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Full Length Research Paper

In vitro immunomodulatory activity of various extracts of Maltese plants from the Asteraceae family

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Various extracts of ten plants from the Asteraceae family were studied for their effects of on human peripheral T-lymphocytes *in vitro*. Five solvent systems were used to extract constituents from these plants. Phytochemical identification of the most prevalent phytochemical classes was carried out, followed by screening for pharmacological activity using the Brine Shrimp Lethality test (BST). One BST-negative and five BST-positive extracts were tested on human lymphocytes. Marked effects were observed in treated lymphocytes with all six extracts. However only two extracts caused lymphocyte activation and pronounced blastogenesis similar to that of phytohaemagglutinin (PHA). The results obtained indicate that in particular the petroleum ether extract of *Calendula arvensis* is relatively non-toxic to peripheral lymphocytes suggesting its potential use as an immune booster.

Key words: Asteraceae, phytochemical analysis, lymphocyte activation, cell proliferation, cytotoxicity

INTRODUCTION

The Asteraceae family is the largest and the most cosmopolitan of the flowering plants and is probably the most widespread in the Mediterranean. This family consists of about 900 genera and some 13,000 species (Trease and Evans, 1978). Plants in this family were widely utilized in the past and are still used today for their medicinal properties. Activities include antibacterial and antifungal (Vajs et al., 2004), digestive properties, antihelminthic and antitussive (Pieroni et al., 2003), antidiabetic, to treat infertility (Burkill, 1985; Adjanahoun et al., 1991) and immunostimulatory (Jiménez-Medina et al., 2006; Rininger et al., 2000) as well as immunosuppressive (Rezaeipoor et al., 1999) and cytotoxic (Hoffmann et al., 1998) activities. These activities are attributed to several phytochemical classes including polysaccharides and proteins (Jiménez-Medina et al., 2006), terpenoids (Namdeo et al., 2006) and flavonoids (Trovato et al., 1996). On the Maltese archipelago, there are about 65 medicinal Asteraceae species out of 458 medicinal taxa (Attard, 2004).

In this present study, an attempt was made to investigate petroleum ether, chloroform, ethanol, 50% aqueous-ethanol and aqueous extracts of ten Asteraceae species for their immunomodulatory effects *in vitro*.

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MATERIALS AND METHODS

Plant Materials

The plant specimens for the study were collected fresh from several localities around Malta between October 2005 and January 2006. The specimens were authenticated by the botanist of the Faculty of Science, University of Malta, and voucher specimens were deposited at the Institute of Agriculture, University of Malta. The aerial parts of the plant were harvested and utilised for extraction.

The following Asteraceae plants were used for the study: *Dittrichia viscosa* (L.) Greuter, *Inula crithmoides* L., *Calendula arvensis* L., *Sonchus oleraceus* L., *Reichardia picroides* (L.) Roth, *Aster squamatus* (Sprengel) Hieron., *Glebionis coronaria* (L.) Tzvelev, *Leontodon tuberosus* L., *Galactites tomentosa* Moench and *Carlina involucrata* Poir.

Preparation of extracts

The freshly collected aerial parts were washed with distilled water, oven dried at 38°C and pulverised to fine powders. Dried powdered material (80-100 g per sample) was macerated in different solvents (200-650 ml) petroleum ether, chloroform, ethanol, 50% aqueous-ethanol and distilled water for 48 h at a speed of 210 rpm. All extracts were filtered through Whatman filter paper no. 41 and dried

Table 1. Percentage extracts (%w/w) for the five solvent extractions carried out on the ten Asteraceae species.

Plant name	Distilled water	Absolute Ethanol	Aqueous ethanol (1:1)	Chlorofom	Petroleum ether
Aster squamatus (Sprengel) Hieron.	8.13	2.56	13.13	1.73	0.45
Glebionis coronaria (L.) Tzvelev	12.14	2.83	13.33	2.89	8.47
Calendula arvensis L.	19.84	3.11	11.43	2.44	2.07
Carlina involucrata Poir.	2.94	2.82	8.29	2.34	9.52
Dittrichia viscosa (L.) Greuter	5.91	5.93	10.91	4.46	5.15
Galactites tomentosa Moench	8.52	3.84	17.68	2.86	8.6
Inula crithmoides L.	19.65	4.44	18.22	4.1	5.78
Leontodon tuberosus L.	8.74	3.69	12.25	2.52	3.91
Reichardia picroides (L.) Roth	9.65	3.09	10.15	2.28	4.94
Sonchus oleraceus L.	16.37	3.86	11.08	3.77	3.61

at 38°C to obtain dried extracts. All extracts were rendered water-soluble by the addition of a few drops of DMSO at a concentration not exceeding 0.25%, a dose that does not influence cell viability (Mirossay et al., 1999).

Phytochemical screening and primary bioactivity determination

A preliminary phytochemical screening of all fifty extracts was carried out using standard phytochemical procedures to determine the presence of flavonoids (Deshpande et al., 1986), terpenoids (Edeoga et al., 2005), alkaloids (Sreevidya and Mehrotra, 2003) and proteins (Friedman, 2004). The primary bioactivity of the extracts was monitored by the brine shrimp lethality test (BST) (McLaughlin et al., 1991). Briefly, 1000, 100 and 10 µg/ml final concentrations for each extract, were tested in triplicates on groups of 10 *Artemia salina* larvae and the median lethal concentration determined using Finney's Probit analysis.

Immunomodulatory effects

Human peripheral blood lymphocytes were isolated from heparinised peripheral blood of healthy human male volunteers using Histopaque®-1077 (Sigma, USA). These were cultured in RPMI 1640 medium supplemented with 15% foetal calf serum and antibiotics at a concentration of 2 x 10^6 cells/ml and distributed in 96-well plates as stated below. The cultures were treated with: (a) ethanol and (b) chloroform extracts of *G. coronaria*, (c) aqueous and (d) petroleum ether extracts of *I. crithmoides*, and (e) aqueous-ethanol and (f) petroleum ether extracts of *C. arvensis* and (g) PHA, m-form (Gibco BRL, UK); all to final concentrations ranging from 1000-10 µg/ml. The cultures were assayed, at the specified time intervals, according to the following procedures performed in triplicate.

Cell proliferation assays

These were performed using the WST-1 tetrazolium (Roche Diagnostics, Germany) assay for mitochondrial activity in flat-bottomed microtiter test plates. Spectrophotometric measurement of optical density at 450/650 nm in an ELISA reader (Statfax 2100, Awareness, U.S.A.) were carried out after 48 h. From the absorbance values, the relative percentage stimulation was calculated as the ratios of the experimental values to the value of the highest concentration for each extract or compound.

Cytotoxicity assays

Cytotoxicity was estimated using the tetrazolium LDH cytotoxicity assay (Roche Diagnostics, Germany) and spectrophotometric measurement of dye absorbance obtained at 492/650 nm in an ELISA reader (Decker and Lohmann-Matthes, 1988). Cell suspensions treated with 100 µl Triton X-100 were used as 'high' controls, while the untreated cultures were used as 'low' control. Cytotoxicity was estimated, at 48h, from the absorbance values using the following equation:

$$\text{Cytotoxicity (\%)} = \frac{A_{\text{exp value}} - A_{\text{untreated control}}}{A_{\text{Triton X control}} - A_{\text{untreated control}}} \times 100$$

Morphological Investigation

Morphological characteristics were observed in lymphocytes stained with Eosin Azure 50 (EA 50) as adopted from the Papanicolau method (Bonn and Drijver, 1986).

Statistical analysis

Numerical data was analyzed using the BMDP/DYNAMIC (v 7.0) (Cork, Ireland) statistical package for one-way analysis of variance (ANOVA), the Bonferroni post-hoc test for comparison of means with the control, one-way analysis of co-variance (ANCOVA) and two-tailed adjusted means T-test. Differences were considered statistically significant at a P value <0.05.

RESULTS AND DISCUSSION

The fifty extracts were prepared and tested for their phytochemical content. Extracts with very high yields include the aqueous extract of *C. arvensis (*19.8 %) and the aqueous extract of *I. crithmoides* (19.7 %), while those with very low yields include the petroleum ether (0.5%) and the chloroform (1.7%) extracts of *A. squamatus* ($p<0.05$) (Table 1). Flavonoids, terpenoids, alkaloids and proteins were found to be present in these extracts as observed by qualitative tests (Table 1). Of the 50 extracts tested, 76% were found to contain flavonoids,

Table 2. Reaction of extracts to Phytochemical assays.

Plant name	Distilled water	Absolute Ethanol	Aqueous ethanol (1:1)	Chloroform	Petroleum ether
Aster squamatus (Sprengel) Hieron.	-	P	AFP	FP	F
Glebionis coronaria (L.) Tzvelev	-	AFPT	T	FPT	FT
Calendula arvensis L.	-	AFP	FPT	APT	F
Carlina involucrata Poir.	-	A	FP	AFPT	FT
Dittrichia viscosa (L.) Greuter	FP	FT	FP	FPT	FP
Galactites tomentosa Moench	-	AP	FPT	FPT	PFT
Inula crithmoides L.	F	FPT	FPT	FPT	FT
Leontodon tuberosus L.	F	FT	FPT	FPT	F
Reichardia picroides (L.) Roth	F	PT	FPT	FPT	FPT
Sonchus oleraceus L.	A	FT	FT	FT	FT

"–" means absence, **A**; Alkaloids, **F**; Flavonoids. **P**; Proteins and T; Terpenoids.

Table 3. The median lethal concentrations, median inhibitory concentrations and cytotoxic activities for the extracts and PHA, for the brine shrimp test (BST), the cell proliferation assay (WST-1) and cytotoxicity assay (LDH), respectively.

Solvent/Plant Combination	LC_{50}, µg/ml (BST)	IC_{50}/SC_{50},[a] µg/ml (WST-1)	LDH[e]
PHA	-	<0.001[b,d]	25.058±5.059
Ethanol/*G. coronaria*	92.919	27.794±5.465	3.191±1.767[c]
Chloroform/*G. coronaria*	131.280	17.677±0.830	3.975±0.612[c]
Aqueous/*I. crithmoides*	>1000[d]	19.714±1.307	15.662±0.556
Aq-Ethanol/*C. arvensis*	62.697	>100[d]	39.042±7.626[c]
Pet. Ether/*I. crithmoides*	562.260	62.132±12.012	59.622±6.177
Pet. Ether/*C. arvensis*	796.274	0.089±0.068[b,d]	22.218±1.239
Aq-Ethanol/*I. crithmoides*	343.727	-	-

Values expressed as means ± S.E.M. [a]IC_{50} or [b]SC_{50}, for immunosuppressant or [‡]immunostimulant activity; [c]p<0.05; [d]p<0.01, v=5; [e]LDH at median inhibitory concentration in WST-1 test

56% contain terpenoids, 54% contain proteins and 16% contain alkaloids (Table 2). The high incidence of flavonoids in extracts agrees with Harborne (1991) who describes flavonoids as being widely distributed in plants. All extracts qualifying to the immunomodulatory assays all tested positive to the acidified vanillin test.

In the preliminary pharmacological testing with the BST, only 6 extracts tested positive with an LC_{50} below 1000 µg/ml, which is an indicator of activity (Alkofahi et al., 1997). The results are tabulated in Table 3. The aqueous extract of *I. crithmoides* gave a value of >1000 µg/ml but was selected in the immunomodulatory study as a negative control (p<0.01, v=5). The WST-1 results demonstrated the immunostimulatory and immunosuppressant activity of some of the Asteraceae extracts employed in the study. In fact, PHA and the petroleum ether extract of *C. arvensis* demonstrated an increase in proliferation (SC_{50} <0.01 µg/ml) as opposed to the other extracts (IC_{50} >10 µg/ml) (p<0.01, v=5), All extracts exhibited a concentration-dependent effect. The LDH assay revealed that at the median proliferative concentration (Table 3), the most toxic extract was the *I. crithmoides* petroleum ether extract (59.622±6.177%), while the least was the *Glebionis coronaria* extract (3.191±1.767 %) (p<0.05, v=5). PHA exhibited some degree of cytotoxicity (25.058±5.059%). However, this is mainly attributed to the fact that the apoptosis-resistant resting lymphocytes become susceptible to apoptosis only after activation (Wesselborg et al., 1993). The morphological studies exhibited various degrees of blastogenesis caused by plant extracts after 48 h incubation with lymphocytes (Figure 1). The qualitative analysis was based on the characteristics of resting and stimulated lymphocytes. The untreated controls showed no sign of lymphocyte stimulation as opposed to the extract and PHA-treated cells. Unstimulated lymphocytes exhibited an average diameter of approximately 7 µm (Roitt et al., 1993) with a high nucleus to cytoplasmic ratio and a condensed nucleus. Activated lymphocytes ranged between 20 and 40 µm in diameter with a central or slightly eccentric, round, euchromatic nucleus and a nucleolus usually present. There was no difference in the

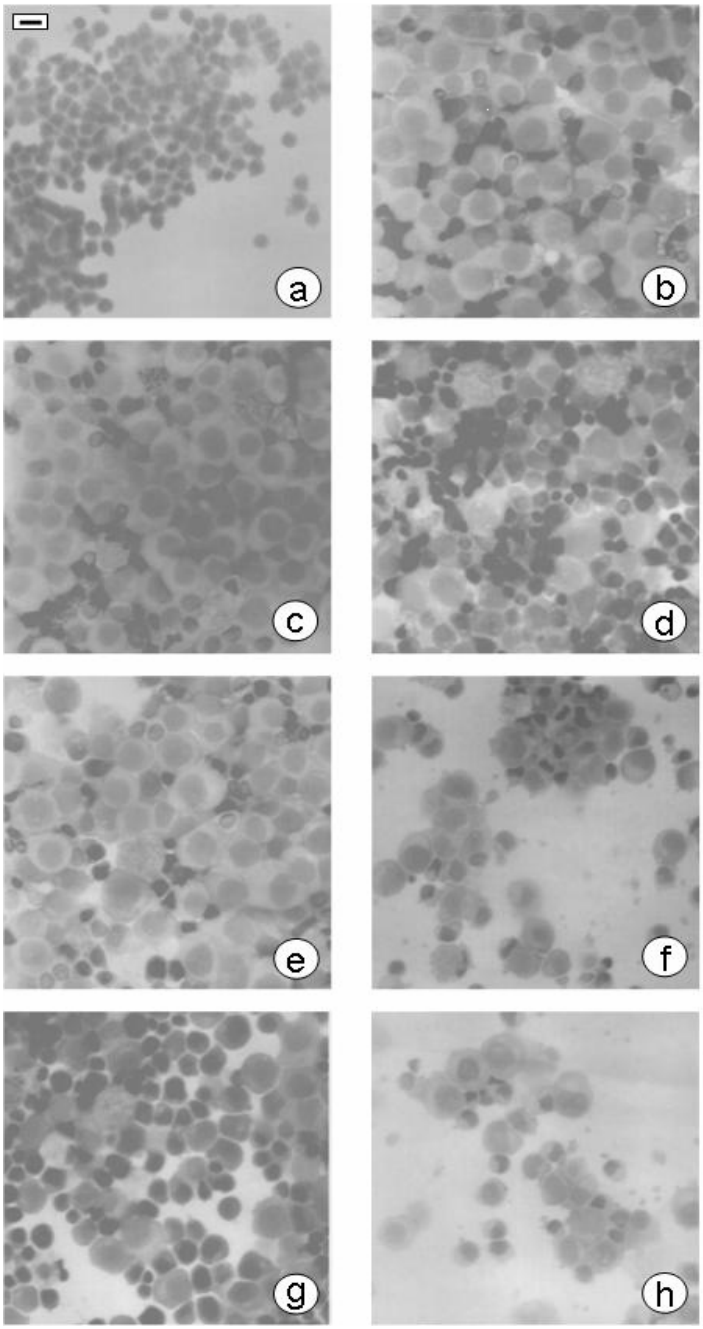

Figure 1. Morphological characteristics of cells as (a) controls (untreated), and treated with 10 µg/ml concentrations of (b) PHA, (c) ethanol and (d) chloroform extracts of *G. coronaria*, (e) aqueous and (f) petroleum ether extracts of *I. crithmoides*, and (g) aqueous-ethanol and (h) petroleum ether extracts of *C. arvensis*. Scale: 10 µm

morphology of activated lymphocytes after treatment with the extracts and PHA, and no immediate evidence of apoptosis was observed. Another qualitative attribute was the ability of lymphocytes to aggregate when stimulated (Frenster and Rogoway, 1968)). This gives evidence that resting lymphocytes are activated and are consequently driven into a state of proliferation.

Although flavonoids exhibit dramatic effects on immune and inflammatory cells, these can be either immunosuppressant (Li et al., 1991) or immunostimulatory (Lang et al., 1988). In some cases, the immunosuppressant effect is not caused by direct cytotoxicity of the flavonoids themselves. Some studies indicate that the effects are possible only when these cells are physiologically-activated (Middleton and Kandaswami, 1992). However, in our study we have shown that lymphocytes can be activated from their resting phase without the use of elicitors or prior treatment with mitogens. We have also shown that the BST assay is only an indicative pharmacological tool for immunomodulatory activity because even 'non-pharmacologically active' extracts may show immunomodulatory effects. Flavonoids have shown to be present in the active extracts. Therefore, this immune boosting activity, of the flavonoids present in the Asteraceae extracts, is worthy of further studies.

Conclusion

Out of the fifty extracts study, only six extracts exhibited an immunomodulatory activity. The most active extract was the petroleum ether extract of *C.* arvensis. This extract exhibited the presence of flavonoids, which have not been characterized under this study. Further work is required in order to isolate the immunomodulatory flavonoids.

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