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Carpobrotus edulis Methanol Extract Inhibits the MDR Efflux Pumps, Enhances Killing of Phagocytosed *S. aureus* and Promotes Immune Modulation

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Although alkaloids from the family Aizoaceae have anticancer activity, species of this family have received little attention. Because these alkaloids also exhibit properties normally associated with compounds that have activity at the level of the plasma membrane, a methanol extract of *Carpobrotus edulis,* a common plant found along the Portuguese coast, was studied for properties normally associated with plasma membrane active compounds. The results of this study show that the extract is non-toxic at concentrations that inhibit a verapamil sensitive efflux pump of L5178 mouse T cell lymphoma cell line thereby rendering these multi-drug resistant cells susceptible to anticancer drugs. These non-toxic concentrations also prime THP-1 human monocyte-derived macrophages to kill ingested *Staphylococcus aureus* and to promote the release of lymphokines associated with cellular immune functions. The extract also induces the proliferation of THP-1 cells within 1 day of exposure to quantities normally associated with phytohaemagglutinin. The potential role of the compound(s) isolated from this plant in cancer biology is intriguing and is currently under investigation. It is supposed that the resistance modifier and immunomodulatory effect of this plant extract can be exploited in the experimental chemotherapy of cancer and bacterial or viral infections. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: Carpobrotus edulis; immune-modulator; efflux pump inhibitor.

INTRODUCTION

Members of the plant family Aizoaceae contain alkaloids known to have narcotic-anxiolytic properties and strong synergism with psychomimetics (Smith *et al*., 1996). These properties are consistent with those presented by other neuro-active compounds whose activities reside primarily at the level of the plasma membrane (Williams *et al*., 2001). Although some of these alkaloids have been reported to have anticancer properties as well (Smith *et al*., 1996) they have received little attention, perhaps due to their reported toxicity.

Along the coast of Portugal one may find a member of the Aizoaceae family that is so prolific that it is considered a nuisance. This plant *Carpobrotus edulis* captured the attention of Professor J. Molnar during his visit to this laboratory (L. Amaral). Subsequent to the plant's identification, it became reasonably clear that the properties reported for members of this family may also be present in this nuisance plant. Furthermore, inasmuch as some inhibitors of efflux pumps responsible for antibiotic resistance in eukaryotic and prokaryotic cells have activities on other plasma membrane functions (Molnar *et al*., 1997; Nacsa *et al*., 1998), this plant was of mutual interest and thus became a subject of mutual study that evaluated any anticancer activity that an extract of this plant might have. This report provides evidence that the methanol extract of *Carpobrotus edulis* inhibits the MDR1-efflux pump of a chemotherapeutic resistant lymphoma cell line, primes a human monocyte-macrophage cell line (THP-1) to kill intracellular *Staphylococcus aureus*, induces these latter cells to secrete those lymphokines associated with cellular immune activity as well induces proliferation of these cells within 24 h. These activities are conducted with concentrations in the extract that are non-toxic and non-apoptotic to the THP-1 cells, human monocytes derived from peripheral blood, sub-populations of human T cells or mouse T cell lymphoma cells.

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MATERIALS AND METHODS

Plant material. There are two species of *Carpobrotus* that grow profusely along the Portuguese coastline, *C. edulis* (L.) N. E. Br. (the most predominant) and *C. chilensis* (Molina) N. E. Br. which, in the absence of recent isoenzyme studies, are difficult to distinguish from each other. In addition, recent studies have shown that hybrids are readily formed at sites where these two species grow, and again, can only be identified by the former studies (Albert *et al.*, 1997). Whether hybrids are to form at any given coastal locale is governed by the salinity of the sandy soil which can effectively act as a barrier since high salinity inhibits the germination of hybrids (Weber and D'Antonio, 1999). We have not made an attempt to employ isoenzymes for identification since expert Portuguese botanists identified the specimen employed in this study to be *C. edulis*.

The genus *Carpobrotus* (Aizoaceae) is a perennial succulent horizontally low-growing sub-shrub, with long prostrate stout branches forming extensive dense mats. It has three sided curved opposite axial leaves, ranging from 8–10 cm long in *C. edulis* to 3–5 cm long in *C. chilensis*, serrated on the inferior curve which assists the plant to hold on to the substrate. The flowers are large (ranging from 8–10 cm in diameter in *C. edulis* to 3–5 cm in *C. chilensis*) and solitary with a long peduncle and petal-like staminodes whose colour ranges from weak to strong yellow or pink, the latter colour may be almost purple in its intensity. The stamen are erect, free standing and numerous. There is a conical ovary, slightly compressed, convex on top with 10–16 carpals and plumose stigmata. The fruit is fleshy, indehiscent in some species, edible and contain long mucilaginous ovoid seeds.

It is non-native (exotic) to the Iberic flora, is much planted along highways and sand banks to control erosion and dune stabilization. Although very well adapted to saline environments it is also found in grass slopes and forest edges away from the coast. *C. edulis* is originally from South Africa and has aggressively colonized the coasts of Southern and Western Europe (Weber and D'Antonio, 1999).

Culture of human monocyte-derived macrophage cell line THP-1 and isolation of human peripheral blood monocyte-derived macrophages (HPBMDM). The macrophage cell line THP-1 was derived from isolated human macrophages and was kindly provided in 1998 by Professor Hazel Dockrell, London School of Hygiene & Tropical Medicine, UK and has been maintained in our laboratory as a monolayer since that time. Short-term cultures of the THP-1 macrophage cell line, 1.0 to 20×10^5 cells/mL were added in triplicate sets to 24-well or 96-well microplates containing RPMI with L-glutamine medium (Gibco Laboratories, Paisley, Scotland, UK) supplemented with 10% human AB serum (Sigma Aldrich Química SA, Madrid, Spain) and incubated at 37 °C in the presence of 5% CO_2. Adhered cells were removed for counting by the addition of 1.0 mL of 0.01% SDS. The number of adherent cells of triplicate wells did not vary by more than 3%. Human peripheral blood monocyte-derived macrophages (HPBMDM) were obtained by methods previously described (Silveira *et al.*, 1997) and employed in these studies in the same manner as that for the THP-1 cell line. It is important to note that the HPBMDM is devoid of lymphocytes since these latter cells do not adhere to the surfaces of the wells and are removed by simple washing. It is also important to note that these washings were sustained separately and were available as potential additions to cultures containing HPBMDM.

Culture of L5178 MDR mouse T cell lymphoma cell line. The L5178 mouse T cell lymphoma cell line was derived from the parental L5178Y mouse T cell lymphoma cell line after the latter was infected with the plasmid pHa that contained the gene MDR1/A thereby rendering the progeny resistant to multiple anticancer drugs as previously described (Aszalos *et al.*, 1995). MDR1 expressing cells were selected with the use of 60 μg/L colchicine (Sigma Aldrich Química SA, Madrid, Spain) that maintains the expression of the MDR phenotype as evident from the ability of the cells to grow in the presence of cancer chemotherapeutics such as epirubicin to which the parental L5178Y cells were susceptible. 1.0 mL of 2.0 million MDR and parental cells per mL were distributed into Eppendorf tubes in McCoy medium (Gibco Laboratories, Paisley, Scotland, UK) and incubated with various concentrations of plant extract (dissolved and diluted in DMSO) for 10 min at room temperature. Then the rhodamine 123 (Sigma Aldrich Química SA, Madrid, Spain) was added to the samples and further incubated for 20 min at 37 °C. The samples were then washed and re-suspended in PBS and their fluorescence was measured in a Becton Dickinson Flow cytometer (Becton-Dickinson, Mountain View, CA, USA). Both parental and derived MDR mouse T cell lines were grown in McCoys 5A medium containing 10% heat-inactivated horse serum and supplemented with L-glutamine, penicillin and gentamicin (Sigma Aldrich Química SA, Madrid, Spain).

Preparation of methanol extract of *Carpobrotus edulis* for evaluation of antimicrobial activity, properties that affect cellular immune functions and anticancer properties. The leaves of *Carpobrotus edulis* were removed, weighed, homogenized in water after which absolute methanol (Sigma Aldrich Química SA, Madrid, Spain) was added to yield a final 70% methanol concentration. The mixture was homogenized again and centrifuged at 10 000 × **g** and the pellet set aside for later study. The methanol extract was filtered and methanol removed in vacuum while maintaining the flask containing the extract at 60 °C and rotating at 150 rpm. The remaining aqueous phase would be used as is, diluted 10x with medium or concentrated 3x. For evaluation of anticancer properties fresh shoots of *Carpobrotus edulis* with leaves and stems were homogenized in a blender and extracted with 70% methanol at room temperature using an ultrasonic bath (3 × 15 min). The extract was filtered and concentrated in vacuum at 50 °C to yield a green oily residue. This extract was dissolved in DMSO and subjected to study for MDR-reversing activity against L5178 mouse lymphoma T cell lines (parental and derived MDR1 progeny).

Determination of *in vitro* activity of the methanol extract against *Staphylococcus aureus*. The *Staphylococcus aureus* strains employed in this study were the

ATCC 25923 that served as the absolute control, and clinical strains of methicillin susceptible (MSSA) which were maintained and characterized in our laboratory with respect to antibiotic profile, response to inhibitors of efflux pumps and electrophoretic profile of proteins extracted from isolated cell wall envelopes. Individual colonies of each strain were obtained from TSA plates (Difco Laboratories, Detroit, USA), transferred to 10 mL of TSB (Difco Laboratories, Detroit, USA) and incubated at 37 °C until they reached their stationary phase (8 h). The *in vitro* activity of the methanol extract against *S. aureus* was performed in 10 mL TSB containing 200 μL of 3x concentrated plant methanol extract by the broth dilution method as previously described (Amaral *et al*., 1992), repeated three times and the values obtained did not differ.

Toxicity of *Carpobrotus edulis* methanol extract. Because plant extracts of the family Aizoaceae have been shown to have toxicity (Smith *et al*., 1996) the plant methanol extract was tested for this *in vitro* activity against THP-1 and HPBMDM as follows:

Naphthol blue-black method: Adherent cells (THP-1 and HPBMDM) were washed with HBSS and 100 μL of medium containing varying concentrations of plant methanol extract were added. After 30 min, 1, 2, 6, 18, 24 h, and throughout the next 6 days, adherent macrophages were assayed for evidence of toxicity by the naphthol blue-black method of Nakagawara and Nathan as previously described (David *et al*., 2001; Ordway *et al.*, 2002). Briefly, after removal of excess liquid, 0.050 mL of the naphthol blue back stock solution containing 1% Triton X-100 (w/v), 0.1 M citric acid, and 0.05% (w/v) naphthol blue black (Sigma Aldrich Química SA, Madrid, Spain), final pH adjusted to 2.0, was added per well and the cells continuously aspirated for 15 min. An aliquot of 0.010 mL was transferred to a haemocytometer and the cells examined and counted for the absence and presence of the dye.

Trypan blue method: Toxicity of the plant methanol extract against adherent THP-1 or HPBMDM cells were determined by the trypan blue exclusion method (Silveira *et al.*, 1997; Ordway *et al.*, 2002). Briefly, after the addition of 100 μL of medium containing various concentrations of plant methanol extract and incubations as described above, the medium of each well was replaced with 0.010 mL of 0.01% SDS and 0.090 mL of 0.2% trypan blue solution (Sigma Aldrich Química SA, Madrid, Spain), and 0.010 mL of the cell suspension placed on a haemocytometer and the cells examined and counted for the absence and presence of the dye.

AnnexinV-binding: Potential causation of apoptosis of THP-1 and HPBMDM by the various concentrations of plant methanol extract in the above cultures was determined by the annexin V method (David *et al*., 2001) and processed according to the AnnexinV-FITC kit (Research & Development Systems, NY, USA).

Phagocytosis and killing activity of strains of *Staphylococcus* aureus by THP-1 and HPBMDM. Bacterial suspensions ranging from 1.0×10^5 to 20.0×10^5 were added to monolayer cultures of THP-1 and HPBMDM and incubated at 37 °C for 30 min. Extracellular bacteria were removed by two consecutive washes with RPMI. The washings were pooled and subjected to CFU counts in order to determine the efficiency of phagocytosis. A third wash was performed and subjected to CFU counts for the verification of the complete absence of non-phagocytosed bacteria. The adhered cells-phagocytosed bacteria cultures were incubated at 37 °C for 0, 1, 2, 6 and 18 h and lysed with the addition of 1.0 mL of 0.01% SDS at the end of each incubation period. Aliquots of 0.1 mL of the lysed cultures were subjected to CFU counting as previously described (Lorian and Amaral, 1987).

Effects of the *Carpobrotus edulis* methanol extract on the killing activity of THP-1 and HPBMDM against *S. aureus*. Macrophages were pre-pulsed with concentrations of the plant methanol extract for 1 h. *S. aureus* was then added to cultures for 30 min at a 1CFU/macrophage ratio and then lysed with the addition of 1.0 mL of 0.01% SDS. Aliquots of 0.1 mL of the lysed cultures were subjected to CFU counting as previously described (Lorian and Amaral, 1987). The results demonstrate that when the extract was added to macrophages prior to infection with *S. aureus*, the ability of the macrophage to phagocytose the bacteria was not significantly effected (Mann-Whitney *U*-test, $p = 0.725$) (data not shown).

One hundred microlitres of RPMI medium lacking (controls) or containing increasing concentrations of the plant extract which were experimentally shown to have no toxic effect on adhered THP-1 cells or HPBMDM were added to cultures of adhered macrophages that have phagocytosed strains of *S. aureus* and the cultures incubated for 0, 1, 2, 6 and 18 h at 37 °C. At the end of each culture period 0.01% SDS was added and the number of viable bacterial cells present in the supernatant of the lysed cultures was determined by CFU counts. In addition to these experiments, the macrophages were incubated with various amounts of plant methanol extract for various periods of time prior to the addition of bacteria. All experiments were conducted in triplicate and performed at least twice.

Stimulatory activity of *Carpobrotus edulis* methanol extract for THP-1 and HPBMDM. *Proliferative activity:* 100 μL of medium containing increasing concentrations of the plant methanol extract were added in triplicate to wells containing the adhered THP-1 cells and HPBMDM and the plates incubated at 37 °C for 0, 1, 3, 5 and 7 days. 1 μCi of ^{3}H-thymidine (Amersham International Plc, Buckinghamshire, UK) in RPMI was added in 0.020 mL volumes to each well. The plates were returned to the incubator for 18 h to allow for the uptake of ^{3}H-thymidine by actively proliferating cells. The plate was harvested on fibre filter paper (Cambridge Technology Inc., Cambridge, MA, USA) using a PHD cell harvester (Cambridge Technology Inc.). The discs containing the cell lysate were placed into Optifluor scintillant (Beckman Coulter, Fullerton, CA, USA) and ^{3}H-thymidine incorporation was measured using an LS 6500 scintillation Counter (Beckman Coulter). Measurement of cell proliferation was expressed as a mean of counts per minute of triplicate wells. Controls with and without the addition of PHA served as a reference of induced proliferation capability of the THP-1 cell line and have been described previously (Silveira *et al*., 1997; Ordway *et al.*, 2002).

Cytokine assays: The effect of plant methanol extract on release of IFN-γ and IL5 by T cells was determined. Non-adherent cells obtained from the processing of

Ficoll-separated monocytes of human peripheral blood were stimulated with PHA and exposed to the concentrations of plant methanol extract. Estimation of IFN-γ and IL5 were conducted with the aid of the Endogen ELISA Kit (Pierce Endogen, Rockford, IL, USA). Cultures contained 100 µL of medium plus 10 µL of either undiluted or diluted extract. Control cultures consisted of two types: Control 1 had no PHA; Control 2 had PHA. Amounts of IFN-γ and IL5 are in pg/mL of medium.

Intracellular cytokine staining and activation markers: The presence of the early activation marker (CD69) on T cells and macrophages and the presence of intracellular IFN-γ was analysed using a commercial IS Ultra Cell-Fix Perm Kit (Immune Source, UK). Briefly, the protocol for intracellular staining of T cells consisted of adding 1×10^6 adherent and non-adherent peripheral blood monocytes to tubes containing 40 µL staining buffer (1% sodium azide in 2% BSA in PBS) and 10 µL of the appropriate surface marker monoclonal antibody or isotype control; the tubes were incubated at 4 °C for 30 min in the dark. The cells were then washed twice in staining buffer (200 µL/tube) and fixed with 100 µL of fixation buffer (4% paraformaldehyde) for 20 min, washed twice with 200 µL permeabilization/wash buffer, re-suspended in 50 µL of permeabilization/wash buffer and 5 µL of PharMingen intracellular cytokine monoclonal antibody or intracellular isotype control (BD-PharMingen, Mountain View, CA, USA), incubated for 30 min at 4 °C in the dark, washed 2x in staining buffer and analysis was carried out using a FACSCalibur (Becton-Dickinson, Mountain View, CA, USA). Parameters were set to measure lymphocytes by forward scatter and side scatter. Fluorescence was analysed by gating on all lymphocytes and measuring fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridimine chlorophyll protein (PerCP) and allophycocyanin (APC) profiles (BD-PharMingen, Mountain View, CA, USA). Cells were analysed on a FACSCalibur (Becton-Dickinson).

Special note: All experiments concerning human monocyte derived macrophages (HPBMDM) were conducted with monocyte-derived macrophages that were freshly prepared from whole blood of healthy donors 30–35 years as well as with the THP-1 cell line. However, some of the experiments were conducted only with HPBMDM since this afforded the opportunity to add to these cultures the non-adherent T cells required for gamma interferon secretion/production.

Determination of effects of *Carpobrotus edulis* methanol extract on drug accumulation of multi-drug resistant mouse lymphoma cells. Cells were adjusted to 2×10^6/mL and re-suspended in serum-free McCoys 5A medium and aliquots of 0.5 mL distributed to Eppendorf centrifuge tubes containing different concentrations of plant extract previously determined in this study not to affect the growth of these cells. The cells were incubated at 25 °C for 10 min after which 10 µL of the indicator rhodamine 123 was added, the tubes incubated for an additional 20 min at 37 °C, centrifuged at 1500 rpm and the pellets re-suspended in 0.5 mL of phosphate buffered saline and washed once more. The amount of fluorescence associated with each of the cultures containing and lacking various compounds and volumes of plant methanol extract was determined with the aid of a Beckton-Dickinson FACScan flowcytometer. The amount of fluorescence reflects the amount of anticancer drug (such epirubicin and many others) retained in the cell when the efflux pump is inhibited by resistance modifier compounds such as verapamil (Sigma Aldrich Química SA, Madrid, Spain) which in this study served as a positive control. Verapamil is widely used for the demonstration of inhibition of MDR1 efflux pumps that account for antibiotic resistance of the cell (Safa *et al*., 1987; Pastan *et al*., 1988). The percentage of mean fluorescence for the parental and MDR-1 containing mouse lymphoma cells served as controls (untreated) versus that of their respective treated (compounds added for evaluation of inhibition activity of efflux pump) was determined and employed by the following equation:

$$R = \frac{\text{mdr treated / mdr control}}{\text{parental treated / parental control}}$$

which yields a measure of activity defining the effectiveness of the compound as an inhibitor of the MDR1 efflux pump.

Statistical analysis. A non-parametric method, the Mann-Whitney *U*-test, which makes no assumptions about the underlying distribution and normality of the data, was used to assess statistical significance between groups of data.

RESULTS

Toxicity and effects of the *Carpobrotus edulis* methanol extract on proliferation of human macrophages

The plant *Carpobrotus* is very familiar to the Portuguese and almost nothing is known about its potential medicinal qualities and therefore this plant merits the description provided in the section of Materials and methods. Figure 1 presents an example of this plant at the time of collection (sea cliffs of Guincho, National Park of Sintra-Cascais, Portugal, December 2000 – all permissions granted). The location and time of the year of collection may be important if whatever is present in the extract is seasonally produced. The toxicity of the plant methanol extract of *C. edulis* on the THP-1 cell line and HPBMDM was non-existent during the first 3 days of culture and evident only after day 5 as shown by Table 1. A volume as high as 20 µL of undiluted

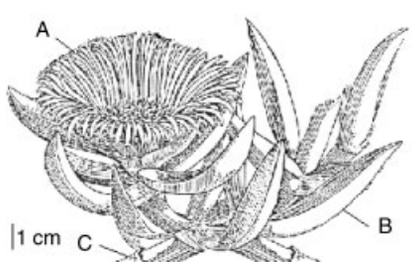

Figure 1. Illustration of *Carpobrotus* sp. Large flowers with petal-like staminodes (A), ranging from 8–10 cm in diameter in *C. edulis* to 3–5 cm in *C. chilensis*, 3 sided curved opposite axial leaves (B), (8–10 cm long in *C. edulis* to 3–5 cm long in *C. chilensis*) and prostrate stout branches (C).

Table 1. Percent toxicity produced by *Carpobrotus edulis* methanol extract against human monocyte derived macrophages (HPBMDM) and THP-1 macrophages

	% Toxicity HPBMDM/ % Toxicity THP-1				
	Day 1	Day 2	Day 3	Day 5	Day 7
Methanol plant extract	0/0	4/2	5/5	30/20	50/50
1:10 dilution of methanol plant extract	0/0	4/3	5/5	25/15	75/85

THP-1 and HPBMDM in wells containing 100 μL of media with and without 10 μL of undiluted or 10 μL of 10x diluted *Carpobrotus edulis* methanol extract. Incubated at 37 °C for a period of 7 days and cultures examined periodically. Cultures that received an equivalent volume of diluent (methanol) served as absolute controls. The percent toxicity was determined by the use of naphthol blue black and trypan blue exclusion methods (Silveira *et al.*, 1997; Ordway *et al.*, 2002). Values of toxicity against HPBMDM/THP-1, respectively.

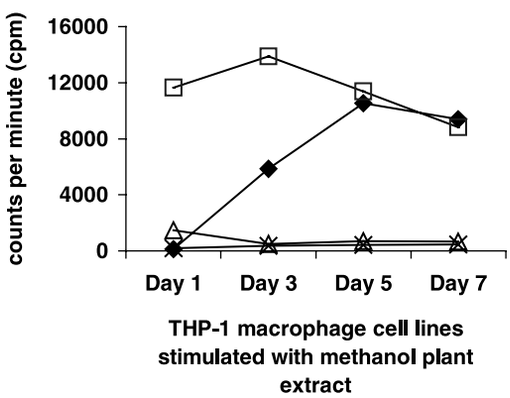

Figure 2. The effect of *Carpobrotus edulis* extract on proliferation of THP-1 macrophages. Ten μl of undiluted *Carpobrotus edulis* methanol (MetOH) extract (□) or diluted 1:10 plant extract (△) was added to sets of triplicate cultures of THP-1 cells. Separate absolute controls received: phytohaemagglutinin (PHA) (◆), no PHA nor plant extract (X). Cultures were incubated for up to 7 days and cellular proliferation was periodically assayed by the use of ^{3}H-thymidine incorporation (Silveira *et al.*, 1997). The results are expressed as the mean from three separate experiments.

methanol extract added to cultures of THP-1 cells did not result in any appreciable intracellular presence of trypan blue nor did it increase the amount of Annexin V binding over that of controls (data not shown). The direct effects of the plant methanol extract on the proliferation of THP-1 cells are summarized by Fig. 2. Briefly, whereas exposure of THP-1 cells to PHA induced proliferation by day 3 reaching a maximum by day 5, exposure of these cells to 10 μL of plant methanol extract induced marked proliferation by the end of day 1, reaching a maximum by the end of day 3. Identical treatment of HPBMDM yielded similar results.

Activity of the *Carpobrotus edulis* methanol extract against *S. aureus in vitro* and phagocytosed by human macrophages

The *in vitro* activity of the methanol extract against *S. aureus* was assayed by the use of step-wise dilution of 10 mL of TSB broth containing 200 μL of 3x concentrated plant methanol extract and the results obtained

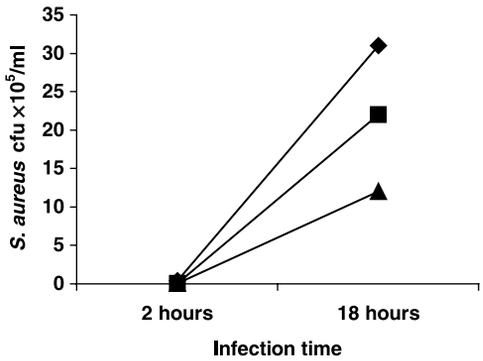

Figure 3. Effect of the *Carpobrotus edulis* extract on intracellular growth of *S. aureus* strains. THP-1 macrophages were infected for 30 min with methicillin sensitive *S. aureus* ATCC strain at concentrations of 1 CFU per macrophage. The methanol (MetOH) (■) and MetOH 3x (▲) concentrated plant extract were added to triplicate cell cultures and then after 2 and 18 h of infection the cells were lysed and the lysates plated for CFU counting on TSB agar. (◆) control-Results are expressed as the mean of three separate experiments.

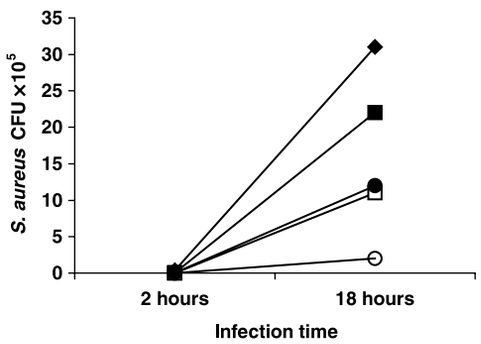

Figure 4. Effect of *Carpobrotus edulis* extract on the killing of *S. aureus* phagocytosed by HPBMDM. Triplicate cultures of human peripheral blood monocyte derived macrophages were infected for 30 min with methicillin sensitive *S. aureus* ATCC at concentrations of 1 CFU per macrophage. Methanol (MetOH) (■) and 3x concentrated (●) MetOH plant extract were added and after 2 and 18 h post-infection, cultures were lysed and the lysates plated for CFU counting on TSB agar. Non-adhered T cells were added to cultures with MetOH (□) and 3x concentrated (○) MetOH plant extract. (◆) Controls received only an equivalent volume of medium. Results are expressed as the mean of three experiments. Similar results were obtained with the use of THP-1 cells.

showed that the growth of *S. aureus* was not affected. The effects of the plant extract on the killing of phagocytosed *Staphylococcus aureus* is presented by Fig. 3. Briefly, the killing of phagocytosed *S. aureus* was enhanced by the presence of plant methanol extract over that shown by THP-1 cells not receiving the extract (control). This killing activity could be substantially increased with the addition of concentrated extract. The effects of the methanol plant extracts on the killing of *S. aureus* previously phagocytosed by HPBMDM was similar (data not shown). However, whereas pre-exposure of THP-1 cultures to plant methanol extract for 30 min followed by phagocytosis of the organism yielded the similar data shown by Fig. 3, similar pre-exposure of HPBMDM to the extract produced significantly more enhancement of killing only if the non-adhered T cells were added to cultures of HPBMDM (Fig. 4). The observation that pre-exposure to

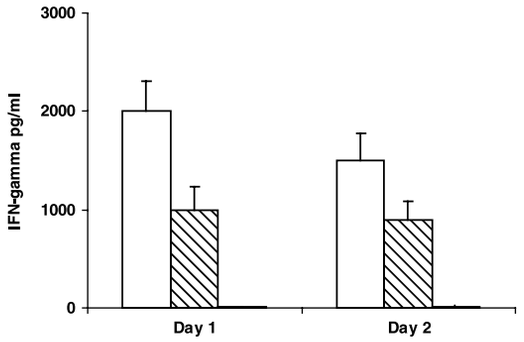

Figure 5. The effects of *Carpobrotus edulis* extract on the production of IFN-γ by PHA stimulated T-cells (non-adherent) from human PBMC. T cells present in the wash containing non-adherent cells of cultures designed to retain HPBMDM were isolated, washed, stimulated with PHA and cultured for 7 days in the presence and absence of (□) *Carpobrotus edulis* methanol (MetOH) and (▨) 10x diluted *Carpobrotus edulis* MetOH extract. (■) Control at the end of each day of incubation supernatantes were removed for estimation of IFN-γ present. Results are expressed as average of IFN-γ pg/mL ±SD measured from 3 donors (n = 3).

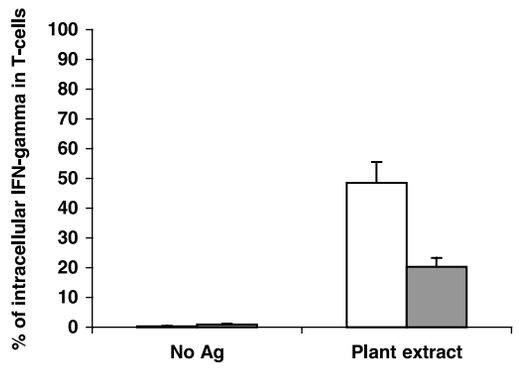

Figure 6. The effect of *Carpobrotus edulis* extract on IFN-γ present within (□) CD4+ and (■) CD8+ cells. Sub-populations of T lymphocytes were isolated from peripheral blood of healthy donors (n = 3) and cultured for 24 h in the presence and absence of methanol plant extract. After 24 h the cultures were harvested and stained with fluorescent antibodies against CD4+ CD8+ T cells for their identification in combination with intracellular IFN-γ. The amount of fluorescence representing intracellular IFN-γ within distinct sub-populations was obtained with the use of the FACSCalibur. Results are expressed as mean % expression of intracellular IFN-γ associated with CD4+, CD8+ T cells.

the plant methanol extract caused HPBMDM cultures supplemented with T cells to yield greater killing over that produced by similar treatment of THP-1 cells suggested that gamma interferon (IFN-γ) might have a role in killing inasmuch as the latter cultures do not contain the cell type which produces this compound. As shown by Fig. 5, both undiluted and diluted plant methanol extracts induced the production of IFN-γ by T cells within 1 day. Although the undiluted extract caused about twice the amount of IFN-γ released over that caused by the 1:10 diluted extract, the difference is not in proportion to the concentration of the methanol extracted material. The production of IFN-γ continues up to day 7 but at a decreasing rate. Again, the differences noted between undiluted and diluted extract containing cultures are not proportional to the concentration of the material contained in the respective extracts. Similar exposure of T lymphocytes to the plant methanol extracts did not affect appreciably the release or production of IL5 (data not shown). The effects of plant methanol extracts on intracellular IFN-γ, as shown by Fig. 6, confirm the results presented above. Briefly, the T cell populations CD4+ and CD8+ exposed to concentrated plant methanol extract presented greater amounts of intracellular IFN-γ without any concomitant increase of IL4 (data not shown).

Table 2. Effects of *Carpobrotus edulis* methanol extract on the MDR1 efflux pump of L1578 mouse T lymphoma cells

	R
L1578 Parent control	1
L1578 MDR-1 control	1
plus Verapamil control	9.6
plus DMSO control	0.93
plus Extract @ 20 µl	2.7
plus Extract @ 200 µl	15.8

Parental L1578 and multi-drug resistant L1578 MDR-1 mouse T lymphoma cells (2×10^6 cells/mL), plus 10 µL of the indicator rhodamine 123, were separately incubated for 20 min at 37 °C with and without verapamil, DMSO, 20 or 200 µL of *Carpobrotus edulis* extract. Verapamil is widely used for the demonstration of inhibition of MDR1 efflux pumps that account for antibiotic resistance of the cell (13, 14). The percentage of mean fluorescence for the parental and MDR-1 mouse T lymphoma cells served as controls (untreated) versus that of their respective treated (compounds added for evaluation of inhibition activity of efflux pump) was determined and employed the following equation; R = [(MDR treated/MDR control)/(parental treated/parental control)], which yields a measure of activity defining the effectiveness of the compound as an inhibitor of the MDR1 efflux pump.

Effects of the *Carpobrotus edulis* methanol extract on MDR1 lymphoma T cells

Exposure of the parental and MDR1 lymphoma T cells to various concentrations of the methanol plant extract did not produce toxicity as shown by the trypan blue exclusion test. The effect of the plant methanol extract on L1578 MDR1 cells is presented by Table 2. Firstly, parental lymphoma T cells accumulate rhodamine (epirubicin analogue) as expected since these cells are sensitive to epirubicin and do not have an efflux pump that excludes the drug. Secondly, MDR1 cells treated with rhodamine in the same manner as the parental cells have little fluorescence due to an active efflux pump that excludes the compound. Thirdly, compounds that are known to inhibit the efflux pump responsible for the resistance of MDR1 cells to epirubicin cause the cells to retain fluorescence. Exposure of MDR1 cells to various amounts of plant methanol extract causes these cells to retain fluorescence in accordance to amount of extract present.

DISCUSSION

The search for sources of anticancer drugs continues inasmuch as to date, no drug has yet been found or developed that retains its effectiveness when employed

in a chronic manner. The loss of effectiveness is due to the activation or mutation of a gene or genes which make the cancer cell resistant to the drug (Molnar *et al*., 2000). When examined closely, much of this ensuing resistance has been attributed to the presence of efflux pumps which extrude the drug from the cell before the drug reaches its more distally located targets (Safa *et al*., 1987; Sharples *et al*., 2001). These efflux pumps are readily demonstrable by the use of inhibitors, some of which serve to characterize the individual efflux pump. Verapamil is such an inhibitor and to this extent it has been used extensively for the demonstration of efflux pumps that extrude one or more antibiotics (Safa *et al*., 1987; Sharples *et al*., 2001). One such efflux pump that is inhibited by verapamil is the MDR-1 pump. The MDR-1 pump represents a highly conserved genetic region present in mammalian (Kerb *et al*., 2001) and bacterial cells (Gouyette, 1994).

The demonstration that cancer cells that become refractory to a given antibiotic contain one or more efflux pumps which account for such resistance has spurred the search for compounds that have inhibitory activity against these pumps. Our chance detection of a common plant whose physical characteristics are those commonly present in plants that are known to have alkaloids with anticancer activity resulted in the investigations described.

The results obtained indicate that the methanol extract has no obvious toxicity against THP-1 or HPB-MDM, or L5178 mouse T cell lymphoma cells. Non-toxic concentrations of the plant extract are shown to inhibit the activity of an efflux pump that is responsible for multi-drug resistance of MDR-1 mouse T cell lymphoma. This inhibition was significantly greater than that produced by verapamil.

The plant methanol extract affects a variety of activities of cells isolated from human peripheral blood or THP-1 cells; it induces these cells to proliferate 2 days earlier than that produced by PHA; it causes the T-cells to secrete gamma interferon and although it has no *in vitro* antimicrobial activity against *S. aureus* it enhances its killing by the macrophage that has phagocytosed the organism. The observation that pre-exposure of the cultures containing T cells to plant methanol extracts further enhances the killing of phagocytosed *S. aureus* is predictable in light of the effects of the extract on the production and release of IFN-γ by T cells and correlates well with the increased presence of intracellular markers IFN-γ in CD4+ and CD8+ cells that are involved in the killing of foreign cells.

Whether one component in the plant extract is responsible for the activities described in this study or whether each activity noted is due to separate entities is of course not known at this time. Nevertheless, the activities rendered by the methanol extract of the plant *Carpobrotus edulis* are intriguing and in light of the need to obtain compounds that inhibit efflux pumps responsible for antibiotic resistance of cancer cells, the extract will be fractionated and the isolated compounds examined as described in this study.

Of further interest is the activity of the extract on cells involved in cellular immunity, especially that on the enhanced killing of bacteria that has been phagocytosed by the human macrophage. Fractionation of the extract will of course be relevant to future studies of such T cell activities.

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