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ISOLATION AND IDENTIFICATION OF A TUMOUR REDUCING COMPONENT FROM MISTLETOE EXTRACT (ISCADOR)

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SUMMARY

Using a combination of gel filtration and paper chromatography, a tumour reducing component from mistletoe extract (Iscador) was isolated and identified to be a peptide of approximate molecular weight 5000. The isolated peptide reduced the solid tumour induced by Dalton's lymphoma ascites tumour cells (DLA cells) in mice. The isolated component was very cytotoxic to the DLA cells but was not cytotoxic to normal lymphocytes, indicating a cell dependent specificity.

Key words: Iscador; Mistletoe; Antitumour drugs.

INTRODUCTION

An extract of mistletoe (*Viscum album*) known by the proprietory name Iscador had been found to be useful in cancer therapy [8,10]. However, due to its inconsistent response, its efficacy is still being questioned [11]. This extract had been found to be cytotoxic to various cells lines [5]. Reduction in some animal tumours, including Sarcoma 180 and Ehrlich ascites tumour, was observed during the administration of Iscador while some other tumours such as L1210 and P388 leukemia were non-responsive. Since L-1210 and P-388 are used for the primary screening of antitumour agents non-responsiveness to these tumours made its action controversial [1].

Recently we reported that Iscador administration reduced ascites tumours and solid tumours induced in mice by Dalton's lymphoma ascites tumour cells [7]. The latter originated from a spontaneously grown tumour of mouse thymus. In the present manuscript we have isolated the cytotoxic and tumour reducing component from Iscador and studied its properties.

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MATERIALS AND METHODS

Iscador M (5%) used in this study was a gift from Dr. Rita Leroi, Varein Fuer Krebsforschung, Switzerland.

In vitro cytotoxicity assay

In vitro cytotoxicity assay was conducted using Dalton's lymphoma ascites tumour cells [6]. Briefly a small aliquot of the extract of isolated fraction was incubated at 37 °C with 1 million cells in 1 ml phosphate buffered saline (PBS) for 3 h. After incubation, the percentage of dead cells was determined using the Trypan Blue exclusion method. Control tubes (without drug) had less than 5% dead cells after 3 h incubation. Cytotoxicity (in vitro) assay was also conducted, using human lymphocytes prepared by the Ficoll-Hypaque method using the same procedure. However, due to the higher percentage of dead cells, mouse spleen cells could not be used.

Tumour reduction experiments

Female Swiss albino mice weighing 16—20 g were used for animal tumour experiments. They were fed with standard mouse chow (Lipton, India) and were housed in ventilated cages in air-conditioned rooms. Dalton's lymphoma ascites tumour cells were propagated in the peritoneal cavity of the mice by injecting 10^6 cells. The cells were aspirated from developed tumours and washed in PBS. Solid tumours were produced in mice by injecting 1 million cells subcutaneously on hind limbs. Isolated component (10 μg) was injected subcutaneously at the same site on the following day and continued on alternate days. Five injections were given in total. Solid tumours were measured from day 6 and the volume was calculated using the formula $V = 4/3\,\pi r_1^2 r_2^2$ where r_1 and r_2 are radii of tumours.

Isolation of cytotoxic and tumour reducing component from Iscador

We have used in vitro cytotoxicity assay during the whole isolation procedure. Fifty millilitres of Iscador M (5%) was lyophilized to 5 ml, loaded on to a Sephadex G-50 (40 × 2 cm) column and eluted with isotonic saline. Two-millilitre fractions were collected and assayed for cytotoxicity. Active fractions (eluted at 23—28) were pooled and concentrated to 2 ml and passed through DE-52 cellulose column (15 × 1 cm) and eluted with saline. Fractions (2 ml) containing active material (eluted at 5—8) were pooled and concentrated to 2 ml and passed through a Sephadex G-10 column (44 × 1 cm) and eluted with water. Fractions (2 ml) containing the cytotoxic component were pooled, concentrated and streaked on Whatman no. 1 paper; the paper was developed with butanol/acetic acid/water (4:1:1). The position of the cytotoxic component, as determined from a guide strip, was eluted with water and concentrated to dryness by lyophilization.

Quantitation of isolated material and yield

Quantitation of isolated material was done by reaction with Folin-Ciocalteu reagent, absorption at 280 nm, and scanning the polyacrylamide

electrophoresis and staining with coomassie blue. Bovine serum albumin was used as standard, yield of isolated material in four different experiments was approximately 3.1 mg/100 ml Iscador.

Molecular weight determination

Molecular weight of the isolated component (without reduction) was determined from its mobility during SDS-polyacrylamide gel electrophoresis (7.5% gel) and comparison with known molecular weight standards. The molecular weight was also compared with insulin which has similar molecular weight of isolated component by SDS-PAGE and Sephadex G-50 column chromatography.

Digestion with proteolytic enzymes

The isolated component (10 μg) was digested with trypsin (500 μg) for 1 h at pH 8.0 and pronase (200 μg) for 1 h at pH 7.2. After the enzymatic treatment, the enzyme was inactivated by keeping in a boiling water bath for 10 min (enzyme solution without drug treated similarly was used as control). Acid hydrolysis was done using 6 N HCl at 90 °C for 1.5 h and excess acid was removed by evaporation. Heat denaturation was done by placing in a boiling water bath for 15 min. Isolated component treated by the above methods was tested for its cytotoxicity on Dalton's lymphoma cells by incubating for 3 h at 37 °C and percentage of dead cells was determined by the trypan blue exclusion method.

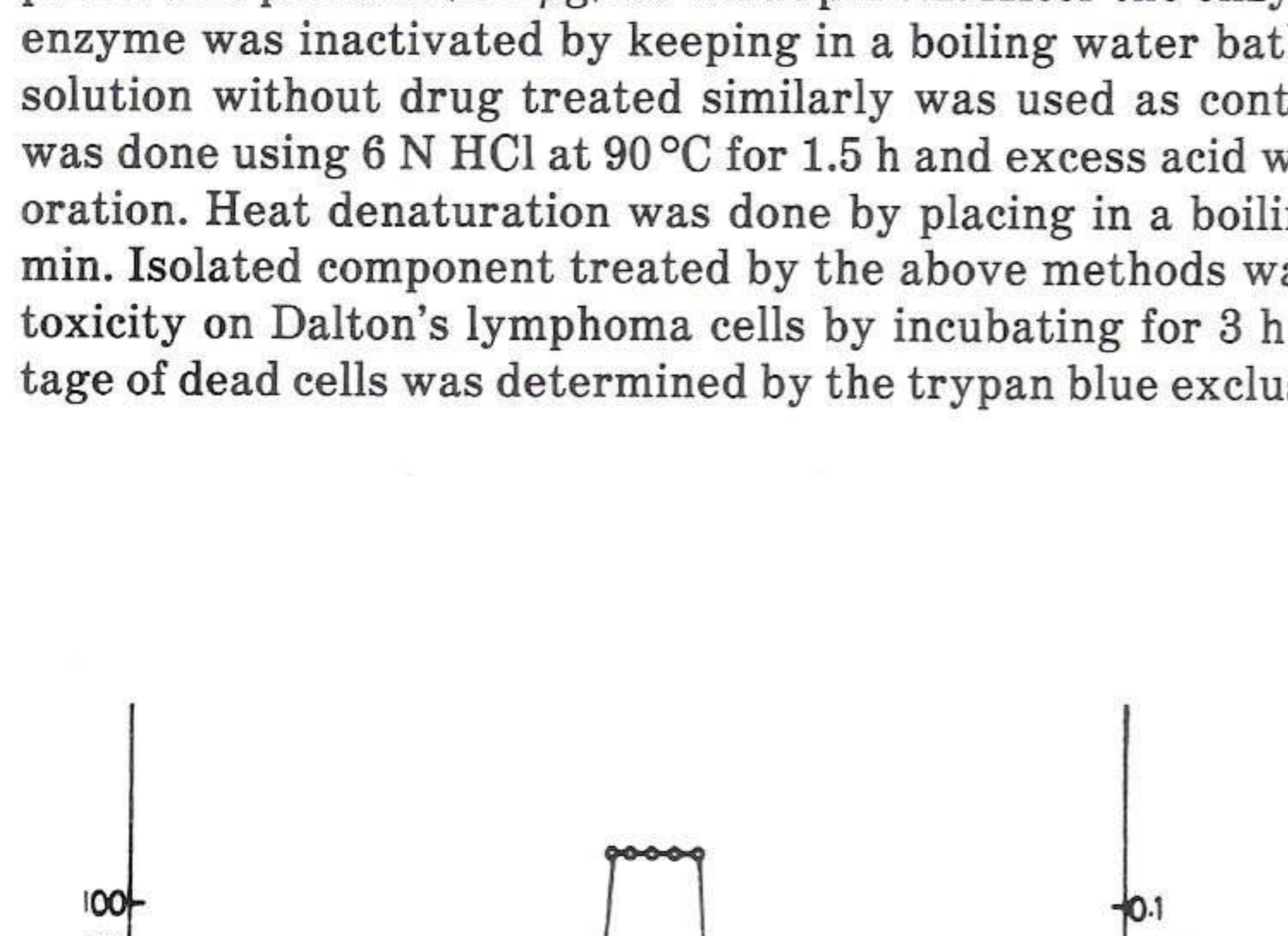

Fig. 1. Sephadex G-50 column chromatography of Iscador. Column (40 × 2 cm) was eluted with isotonic saline. Cytotoxicity of the fractions were determined by using Dalton's lymphoma ascites tumour cells and agglutination by sheep red blood cells. Marked area (× × ×) indicates the position of agglutinating component, O———O cytotoxicity, ●———● protein.

RESULTS

Initial column chromatography of Iscador on Sephadex G-50 indicated that the active material was eluted in late inner volume of the column indicating a rather low molecular weight (Fig. 1). When Insulin (molecular weight 6000) was chromatographed on the same column it was eluted in a similar position indicating a similar molecular weight for the isolated component. This was also confirmed by SDS-PAGE in which it moved in the same position of insulin (see later).

Fractionation of Sephadex G-50 separated the active component from a hemagglutinating component which was eluted much later. The active component was found to react with Folin-Ciocalteu reagent and had maximum absorbance at 280 nm indicative of peptidyl nature, only very little sugar was associated with these fractions.

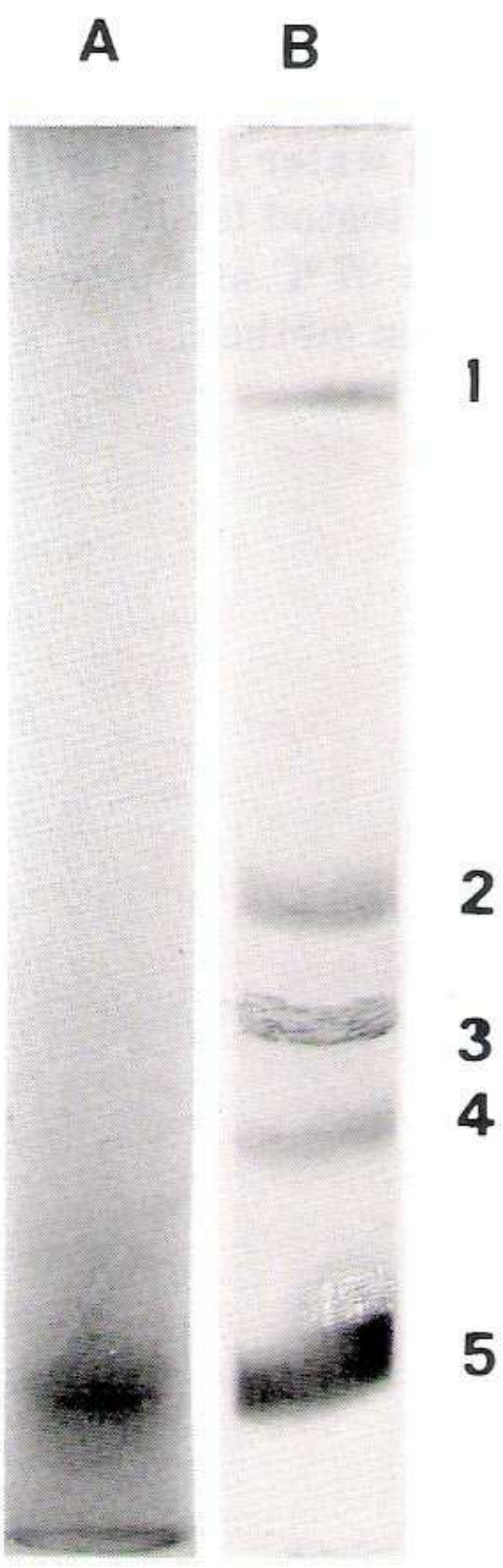

Fig. 2. SDS polyacrylamide gel (5%) electrophoresis of isolated component from Iscador. (A) Iscador component, (B) protein standards. (1) Ferritin; (2) catalase; (3) aldolase; (4) bovine serum albumin; (5) insulin.

The active material did not bind on DE-52 cellulose column, indicative of its basic nature. Further Sephadex G-10 column chromatography and paper chromatographic separation isolated the material into a pure component free from large molecular weight protein, free from lectin activity and free from small molecular weight amino acids and no detectable sugar. SDS-gel electrophoresis of the isolated component and further staining with Coomassie Blue and comparison with standards indicated that this component is a low molecular weight peptide (molecular weight approx. 5000). This was also confirmed by comparing its mobility on SDS-PAGE and Sephadex gel filtration profiles with that of insulin which has a similar molecular weight (Fig. 2).

Properties of the isolated material

The cytotoxic activity of the isolated component was stable to heat denaturation, trypsin and pronase digestion (Table 1). It had a maximum absorption at 280 nm and upon acid hydrolysis it liberated amino acids indicative of a peptide.

Cytotoxicity

There was a dose dependent cytotoxicity to Dalton's lymphoma tumour cells in vitro (Fig. 3). The cytotoxicity was also time dependent and needed nearly 3 h for its maximum effect. Concentration needed for 50% cytotoxicity was found to be 0.23 μg/ml in vitro to these cells. Cytotoxicity was reduced at 4 °C by 50%. It was found that treatment with isolated component produced a complete disintegration of Dalton's lymphoma ascites cells.

The isolated component did not produce any cytotoxicity to lymphocytes

TABLE 1

EFFECT OF TREATMENT OF ISOLATED COMPONENT WITH PROTEOLYTIC ENZYMES, HEAT AND ACID

Isolated material was digested with either trypsin pronase or had undergone acid hydrolysis or heat denaturation. After the treatments the enzyme was inactivated by boiling and acid removed by evaporation. The cytotoxicity of the treated material was determined in vitro using Dalton's lymphoma ascites cells (see Materials and Methods for details).

Treatment	Percentage of dead cells
Control cells	5
Treated with isolated component	100
Treated with trypsin digested isolated component	100
Treated with pronase treated isolated component	100
Treated with heat-denatured isolated component	100
Treated with acid hydrolysed isolated component	5

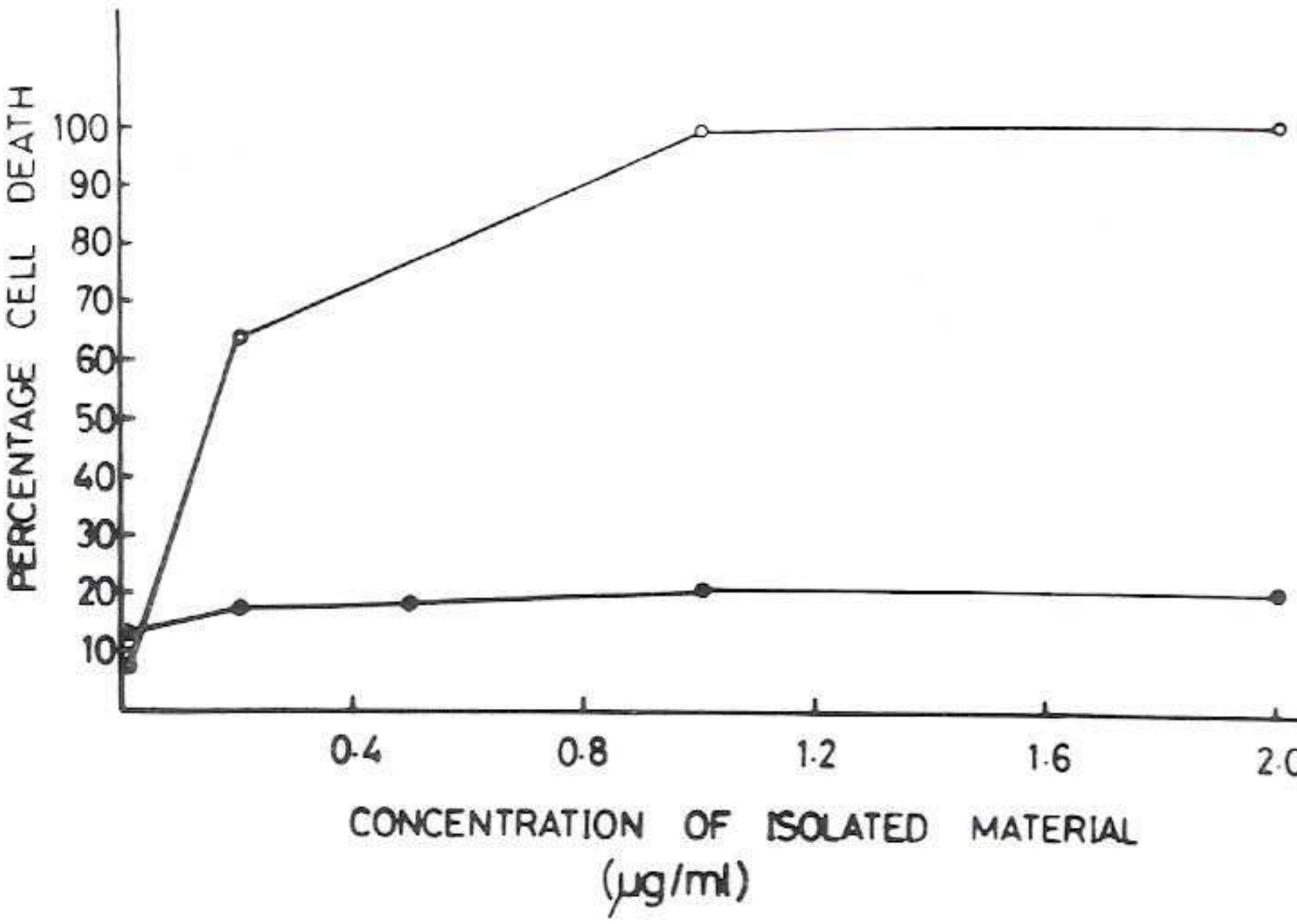

Fig. 3. Effect of various concentrations of isolated component (0.25—2 µg/ml) on cytotoxicity to Dalton's lymphoma tumour cells (○————○) and normal human lymphocytes (●————●). Ten times concentrated solution of isolated sample was used for lymphocytes (2—20 µg/ml).

(isolated from human volunteers by the Ficoll-Hypaque method). It was found that concentrations (20 µg/ml) which is 100 times more than needed for tumour cells for 50% cytotoxicity did not produce any effect on lymphocytes in vitro (Fig. 3). Due to the higher number of dead cells (> 50%) in controls mouse spleen cells could not be used in this assay method.

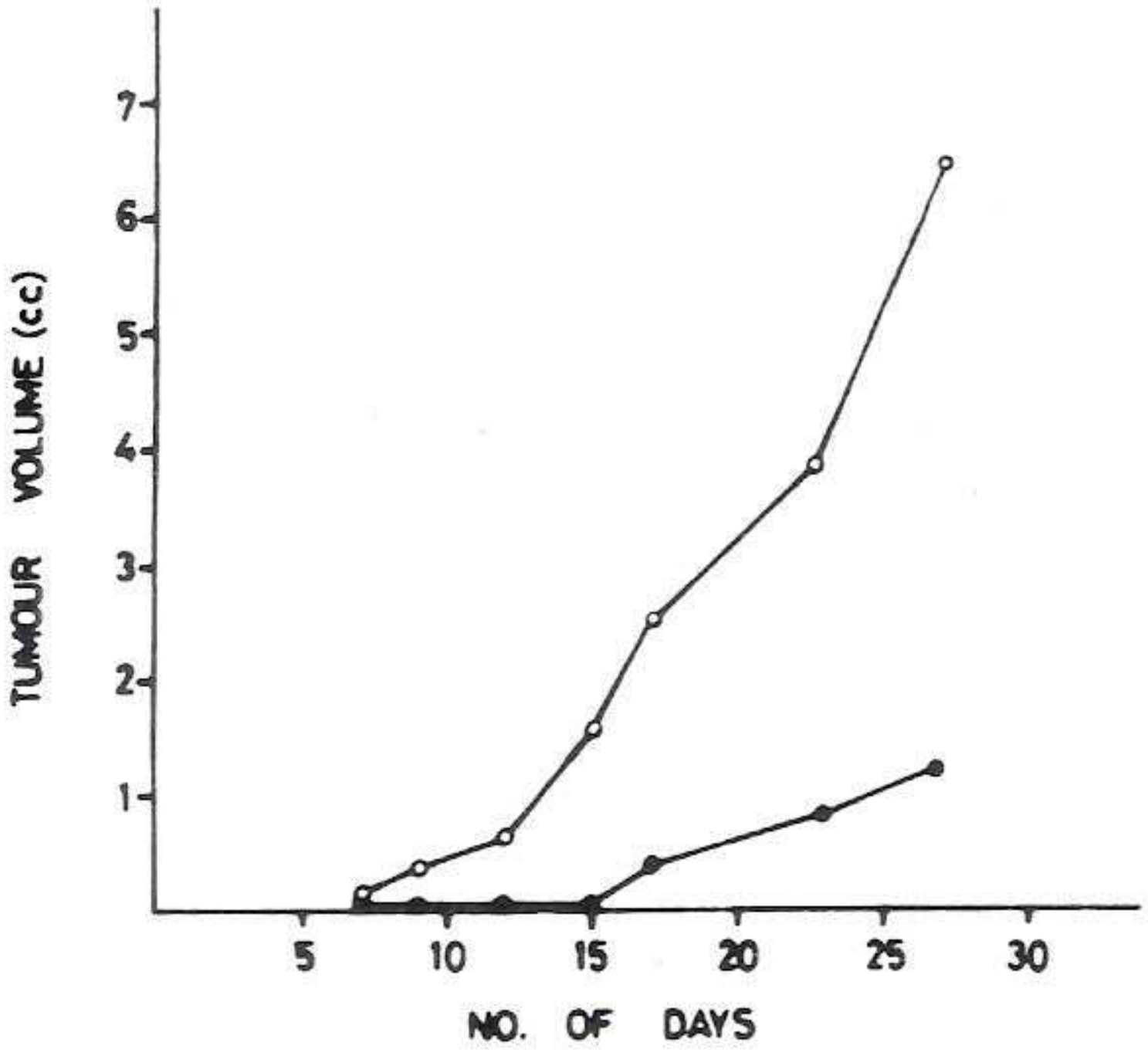

Fig. 4. Effect of injection of isolated component on solid tumour reduction in mice induced with Dalton's lymphoma ascites tumour cells. Values are average of 6 animals in each group. Standard deviation was less than 10%. ○————○, tumour volume of non-treated animals; ●————●, tumour volume of treated mice.

Tumour reduction

The effect of isolated component in tumour reduction is shown in Fig. 4. Injection of isolated component reduced solid tumour volume in mice considerably. For example, tumour volume of untreated mice was 0.5 ml on the 10th day, 1.5 ml on the 15th day, 3 ml on the 20th day, 3.75 ml on the 25th day and 6.5 ml on the 27th day while for the treated mice the tumour volume was nil on the 10th day, nil on the 15th day, 0.5 ml on the 20th day, 1 ml on the 25th day and 1.25 ml on the 27th day. These data indicate a considerable reduction in tumour volume during the treatment with isolated component from Iscador.

DISCUSSION

Viscum album as well as Iscador, the proprietory extract prepared from *Viscum album* has been shown to produce a regression of several kinds of tumours in experimental animals as well as clinically [2,8,10]. However, the tumour reducing component in Iscador has not been characterized. Several types of compounds have been isolated. This includes lectins which have been classified as ML I, ML II and ML III [3]. Viscotoxins [2], Vester protein complex [10], alkaloids [5], polysaccharides [4] and polyphenolic substances [13]. Vester found that both non-protein and protein components in *Viscum album* was found to have anticancer activity. But the exact nature of the component has not been determined. Other components such as viscotoxins and lectins were found to be cytotoxic to tumour cells but their anticancer activity has not been reported. In fact some of the immunomodulatory activity that is produced by *Viscum album* has been suggested to be mediated by the lectins or its component side chain [3].

In the present manuscript we have identified a tumour reducing component from Iscador as a peptide of molecular weight approximately 5000. Identification of the tumour reducing component as a low molecular weight peptide is not unexpected as many peptides of known antitumour properties have been reported recently. This list includes interferon, interleukins and several lymphokines. The molecular weight profiles, resistance to trypsin, pronase and heat resistance indicate that the isolated peptide is similar to viscotoxin [9] in its properties. However, at present we do not know whether this is the only tumour reducing component present in Iscador. Its role in tumour prophylaxis is also not known at present.

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REFERENCES

1 Berger, M. and Schmahl, D. (1983) Studies on tumour-inhibiting efficiency of Iscador in experimental tumours: J. Can. Res. Clin. Oncol., 105, 262–265.

2 Evans, M.R. and Preece, A.W. (1973) Viscum album — a possible treatment for cancer? Bristol. Med. Chir. J., 88, 17—20.
3 Franz, H. (1986) Mistletoe lectins and their A & B chains. Oncology, 43, suppl. 1, 23—34.
4 Jordan, E. and Wagner, H. (1986) Structure and properties of polysaccharides from Viscum album L. Oncology, 43, suppl. 1, 8—15.
5 Khwaja, T.A., Dias, C.B. and Pentecost, S. (1986) Recent studies on the anticancer activities of mistletoe (Viscum album) and its alkaloids. Oncology, 43, Suppl. 1, 42—50.
6 Kuttan, R., Bhanumathy, P., Nirmala, K. and George, M.C. (1985) Potential anticancer activity of turmeric (*Curcuma longa*). Cancer Letters, 29, 197—202.
7 Kuttan, G., Vasudevan, D.M. and Kuttan, R. (1986) Anticancer activity of an extract from Viscum albumin. Soc. Biol. Chem. 55th Annual Meeting 4, 105.
8 Leroi, R. (1975) Clinical Experience with Mistletoe Preparation, Iscador. Vittorio Klostermann, Frankfurt.
9 Samuelson, G. and Petterson, B.M. (1971) The amino acid sequence of Viscotoxin B from European mistletoe. Eur. J. Biochem., 2, 86—89.
10 Steiner, R. (1920) Spiritual Sciences and Medicine. Rudolf Steiner Publishing Co., London.
11 Unproven methods of cancer management, Iscador (1983) Can. J. Clin., 33, 186—188.
12 Vester, F. and Neinhaus, J. (1965) Cancerostatuche Protein Komponenten and Viscum album. Experientia, 21, 197—199.
13 Wagner, H., Jordan, F. and Fcel, B. (1986) Studies on the standardization of Mistletoe preparation. Oncology, 43, suppl. 1, 16—22.