 72 hours

Time-dependent inhibition was demonstrated for all the cancer cell lines from the MTT assays as seen in Table 4 (as well as Figure 4).

Differential response between cell lines

Six human cell lines were used in this study, namely: two melanoma, one breast, one colon, one ovarian cancer and one leukaemia cell line.

The p values indicated a statistically significant difference with the mean IC₅₀ of SK-MEL-28 being less than that of PBMC, MCF7, COLO 679, OAW42 and HT29. That of PBMC was also significantly less than that of K562, COLO 679 and HT29 (Table 4).

At 48 hours of exposure to the propolis 1 methanol extract, the IC₅₀ for the SK-MEL-28 melanoma cell line was much greater than for any of the other cell lines.

Table 4 Mean IC₅₀ values and standard error of the mean obtained from the dose response plots for the drug sensitivity assays when adding propolis 1 to different cell lines after 24, 48 and 72 hours of exposure to propolis 1. Means are calculated from IC₅₀ of at least three replicate experiments.

Cell line	Mean IC ₅₀ ± SEM (µg/ml)		
	T ₂₄	T ₄₈	T ₇₂
OAW42	74.00 ± 9.45	31.67 ± 7.26	20.67 ± 6.36
MCF7	67.33 ± 8.82	35.67 ± 8.95	21.33 ± 7.31
HT29	79.00 ± 6.66	37.33 ± 2.91	30.00 ± 5.77
COLO 679	46.33 ± 0.67	39.00 ± 2.08	19.33 ± 0.67
K562	71.33 ± 8.67	39.67 ± 0.33	19.67 ± 4.18
SK-MEL-28	95.00 ± 10.41	78.33 ± 2.03	62.67 ± 9.60
PBMC		51.67 ± 3.33	

The propolis 1 IC₅₀ values, obtained for all the other cancer cell lines tested are lower than that for normal PBMCs suggesting that the sensitivity of most cancer cell lines the cytotoxic effects of the Maltese propolis methanol extract may be greater than that of healthy blood cells. SK-MEL-28 is a melanoma cell line and melanoma cells are notoriously difficult to treat with chemotherapy [24], which may explain its much greater resistance to the cytotoxic Maltese propolis extract.

Comparison of cytotoxicity of propolis extracts from different areas in Malta

Propolis 1 and 2 extracts show the greatest toxicity on the cancer cell lines tested, as is seen from Table 3 as well as in Figure 3. Propolis 3 and 4 are much less cytotoxic to leukaemia and melanoma cells than propolis 1 and 2, which have a much higher relative abundance of totarol. Totarol and associated compounds were shown to exhibit cytotoxic activity against an ovarian cancer cell line and a human breast tumour cell line [25, 26] so the differences concentrations of this diterpenoid most probably explain the variably cytotoxicity between these two groups. At very low doses, propolis 2 extract is much more effective on the COLO 679 melanoma cell line than is propolis 1. Additional components found at much lower concentrations may play a role in this difference since both extracts have very high totarol abundance.

Studies have shown geographic variability in the contents of propolis within large countries such as Brazil and in the extract's resultant bioactivity [9, 27, 28].

In our study, there was considerable constituent variability as between different propolis samples collected within a range of only a few square kilometres and over different seasons. Also, within this small area, propolis extracts with cytotoxicity more than an order of magnitude greater than extracts derived from other propolis in the area was identified.

Different times of collection (propolis 1 and propolis 2) also showed differences in cytotoxicity though this variability was less marked. This indicates the significance of flora foraged, collection point and collection time to the bio-activity of any propolis-derived products.

CONCLUSION

This study shows that Maltese propolis crude methanol extract is differently cytotoxic to a variety of neoplastic cell lines in a dose- and time-dependent fashion. This cytotoxicity appears to strongly correlate with the totarol content of the propolis extracts as assayed by GC-MS. Peripheral blood mononuclear cells are less sensitive to the extract of propolis than most of the cancer cell lines tested, with the exception of the SK-MEL-28 melanoma cell line. Interestingly, extracts made from propolis collected within as little as 1 km distance of each other showed an order of magnitude difference in cytotoxicity, indicating the importance of functional studies for propolis bio products, even if collected in the same area.

ACKNOWLEDGEMENTS

We are grateful to Mr. Arnold J. Grech, apiculture consultant, for the kind gift of raw Maltese propolis. We also would like to acknowledge Prof. A. Vella at the Chemistry Department, University of Malta, for use of facilities to carry out the propolis extractions.

REFERENCES

- [1] Gojmerac W. Bees, Beekeeping, Honey and Polination. A VI publishing company, Westport, CT, 1980.
- [2] Valcic S, Montenegro G, Mujica AM, Avila G, Franzblau S, Singh MP, et al. Z Naturforsch C 1999; 54: 406-416.
- [3] Elkins R. Woodland Publishing Co, Utah, 1996.
- [4] Marcucci MC. Apidologie 1995; 83-99.
- [5] Greenaway WST and Whatley FR. Bee World 1990; 107-118.
- [6] Usia T, Banskota AH, Tezuka Y, Midorikawa K, Matsushige K, and Kadota S. J Nat Prod 2002; 65: 673-676.
- [7] Burdock GA. Food Chem Toxicol 1998; 36: 347-363.
- [8] Banskota AH, Tezuka Y, and Kadota S. Phytother Res 2001; 15: 561-571.
- [9] Marcucci MC, Ferreres F, Custodio AR, Ferreira MM, Bankova VS, Garcia-Viguera C et al. Z Naturforsch C 2000; 55 76-81.
- [10] Hegazi AG and Abd El Hady FK. Z Naturforsch C 2002; 57: 395-402.
- [11] Crane E. Beekeeping: Science, practice and World Resources. London, 1988.
- [12] Kartal M, Kaya S, and Kurucu S. Z Naturforsch C 2002; 57: 905-909.
- [13] Midorikawa K, Banskota AH, Tezuka Y, Nagaoka T, Matsushige K, Message D, et al. Phytochem Anal 2001; 366-373.
- [14] Suzuki I, Hayashi I, Takaki T, Groveman DS, and Fujimiya Y. Cancer Biother Radiopharm 2002; 17: 553-562.
- [15] Jagetia GC and Baliga MS. J Ethnopharmacol 2005; 96: 37-42.
- [16] Lavoie JM and Stevanovic T. J Agric Food Chem 2005; 53: 4747-4756.



- [17] Amari M. Biblioteca Arabo-Sicula.
- [18] Schembri PJ. Natural Heritage. Malta Culture and Identity. Frendo H and Friggieri O, Valletta, Malta, 1994.
- [19] Popova M, Trusheva B, Antonova D, Cutajar S, Mifsud D, Farrugia C, et al. Food Chemistry 2011; 126: 1431 - 1435.
- [20] Bankova V, Popova M, Bogdanov S, and Sabatini A G. Z Naturforsch C 2002; 57: 530-533.
- [21] Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R, and Popov S. J Ethnopharmacol 1999; 64: 235-240.
- [22] Trusheva B, Popova M, Bankova V, Tsvetkova I, Naydensk C, and Sabatini A. Revista Italiana EPPOS 2003; 36: 3-7.
- [23] Kimoto T, Aga M, Hino K, Koya-Miyata S, Yamamoto Y, Micallef MJ et al. Anticancer Res 2001; 21: 221-228.
- [24] Satyamoorthy K, Bogenrieder T, and Herlyn M. (2001). Trends Mol Med 2001; 7: 191-194.
- [25] Pettit GR, Tan R, Northen JS, Herald DL, Chapuis JC, Pettit RK. J Nat Prod 2004; 67:1476-82.

- [26] Reynolds M, Chaturvedula GS, Ratovoson F, Andriantsiferana R, Rasamison VE, Guza RC, Kingston DG. Nat Prod Res 2006; 20: 606-10.
- [27] Velikova M, Bankova V, Marcucci MC, Tsvetkova I and Kujumgiev A. Z Naturforsch C 2000; 55: 785-789.
- [28] Park YK, Alencar SM, and Aguiar CL. J Agric Food Chem 2002; 50: 2502-2506.