Evaluation Of New Zealand Velvet Antler Efficacy And Diagnostic Testing

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Abstract

New Zealand velvet antler has been sold in increasing quantities in Asia since the start of deer farming in the early 1970s. New Zealand velvet antler has had to compete with velvet antler from the traditional suppliers, Russia and China. The aims of this paper are to describe an extensive series of studies, which were designed to test the efficacy of New Zealand velvet antler and, to report on progress toward diagnostic testing for velvet antler.

A range of efficacy measures were selected. These were intended to test New Zealand velvet antler for many of the principle traditional uses of velvet antler. The growth promoting activity was tested by feeding rats an aqueous velvet antler extract. The velvet antler caused a dose dependent increase in body weight. Inactivation of the extract with high heat prevented this growth stimulation. An anabolic function for New Zealand velvet antler has thus been shown. Immunopotentiation probably underlies the use of velvet antler as a tonic to ward off infectious disease. The efficacy of New Zealand velvet antler to cause proliferation of human white blood cells (lymphocytes) in culture was therefore measured as an index of this function. Aqueous extracts of New Zealand velvet antler were highly potent at causing the proliferation of lymphocytes. We can conclude that New Zealand velvet antler has efficacy as an immunopotentiating agent. The anti-inflammatory activity was tested using an *in vivo* model of peritoneal inflammation in mice. Extracts of New Zealand velvet antler were shown to be potent reducers of the inflammatory response, because they reduced to almost zero the number of neutrophils stimulated by an inflammatory challenge. New Zealand velvet antler thus has at least two potential modes of action on immune function. The efficacy of velvet antler to reduce the debilitating effects of anti-cancer drugs was also shown.

In vitro assays of growth and cytotoxic efficacy have been set up to amplify and complement the *in vivo* studies. The growth assays have highlighted the crucial relationship between yield and efficacy. Thus the velvet antler tip has higher growth activity than the upper part and so on down the antler. Velvet antler removed between 30-75 days of growth has similar growth promoting activity *in vitro*, but after 80 days of growth the growth efficacy decreases substantially. In contrast cytotoxic activity is maximal in the upper parts of velvet antler removed late, between 70-100 days after removal of the previous hard antler. Commercial processing of velvet antler reduces growth efficacy but amplifies or enhances cytotoxic activity compared with freeze drying. Overall the *in vitro* assays are proving useful as indicators of *in vivo* activity.

Diagnostic tests are required to prove the presence of velvet antler in extracts and unknown powders. Two approaches have been taken to develop diagnostic tests. Antibodies have been

raised in rabbits against an aqueous extract of velvet antler. These antibodies selectively recognise antler extracts and have promise for development into a diagnostic kit. Unique lipids or combinations of lipids have been found in the antler and their application as diagnostic markers are also being evaluated.

Overall these studies show that New Zealand velvet antler is efficacious in a wide range of traditional and novel applications. New Zealand velvet antler can confidently be used for traditional applications. Diagnostic tests are being developed.

Introduction

In Asia velvet antlers of Russian and Chinese origin are traditionally regarded as effective Traditional Chinese Medicines (TCM). This tradition is supported by an extensive scientific literature from Russia, Korea and China (see Brechman, undated; Kim, 1994; Wang, 1996 for reviews). These studies have shown that the efficacy of velvet antler in many of its traditional uses can be proven, scientifically. In particular Russian studies using pantocrine, an alcohol/water extract, have shown that velvet antler has hypotensive, erthyropoietic, anti-stress, stimulating, anti-inflammatory, gonadotrophic, growth and metabolic effects (Brechman, undated). These effects are consistent with the traditional uses of velvet antler as a warming tonic before winter, for children, for blood loss, for weakness and for chronic joint pain (Wang, 1996). As New Zealand velvet antler does not vary a great deal in composition compared with Russian or Chinese velvet antler (Suttie, 1995), it would be anticipated that New Zealand velvet antler would be equally effective. It remains to confirm that New Zealand velvet antler is efficacious in a clinical context. This is the first aim of the present paper.

In choosing suitable clinical tests to demonstrate the effectiveness of New Zealand velvet antler the New Zealand velvet industry was guided by two main concerns, to use simple tests which would give unambiguous results and to use tests which conformed, where possible, to traditional use. The uses of velvet antler for enhancement of growth, immunopotention, anti-inflammatory and amelioration of anti-cancer drug side effects were chosen for study. In the paper presented by the New Zealand group to the first Cervi Parvum Cornu Symposium in 1994 (Suttie *et al*, 1994) we described the use of two *in vitro* tests of efficacy, namely growth and cytotoxic activities. Since that time these tests have been improved and their use has been extended. These new findings are also reported in the present paper.

The traditional way of selling velvet antler was in the whole stick form. The whole stick was sliced at the point of retail and the slices sold to consumers who made their own medicine at home, usually in a mixture with other traditional medicines. Both buyer and seller of the slices could be confident that the purchase was genuinely antler, because they could see it. They could also make a judgement on the quality by observing the size, colour, smell and appearance of the whole stick and slices. Recently velvet antler extracts ground velvet antler powders have become available. These present dilemmas to the consumer as neither the genuine identity nor the quality of the velvet antler can be viewed. For these extract and powdered products to be successful, rigorous tests are required which will convince the consumer that their purchase is genuine. Currently no such tests are available, and this limits the application and commercial development of extracts and powders. The tests are required for two purposes, to identify the presence of velvet antler and to provide a standard, upon

which informed consumers can make purchasing decisions based on the level of that standard. It is likely that any standard would be based on the level of an active ingredient. At Invermay we have initiated several approaches to the development of diagnostic tests and the second aim of this paper is to describe the progress we have made in developing these for the presence of velvet antler in extracts or unknown powders.

Rat Growth

Velvet antler powder and velvet antler extract have been shown to increase growth in a dose dependent manner in mice (Mineshita, 1937), chickens (Bae, 1975, 1976) and rabbits (Gavrin, 1976). In New Zealand *in vitro* studies have revealed that New Zealand velvet antler extracts stimulate the growth of antler cells in a dose dependent manner (Suttie *et al*, 1994). Consequently it can be concluded that growth stimulation is a consistent effect of velvet antler.

The aim of this study was to determine whether feeding an aqueous extract of New Zealand velvet antler to young male Wistar rats could stimulate growth. A secondary objective was to investigate whether heat inactivation of the extract would influence the extent of any growth stimulation. The rationale for this was that although the active ingredients for growth were not known, it was hypothesised that they might be heat labile polypeptide growth factors, for example insulin-like growth factor 1 (IGF 1) (Sadighi *et al*, 1994). If heat inactivation blocked activity, them some informed speculation on likely active ingredients could be made.

Materials and Methods

Animals

70 Male albino Wistar rats of about six weeks of age and weighing about 105g were randomly allocated to one of seven treatment groups (see below). The animals were housed in group cages with 4-6 rats per cage. The rats were fed *ad libitum* a purified casein based diet with either none or different levels of velvet antler extract added (see below). Deionised water was available for drinking at all times.

Treatments

An aqueous based velvet antler extract was prepared and half of it was inactivated by raising its temperature to 120°C for two hours. The active and inactive velvet antler extracts were added daily for six weeks to the purified casein diets at one of three rates, to provide 10, 30 or 100mg/kg bodyweight.

The treatment groups were as follows:

1. Control
2. Low (10mg/kg bodyweight), heat inactivated velvet antler extract
3. Low (10mg/kg bodyweight), active velvet antler extract
4. Medium (30mg/kg bodyweight), heat inactivated velvet antler extract
5. Medium (30mg/kg bodyweight), active velvet antler extract
6. High (100mg/kg bodyweight), heat inactivated velvet antler extract
7. High (100mg/kg bodyweight), active velvet antler extract

Measurements

The rats were each weighed at the start of the study and weekly thereafter. At the close of the study the rats were killed and body organs were weighed and the femur was dissected for total calcium analysis. During the final week of the study each rat was individually confined for 24 hours, in a metabolism cage, for the collection of urine samples. These samples were used to measure calcium and hydroxyproline excretion.

Biometric Analysis

Weight, body component and urinary calcium and hydroxyproline values were analysed using analysis of variance (ANOVA) with control, extract activity status and dose as main effects. ANOVA on weight data was repeated with initial weight as a covariate, but this gave no advantage to the analysis.

Results

The rats were healthy throughout the trial and no health concerns were noted. All 70 rats completed the trial.

The mean body weights of each group of rats, expressed as percentages of the mean body weights of the control animals, is shown for each week of the study in Figure 1. The group fed the high level of active velvet antler extract grew significantly more throughout the study than the control group or the groups fed the heat inactivated extracts. During the first three weeks they rapidly achieved a 12% body weight advantage over the control animals, and then maintained most of this advantage during the rest of the study. The group fed the medium level of active velvet extract also grew significantly more during the final three weeks of the study compared with the control group or those groups fed the diets containing the heat inactivated extract. The overall weight gain also differed significantly (Table 1) among the groups. The groups fed the medium and high doses of active velvet antler extract grew significantly more in a dose dependent manner than the control group. In contrast those groups fed the heat inactivated extracts did not grow faster than the control and no dose response was observed.

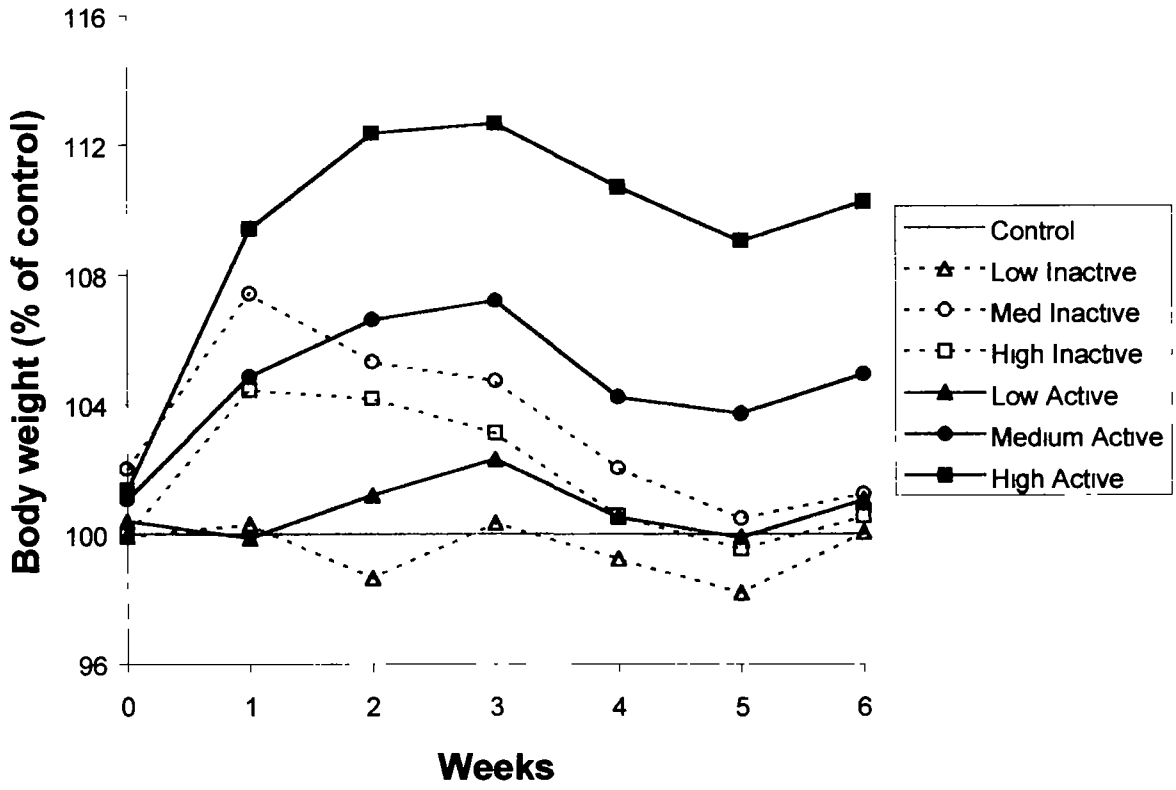
TABLE 1. The effect of feeding diets either unsupplemented (control) or supplemented with graded doses of heat inactivated or active aqueous New Zealand velvet antler extracts on weight gain, body organs, femur calcium and urinary excretion of calcium and hydroxyproline. The weight gain was measured over a six week period. The organ weights were recorded at slaughter. The femur was removed after slaughter. The calcium and hydroxyproline excretion measures were made during the week prior to slaughter. SED is the standard error of the difference between any two means.

	Control	Inactive			Active			SED
		Low 10mg/kg	Medium 30mg/kg	High 100mg/kg	Low 10mg/kg	Medium 30mg/kg	High 100mg/kg	
Wt gain[1]	221	221	223	223	224	236	253	11
Liver wt[1]	13 7	14 3	13 2	13.9	13 5	15 2	17 1	1 0
Testes wt[1]	3 12	3 21	3 11	3 13	3 07	3 13	3 25	0 13

	Control	Inactive			Active			SED
		Low 10mg/kg	Medium 30mg/kg	High 100mg/kg	Low 10mg/kg	Medium 30mg/kg	High 100mg/kg	
Femur calcium[2]	129	129	132	132	131	130	144	4
Hydroxyproline[3]	5 1	4 4	5 2	3 4	5 6	3 7	3 9	0 5
Urinary calcium[3]	2 0	2 4	2 8	3 2	2 9	3 0	1 6	0 4

[1] g
[2] mg
[3] μmol/L

FIGURE 1. Percentage changes, relative to control animals, of the bodyweights of groups of male rats fed active or heat inactivated New Zealand aqueous velvet antler extract throughout the study.



The liver weight was significantly heavier in the groups fed the medium and high doses of active velvet antler extract, but there were no significant differences in testes weight (Table 1). The calcium content of the femur was significantly higher in the group fed the high dose of active velvet antler extract.

Hydroxyproline urinary levels were lower in the groups fed the medium and high doses of active velvet antler extract and also the group fed the high dose of inactive antler extract. In contrast urinary calcium was significantly lower only in the group fed the high dose of active extract (Table 1).

Discussion

Feeding graded doses of active velvet antler extract resulted in dose dependent increases in weight gain. Heat inactivation abolished the positive effect of the extract on weight gain. It can therefore be considered that the growth response is due to the presence of heat labile substances, possibly growth factors, present in the velvet antler. The most complete published study on the effects of velvet antler extract on growth is by Bae (1975). He fed chickens which weighed 1.7-1.8kg at the close of an eight week study, 3.75-70mg of velvet antler extract per day. This is the equivalent of about 2-40mg/kg. He found that all doses were effective but the 18.75mg dose (about 11mg/kg) gave the highest response, and resulted in an increase in weight gain of about 6%. In the present study a dose of 30mg/kg (medium active treatment) gave a 7% increase in weight gain. The comparisons are not perfect as the chickens were only 41g at the start of Bae's study and gained 1.7-1.8kg in eight weeks, whereas the rats in the present study gained only 221-225g in six weeks. In addition the effective dose of velvet antler extract given to the chicks at the start of Bae's trial was 91-1820mg/kg, as he did not administer his extract on a bodyweight basis. Hence, given these differences, the results of the present study are in broad agreement with previous data.

In the present study liver weight but not testes weight was significantly increased by treatment with active velvet antler extract. In contrast Bae (1976) found, in chicken, that testes but not liver weight were increased in weight. Whether this represents a difference between rats and chickens or whether the composition of the extracts differed is not known. In the present study the liver effect is consistent with an overall anabolic effect. The lack of effect on the rat testes could be due to their young age or the specific velvet antler extract itself could contain no gonadotrophic as opposed to anabolic factors. As indicated in the discussion of the weight gain data, the anabolic responses could be interpreted as being caused by growth factors. If that is the case, it would explain the lack of gonadotrophic effects, which would be more likely to be caused by steroids. A dried New Zealand velvet antler powder, in contrast to the extract used, would be expected to have gonadotrophic effects.

Taken together the urinary calcium excretion and femur calcium data suggest that the high dose of active velvet antler extract increased calcium deposition and decreased urinary excretion. In data not shown, the mineral density of the bone was not altered by velvet antler extract treatment, so the most likely interpretation of the urinary calcium and femur calcium data is that it simply reflects an overall anabolic effect rather than a specific effect on bone.

The urinary hydroxyproline data is quite striking in that the high dose of inactive velvet antler extract as well as the medium and high doses of active extract reduced excretion. This points to a possible heat stable factor having some influence on bone metabolism. A decrease in hydroxyproline excretion would reflect an increase in bone matrix synthesis; conceivably this could be due to a small heat stable peptide or even a polar steroid.

In conclusion, an extract of New Zealand velvet antler increased growth in laboratory animals. This supports New Zealand velvet antler having true clinical activity, for growth.

Immunopotentiation Effects Of New Zealand Velvet Antler

In TCM velvet antler is frequently prescribed as a tonic in cases of stress and fatigue and for prevention of disease. Wang (1996) showed that pantocrine at a dose of 0.5-2mg/kg could stimulate the phagocytic function of macrophages in both normal and immune deficient mice. Taking the traditional use together with the Chinese data it could be considered that velvet antler can act as a stimulator of the immune system. In as much as the peak time of year for taking velvet antler is traditionally in winter, when infections are most common, it seemed logical to determine whether New Zealand velvet antler could act as a potentiation factor for the immune system. We chose to use a cell culture system based on the proliferation of human peripheral lymphocytes to evaluate extracts of New Zealand velvet antler.

The aim of this study was to investigate the potency of velvet antler extracts on the proliferation of human white blood cells in comparison with human recombinant interleukin 2 (IL-2) as a positive control.

Materials and Methods

Aqueous extracts were prepared from the base and upper portions of New Zealand velvet antlers removed 40, 60 and 85 days after casting of the previous hard antler. These dates roughly represent early, mid and late velvet antler growth and were selected in an effort to determine the optimal time of antler removal for separate efficacy functions.

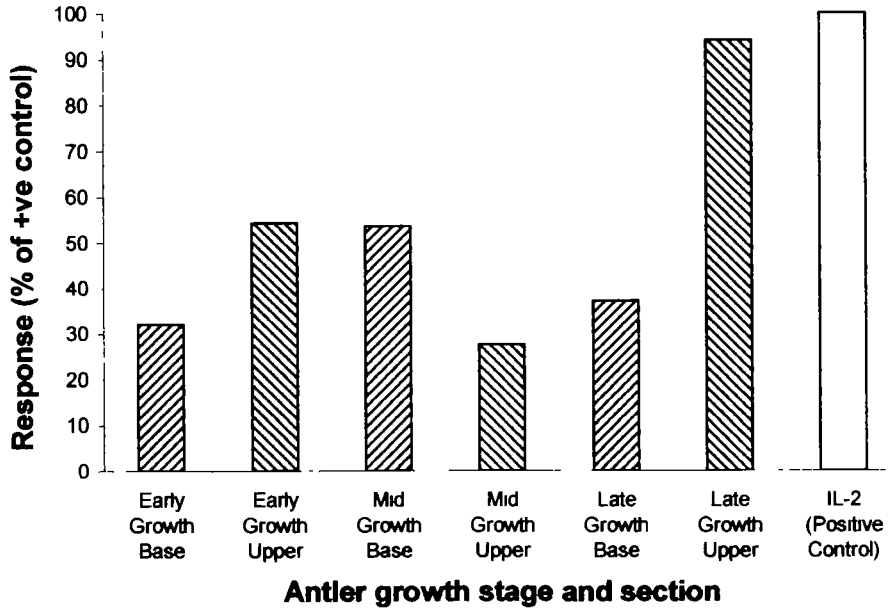
Human peripheral lymphocytes which had been sub-optimally stimulated with a mitogen (Con-A) were treated with increasing dilutions of each of the six extracts from 15-500µg/ml. The positive control, IL-2, which is the natural growth factor for T lymphocytes, was used at a dilution of 5µg/ml. Following incubation, proliferation of the lymphocytes was measured by the extent to which they incorporated tritiated thymidine.

Data are expressed as a percentage of the counts per minute of the positive control.

Results

All of the velvet antler extracts stimulated proliferation of the human lymphocytes at each of the dilutions. There was a peak of proliferative activity at 62µg/ml, and this has been illustrated in Figure 2. The extracts from the upper section of the early and late cut velvet antlers were more potent for immuno-stimulation than the base, but the opposite was true for the antler removed about mid growth. This is about the time of commercial velvet harvest in New Zealand. By far the most potent velvet antler extract was from the upper part of the velvet antler harvested at 85 days, which was removed very late by New Zealand standards.

FIGURE 2. Immunopotentiating activity of antler extracts on human peripheral lymphocytes. Data are expressed as a percentage of the positive control, which was IL-2 (5μg/ml).



Discussion

Extracts of New Zealand velvet antler showed clear immunopotentiation as measured by the proliferation of human peripheral lymphocytes. Thus it could be concluded that the traditional use of velvet antler in winter can be partly explained by its immunostimulatory activity. As only one extract for each velvet antler growth stage and section was tested it would be premature to discuss in detail the variations in efficacy which have been found. Nevertheless the data does point to a possibility that therapeutic functions of velvet antler extracts are variable dependent on time of removal and section of the antler. In this respect it is of interest to note that peak activity for immunostimulation was recorded from the upper portion of an antler which the traditional market would perceive as over-calcified.

In conclusion New Zealand velvet antler has been shown to possess immunostimulatory activity.

Anti-Inflammatory Activity Of New Zealand Velvet Antler

There are reports in the literature that velvet antler extracts have anti-inflammatory activity. Yudin and Dobryakov (1974) concluded that pantocrine showed marked anti-inflammatory activity. Wang Ben-Xiang (1996) has purified a potent anti-inflammatory fraction from hard antler which is particularly effective in mammary hyperplasia. Wang considers that a polysaccharide is responsible for this activity. In contrast Takikawa and Imai (1977) failed to find anti-inflammatory activity with graded doses of pantocrine in rats.

We decided to test whether New Zealand velvet antler had anti-inflammatory efficacy because activity such as this could explain the use of velvet antlers as a tonic and for the promotion of well being. The model used was an *in vivo* bioassay of mouse peritoneal inflammation.

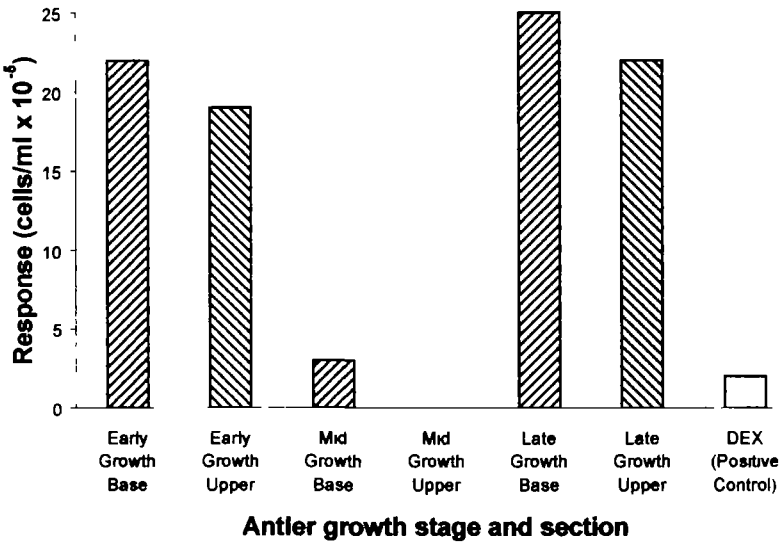
Materials and Methods

The same six aqueous extracts as used in the previous section on immunopotentiation were available for study. Inflammation was induced in mice by intraperitoneal administration of thioglycollate. This treatment induces an influx of neutrophils which peaks 18-24 hours after administration. Groups of three mice were treated with each antler extract or a positive control, dexamethasone, and then thioglycollate. After 24 hours the mice were killed and the peritoneal cavity was lavaged with 5ml of phosphate buffered saline. The number of white blood cells in the lavage fluid was determined. The suppressive effect of the antler extracts was compared to that of the positive control, and to the group given thioglycollate only.

Results

Extracts from velvet antler removed mid way through growth (about the time of normal antler harvest) markedly suppressed neutrophil production (Figure 3). In contrast none of the other extracts demonstrated any suppression of inflammation. To test whether the suppression was due to a cytotoxic response, rather than a true anti-inflammatory response, healthy peritoneal macrophages were cultured with the antler extracts. No cytotoxic effects were noted, (data not shown).

FIGURE 3. Anti-inflammatory activity of antler extracts. The bioassay was an *in vivo* model of peritoneal inflammation in mice. The positive control was dexamethasone. The response is the number of white blood cells collected by intraperitoneal lavage after treatment.



Discussion

Extracts from New Zealand velvet antler removed at the normal time in the New Zealand deer industry show strong anti-inflammatory effects, which are not due to cytotoxic activity, in a mouse peritoneal inflammation bioassay. This is a critical finding as another efficacy parameter can be confirmed for New Zealand velvet antler. The mechanism of this action is unknown. The antler is known to produce a number of cytokines (Brown-Smith *et al*, unpublished observations) and they could conceivably be responsible. Further studies are in progress.

The Development And Application Of *In Vitro* Efficacy Assays For Velvet Antler

Although it is widely recognised that different parts of the antler have both separate therapeutic activities, and levels of activity, few studies have systematically examined this variation. Russian studies, reviewed by Brechman (undated), consider only the effects of pantocrine, an alcohol/water extract. One reason why few comparative studies have taken place has been the lack of screening assays for the efficacy of velvet antler. It would be useful to have *in vitro* assays which would permit the evaluation, for example, of the effects of stage of antler at removal, processing (drying) and part of the antler. Such assays could rapidly and decisively assess the efficacy of velvet antler - and predict its clinical efficacy. Such knowledge could be extended in New Zealand, eventually, to genetic selection of stags with specific velvet antler composition and high levels of activity. In the paper presented to the first Cervi Parvum Cornu Symposium (Suttie *et al*, 1994) the preliminary development of these screening assays was discussed. These have been substantially extended over the last three years and the aim of this part of the paper is to report on progress using the techniques and to describe interesting and challenging findings. Two techniques have been developed, one to measure the growth promoting potential of velvet antler and the other to measure cytotoxic activity. The effects of processing techniques and stage of antler growth on *in vitro* efficacy have been measured.

Materials and Methods

Growth Efficacy

The efficacy of aqueous extracts of velvet antler on the growth of cells in culture was determined using 3T3 cells (BALB/C mouse embryo, ATCC No CCL 163). The cells were kept in continuous culture in $75cm^3$ flasks containing Dulbecco's Modified Eagle's medium (DME 90%), calf serum (CS 10%), penicillin (100U/ml) and streptomycin (100µg/ml).

Extracts were dissolved in serum free media (SFM) for 60 minutes at room temperature before vortexing. The antler extract solutions were sonicated for 2 minutes in a water bath at room temperature and were sterilised by filtration through a 0.22µm Durapore membrane (Millipore). Each antler extract solution was prepared in triplicate and was used within 12 hours of preparation.

The 3T3 mouse cells were seeded in 96 well plates at a density of $2x10^3$cells/cm^2. The cells were incubated for 48 hours after which the media was changed to SFM and the cells were incubated for a further 24 hours. This was followed by incubation in either 5% CS or SFM or in 0.625mg/ml antler extract solutions for 24 hours at 37°C. All media combinations contained [^{3}H]thymidine (5Ci/mmol; Amersham) at a concentration of 2.5μCi/ml. Following incubation, radioactive media were removed, 10% trichloroacetic acid (TCA) was added and the cells were washed twice with further TCA (10%). The cells were solubilised in 1M sodium hydroxide and the [^{3}H]thymidine was counted in HiSafe 3 scintillant (LKB). The results of these experiments were expressed as uptake of [^{3}H]thymidine (dpm/well) and were calculated as the means of duplicate wells of triplicate samples. Results were then scaled using the statistics software package GENSTAT relative to a standard value of 100% for the positive control, 5% CS.

Cytotoxic Efficacy

The cytotoxic efficacy of organic extracts of velvet antler has been determined using P388 (murine leukaemia) cells. The cytotoxicity assay utilised 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is converted enzymatically by viable healthy P388 cells to a purple dye, MTT formazan. For this assay a two-fold dilution series of the samples of interest was incubated for 72 hours with P388 cells. The concentration of sample required to reduce the P388 cell growth by 50% (compared to control cells) was determined by measuring the absorbance of the purple MTT formazan at 540nm. The control cells included a sample blank, in which no P388 cells were present. The intention of the blank was to ascertain whether the sample itself was capable of reducing MTT. The results were expressed as an IC_{50}, in ng/ml; that is the sample concentration required to inhibit the growth of P388 cells by 50% compared to the untreated control wells. These values were transformed as the logarithms to the base 10 prior to biometric analysis.

Extracts Evaluated

Three sets of experimental antlers were available to provide a range of extracts for the evaluation of the *in vitro* methods.

Baseline Experiment : 20 antlers of A, B or C grade were removed 60 days after casting of the previous hard antlers. Separate pooled aqueous extracts of the A and B grade, and of the C grade antlers were prepared for analysis.

Stage Comparison: 12 adult New Zealand red deer stags had one antler (randomly, left or right) removed 60 days after casting of the previous hard antler. The stags were randomly allocated to removal of the contralateral antler at 30-100 days after casting of the previous hard antler. The velvet was freeze dried and aqueous and alcohol extracts were prepared from the tip, upper, mid and base portions. For biometric analysis, efficacy data from the velvet antlers were grouped into the following time periods 30-50 days, 55-65 days, 70-75 days and 80-100 days after casting of the previous hard antler. These periods can be described as "early", "normal harvest", "late harvest" and "mature", respectively. They were chosen to reflect both biological variation and the range of commercial practice.

Process Comparison: 12 adult New Zealand red deer stags had both antlers removed 60 days after casting of the previous hard antler. One of each pair of antlers was randomly allocated to freeze drying and the contralateral antler was allocated to processing at one of two commercial processing plants. Aqueous and alcohol extracts of the upper and base portions of the antler were prepared.

Results

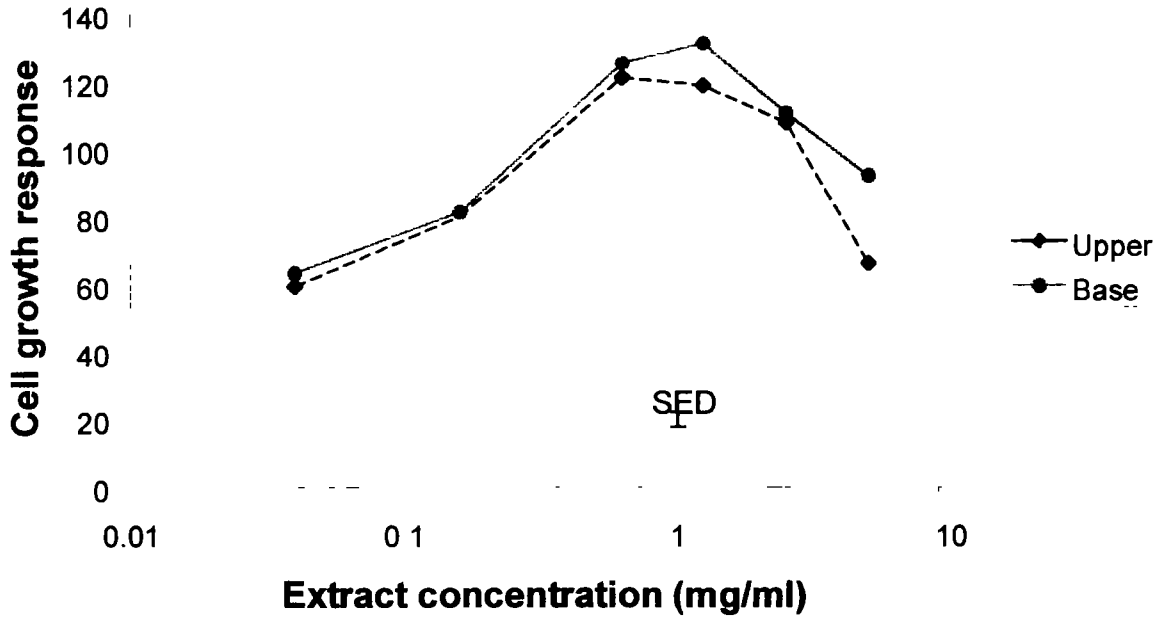
The *in vitro* efficacy data were analysed both with and without adjustment for the yield of the individual extracts. Adjusted data represent the activities of the tissues from which the extracts were prepared, while the unadjusted data is that of the extracts themselves, on a weight for weight basis. In other words, the adjustment for yield allows data obtained using extracts to be extrapolated back to the antler tissues of origin.

In Figure 4(a) the growth data for a range of concentrations of the upper and the base aqueous extracts of the Baseline Experiment antlers are presented. The extracts from the two portions of the antler show similar patterns of growth response, with the base extract of apparently slightly greater activity. However, as can be seen in Figure 4(b), when the data is adjusted for yield a different pattern emerges; a much lower amount of velvet antler tissue from the upper part is required for peak potency than is required from the base for the same growth response. The TCM tonic effect of the upper portion of antler tissue would thus be expected to be much greater than the base.

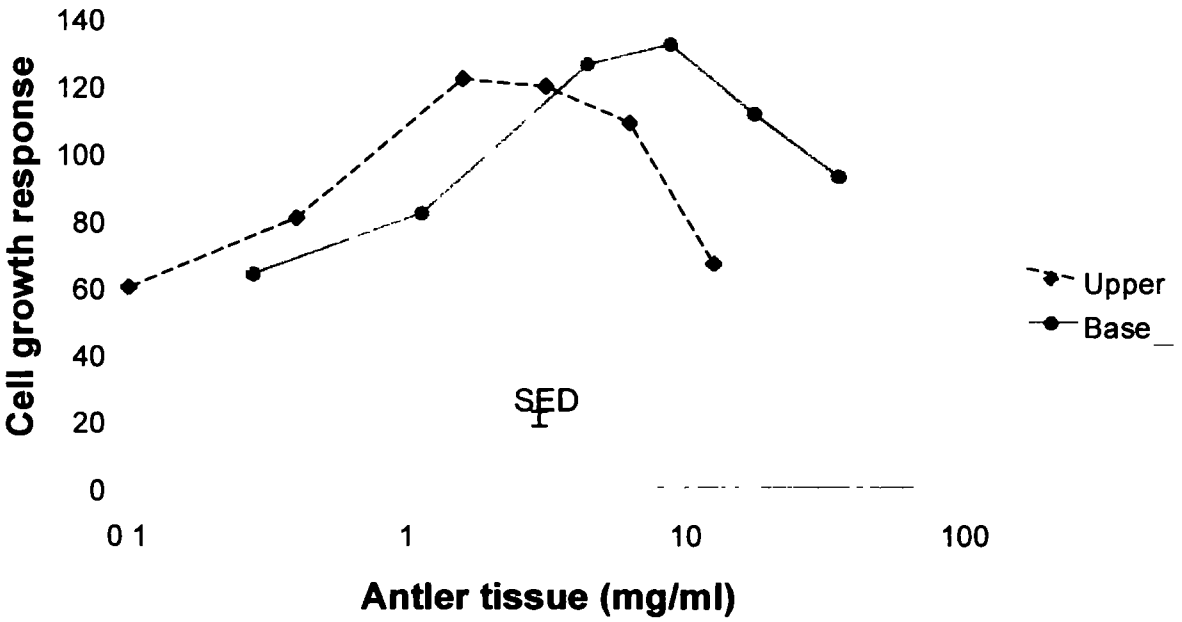
For the studies described below the cell growth activities were determined using extract concentrations of 0.625mg/ml, at or near the concentration required for peak response. Results were scaled relative to the response of 5% CS, which was the positive control and was given the arbitrary value of 100%.

FIGURE 4. The effect of (a) aqueous antler extract or (b) antler tissue on the *in vitro* cell growth response. Data for the antler tissues were derived from that of corresponding extracts by biometric adjustment for the extract yields. Results for aqueous extracts from the upper and base portions of the antlers are presented. SED, standard error of the difference.

(a)



(b)



The relationship between the yields of extract obtained from the Stage Comparison velvet antlers and the growth efficacy of the tissue was examined by plotting the adjusted growth data against the extract yields (Figure 5). This revealed that the greatest growth activity was exhibited by tissues from the tips of antlers cut prior to the "mature" period, which also gave the highest yields of aqueous extract. In contrast, when the unadjusted data are plotted [Figure 6(a)] in relation to days of velvet antler growth and to section of the antler, it is evident that extracts made from the tip of the antler are the least effective at stimulating cell growth, and those from the base the most active. Overall, growth activity of the extracts did not change throughout the period 30-100 days following casting of the previous hard antler. However, the corresponding graph of the adjusted data [Figure 6(b)] clearly shows that from 30-75 days of growth the tip was the most growth promoting tissue, followed by the upper, mid and base portions in that order. Tissue growth efficacy was constant during that period for each type of tissue, but significantly declined during the 80-100 day period.

FIGURE 5. The relationship between *in vitro* cell growth response of antler tissue and yield of aqueous antler extract (as a percentage of tissue dry matter). Data for the antler tissues were derived from that of corresponding extracts by biometric adjustment for the extract yields and are from portions of antler from the Stage Comparison series.

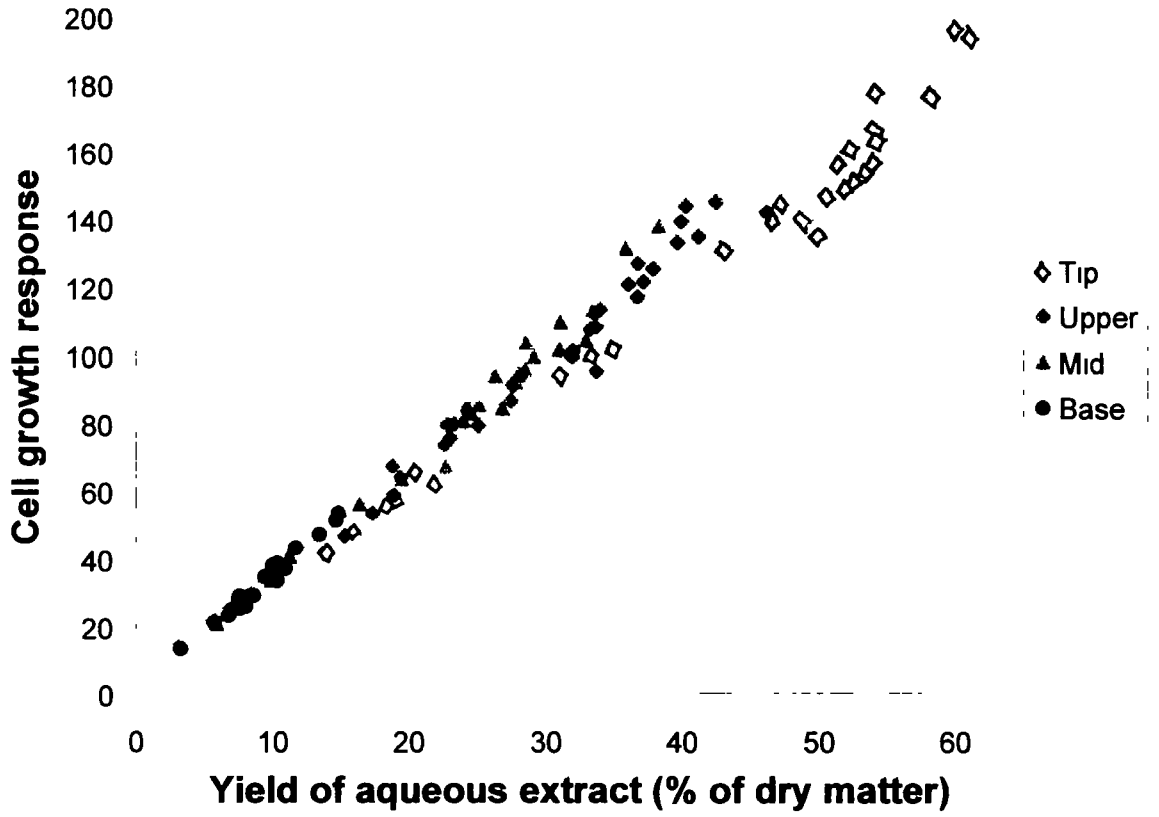
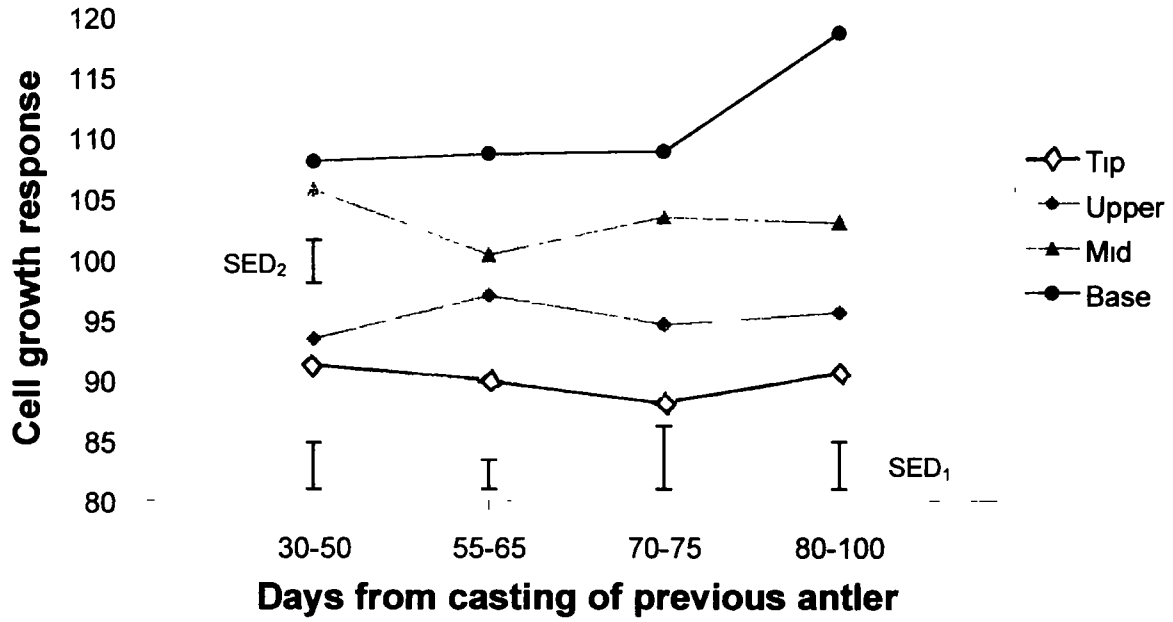
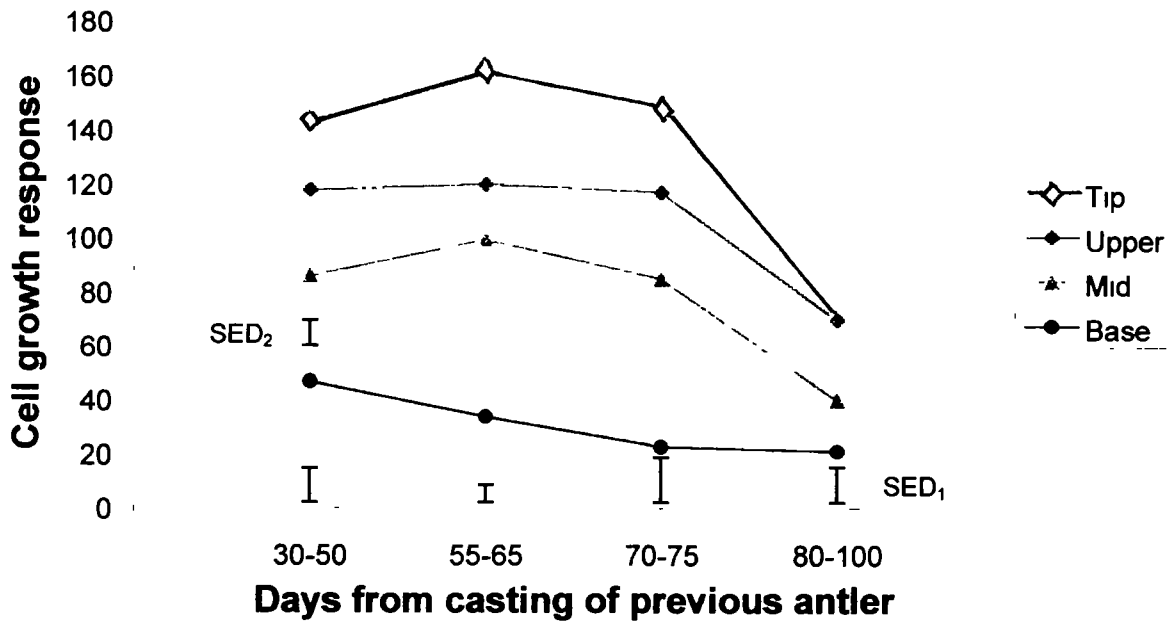


FIGURE 6. The effect of days from casting of the previous hard antler on the *in vitro* cell growth response of (a) aqueous antler extract and (b) antler tissue. Data for the antler tissues were derived from that of corresponding extracts by biometric adjustment for the extract yields. Tip, upper, mid and base parts of the antler were studied. SED_1, stand error of the difference for comparisons within antler ages; SED_2, stand error of the difference for comparisons within portions of the antler.

(a)

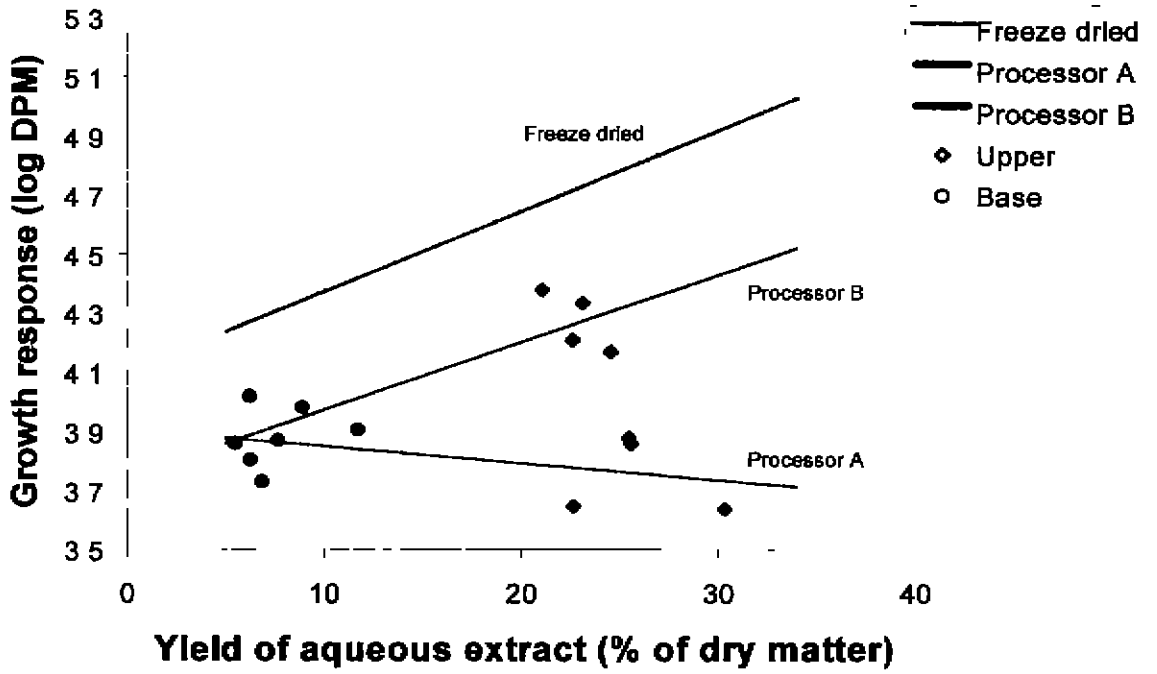


(b)



The effect of antler processing technique on the relationship between tissue growth efficacy and extract yield was studied using the upper and base portions of the Process Comparison antlers (Figure 7) As before, this data was obtained using extracts and was then adjusted for yield of extract. Freeze dried antlers had the greatest activity, at any yield Both commercial drying processes produced antlers which gave lower yields of extract than freeze dried antlers but their activities varied dramatically Processor B's velvet antler showed a similar pattern of growth response to the freeze dried antler, but at a lower level. In contrast, Processor A produced velvet antler of generally very low activity, although the activity of the base portion of the velvet antler appeared to be less affected than the upper portion. This finding emphasises the crucial importance of the processing technique used, in ensuring the retention of high activity Although freeze drying does not produce antler of good colour, these data point strongly to the usefulness of this technique for the production of velvet antler with high growth activity

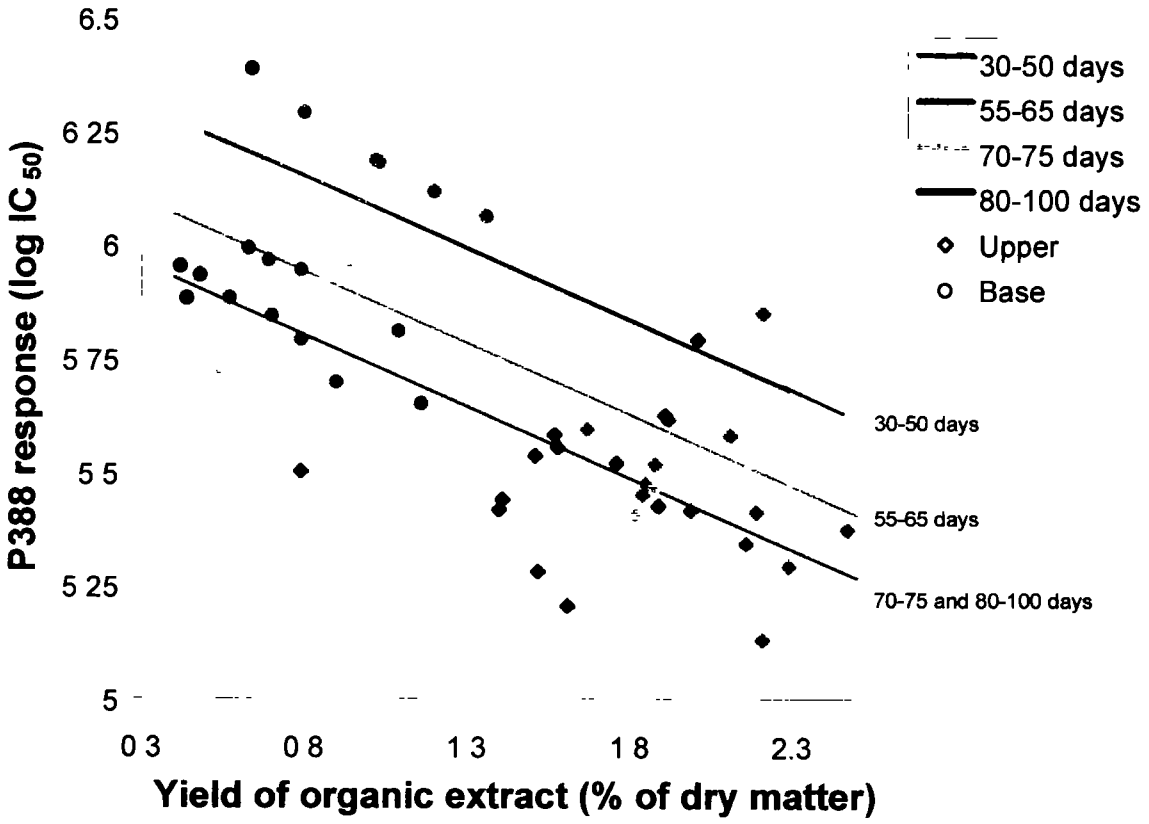
FIGURE 7. Effect of processing technique on the *in vitro* cell growth response of velvet antler tissue Data for the antler tissues were derived from that of corresponding extracts by biometric adjustment for the extract yields Velvet antlers were either freeze dried or dried commercially by either of two processors A or B. Upper and base parts of the velvet antler were studied



The cytotoxic assay results are also presented relative to the yield of organic solvent extract The P388 response is expressed as the log IC_{50} - that is, lower numbers indicate higher cytotoxic activity Figure 8 illustrates the effect of part of the antler (upper or base) on the cytotoxic activity of antlers removed at different stages of maturity (days from casting). The

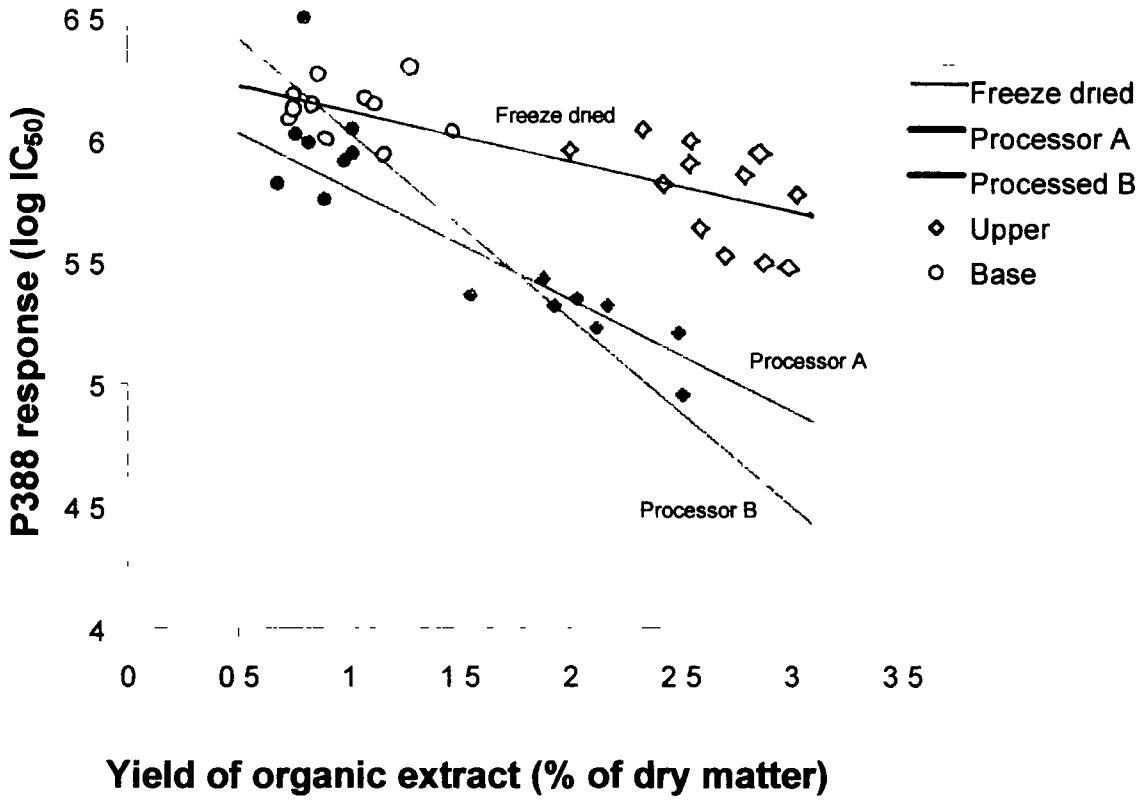
regression lines have been fitted to a common slope but the intercepts vary. Interestingly the most mature antler, removed between 70-100 days from casting, had the highest activity. The activity was least from the earliest velvet antler cut at 30-55 days. As for the growth assay, yield was a good predictor of cytotoxic activity and higher yields corresponded to slightly higher activities. Consequently, upper portions were of higher activity than bases.

FIGURE 8. The relationship between cytotoxic (P388) response and yield of organic extract (as a percentage of tissue dry matter) of velvet antlers removed at different stages from casting of the previous hard antler. Data for the antler tissues were derived from that of corresponding extracts by biometric adjustment for the extract yields. Upper and base parts of the antler were studied.



The effect of processing on cytotoxic activity of antler tissue is presented in Figure 9. There was again a good relationship between yield and activity. The freeze dried velvet antler showed the least activity, and the upper part was slightly more active than the base. In contrast, velvet antlers from both commercial processors were considerably more active than the freeze dried antler, particularly in the upper part.

FIGURE 9. Effect of processing technique on the cytotoxic (P388) response of velvet antler tissue. Data for the antler tissues were derived from that of corresponding extracts by biometric adjustment for the extract yields. Velvet antlers were either freeze dried or dried commercially by either of two processors A or B. Upper and base parts of the velvet antler were studied.



Discussion

Two screening assays have been developed and these have been applied to investigate variation in efficacy due to portion of the antler, stage of maturity (days from casting) and processing technique. The data have been adjusted for yield, in order to express results relative to weights of the tissues of origin rather than extracts, and this correction impacts greatly on interpretation. Extracts of base portions of the antler appear to be quite active in the growth assay, compared to extracts of other portions used at the same concentrations, but this advantage is lost when the comparison is made relative to equal amounts of antler tissue. Yield of extract is always much lower at the base than tip, upper and mid portions, so a greater amount of tissue from the base is required to achieve a similar level of efficacy as the other tissues.

Stage of development does not influence growth activity greatly from 30-75 days of growth but, in contrast, section of the antler has a large effect. Tip and upper portions of the antler have consistently higher activity than lower portions. It is not surprising that growth activity follows this pattern because, as the antler grows from the tip it is reasonable to expect most

growth factors will be present in this region. As the antler tissue differentiates and become organised into bone, distally, then growth factor levels might be expected to reduce; this is borne out by the data. Likewise it is reasonable to expect that as the rate of antler growth slows after 80 days, growth factor levels will decrease. It is also likely that the composition of growth factors differs among the portions of the antler. This was demonstrated by the fact that the extracts from the lower portions of the antler were more active on a weight for weight basis than those from the upper and tip portions. Thus the variations in growth efficacy are qualitative as well as quantitative.

Antler processing technique had a considerable effect on growth efficacy in the *in vitro* assay. One processor appeared to have lost a great deal of growth activity. It is critical then, when considering the efficacy of antlers that arrive in the Traditional Medicine marketplace from different sources or regions, to consider their method of processing. Some processors will be more successful at retaining - or improving - any specific activity than others. The fact that the cytotoxic activity was indeed higher in the commercially processed antlers compared with the freeze dried antlers indicates that specific activity and processing method are linked in a complex way. In the future it may be necessary to process antler in particular ways to maximise specific activities for any given application.

The cytotoxic activity data of the velvet antler extracts present a cohesive picture of the effects of the portion of the antler, the stage of growth and the processing technique on the *in vitro* measure of efficacy. The velvet antler extracts tended to increase in cytotoxic potency with maturity of the antlers, and highest levels were seen in antlers which were removed much later than normal farming practice. This finding brings a further possibility; in the future antlers will be removed when their required therapeutic potential is at the highest. In addition, they will be processed in specific ways to retain/maximise activity.

Our aim is now to continue to use these assays to screen antlers and extracts from different sources. We cannot of course be absolutely sure that *in vivo* clinical activity and *in vitro* activity are precisely the same - that indeed would be unlikely - but we can use the assays to make rational decisions on the choice of velvet antler to use for particular applications. It is encouraging, though, to note that the aqueous velvet extract that increased the growth of rats also had pronounced activity in the *in vitro* cell growth assay (data not shown). The assays are cheaper than full scale clinical trials and have the advantage that large numbers of variables can be included in an experiment. It is realistic to suggest that assays such as these could be used by a purchaser of antler for evaluation prior to allocating it to a specific use. It is also likely that stags which produce antlers with high levels of activity can be selected, but the heritability of such traits are unknown, at this time.

Overall, these assays, although simple, have enormous potential for routine application.

DIAGNOSTIC TESTS FOR VELVET ANTLER

Consumers in most countries today are looking for more "natural" health products that are convenient to take. Velvet antler, with its long tradition of use in Traditional Chinese Medicine, is ideally suited to capture a sizeable share of this developing market. First, however, velvet needs to be presented in forms which are convenient to use. As a

consequence velvet extracts have considerable potential, because of their ability to readily be mixed with other components and to dissolve in water or alcoholic beverages.

There is, however, a considerable impediment to this shift from traditional marketing of velvet towards that of new added value products; namely the current inability to prove that velvet really is a component of preparations that claim to contain it. For this reason, one of the key objectives of the Velvet Antler Research Programme in New Zealand is the development of diagnostic tests that will conclusively identify velvet antler and extracts in mixtures. Initially the focus has been qualitative in nature, seeking the ability to confirm the presence or absence of velvet antler, but the development of quantitative assays is also seen as being of crucial importance.

Two approaches have thus far been adopted. First, antibodies to velvet extracts have been raised in rabbits, and the potential of these antisera for use in diagnostic tests have been evaluated using gel double diffusion (GDD) techniques. Second, the thin layer chromatography (TLC) profiles of the glycolipid fraction of organic solvent extracts of velvet antler have been examined. The objective of this work is to use the distinctive patterns formed on TLC plates as a "fingerprint" that will enable the identification of velvet antler.

Antibody Test

Materials and Methods

Tissue Samples

Samples of various tissues from deer, sheep and rabbits were collected for comparison with velvet antler tissue. These included ligament, lung, heart, testicle, ovary, spleen, brain, kidney and liver samples from deer, sheep (hoggets and two tooth animals), and rabbits. Pooled samples of minced frozen antler, harvested in 1995 from Invermay 3 year old stags, 60 and 90 days following casting of previous antlers, were used for most of the comparisons. Samples of deer blood, of freeze dried velvet antler tissue, and of antler from two separate processors were also available for testing.

Immunisations

Four New Zealand white rabbits were each treated with 2mg of an aqueous velvet extract in 0.8ml of an emulsion containing the adjuvant STM (Span, Tween, and Marcol), given as subcutaneous injections in multiple sites on the back. Similar booster injections were intramuscularly administered after six weeks and again after a further two months. Three weeks following the final booster injection, each rabbit was sacrificed by exsanguination and the antiserum obtained was stored at -20°C in a freezer until required for screening.

Test Tube Screening of Rabbit Antisera

Dilutions of each antiserum (0.25ml) were mixed with equal volumes of solutions containing antigen (velvet extract) at varying concentrations, in the presence of 3% polyethylene glycol to assist antibody-antigen complex precipitation. The mixtures were incubated at 37°C for 1 hour, and were then monitored for 24 hours for the appearance of precipitates. Combinations

of antibody and antigen dilutions that exhibited precipitate formation were used as starting points in performing the agarose gel double diffusion (GDD) experiments.

Gel Double Diffusion Experiments

1.5% agarose plates were prepared at a pH of approximately 7. Circular wells, 3mm in diameter, were cut in the gel and were plugged with molten agarose. Aqueous solutions of velvet extract or tissue homogenates were prepared and were adjusted to 1.0mg/ml or to 0.2mg/ml on the basis of their UV absorbance at 280nm. These solutions (5-8µl) were then added to the centre wells of agarose gel plates by use of a syringe, while dilutions of rabbit antisera were similarly added to the outer wells. The plates were allowed to stand at ambient temperature in a humid atmosphere to permit the reagents to diffuse together, and were monitored for up to three weeks for the appearance of precipitin (i.e. insoluble antibody-antigen complex) bands.

The agarose gel plates were covered with wet filter paper and were incubated at 50°C until dry. After being washed with phosphate buffered saline, the drying process was repeated. The gels were covered with a solution of 0.25% Coomassie Blue dye in 50% trichloroacetic acid, and were incubated and gently shaken at ambient temperature. The dye solution was drained, and the gels were washed with water, before a destaining solution of 25% methanol/7% acetic acid was added. The destaining solution was changed as necessary until the plates were sufficiently decolourised to be able to readily visualise the precipitin bands, if present.

Results

Test tube precipitin reactions were used to identify dilutions of rabbit antisera and tissue extracts to use for agarose gel double diffusion (GDD) experiments. Antibody-antigen complex precipitation was observed to occur when 0-20 fold dilutions of antisera were mixed with equal volumes of aqueous antler extract at concentrations in the range 0.02-1mg/ml.

Antisera were then tested by GDD. In this technique, antibodies and antigens diffuse through the agarose gel forming concentration gradients which overlap between the wells to which they are added. In the region(s) of optimum relative concentrations, one or more precipitin bands (consisting of insoluble complexes of antibody and antigen) form and may be visualised with Coomassie Blue dye.

Most effort has centred on the antiserum from rabbit H51. This antiserum gave a single strong precipitin band at 1:10 and at 1:20 dilution on a GDD plate, when the extract against which it was raised was added to the centre well at 1mg/ml (Figure 10). When the velvet extract concentration was lowered to 0.2mg/ml, an additional precipitin band which occurred over the full dilution range (1:10 to 1:100) of the antiserum was observed (Figure 11).

FIGURE 10. Gel double diffusion plate with 1mg/ml aqueous velvet antler extract ("VAX") in the centre well, and the indicated dilutions of rabbit antibody H51 in the outer wells.

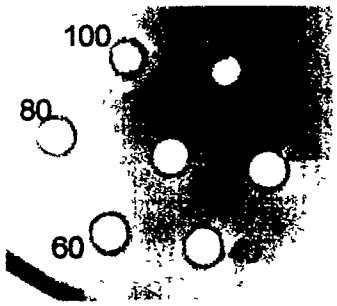
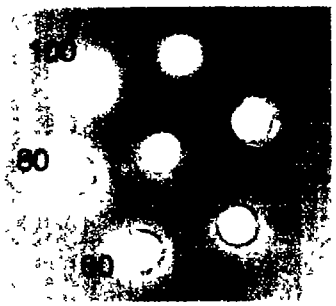


FIGURE 11. Gel double diffusion plate with 0.2mg/ml aqueous velvet antler extract ("VAX") in the centre well, and the indicated dilutions of rabbit antibody H51 in the outer wells.



The selectivity of antiserum H51 has been tested by performing GDD experiments using a variety of velvet antler and other tissues, and deer blood (Table 2). Velvet antler tissue homogenate reacted the same as velvet antler extract and gave a single precipitin band at a concentration of 1mg/ml and a double band at 0.2mg/ml. This was true of antler that had been commercially processed as well as dried by freeze drying. In contrast, although some other tissues from sheep, deer or rabbit gave a single or a double band at concentrations of 1mg/ml, none gave any bands at 0.2mg/ml. Similarly, deer blood gave a single precipitin band at 1mg/ml and none at 0.2mg/ml.

TABLE 2. Summary of bands displayed on 1.5% agarose gel plates following gel double diffusion experiments using rabbit antiserum H51. Precipitin bands were visualised by staining with 0.25% Coomassie Blue dye in 50% trichloroacetic acid.

Sample	Bands at 1mg/ml	Bands at 0.2mg/ml
Aqueous velvet extract	Single	Double
Velvet antler homogenate	Single	Double
Blood (deer)	Single	None
Liver (deer, sheep, rabbit)	None	None
Lung (deer)	Double/None	None
Lung (deer)	Double	None
Lung (sheep)	Single	None
Kidney (deer)	Single	None
Kidney (sheep)	Double	None
Heart (deer)	None	None

Discussion

The GDD experiments have confirmed that antisera to an aqueous extract of velvet antler have been successfully raised in rabbits. Since two precipitin bands were observed under some conditions, it is concluded that the antisera recognise at least two separate antigens. The most promising of these antisera has been shown to selectively recognise velvet antler tissue or aqueous extract when the test sample is added at a concentration of 0.2mg/ml. Significantly, blood does not produce any precipitin bands at all under these conditions. It is thus concluded that the antigens recognised by the antiserum are not just blood serum proteins present in the antler samples. The fact that other tissues produced bands when presented at a higher concentration than velvet antler indicates that the antiserum will cross react with antigens present in these tissues, but this critical concentration difference should allow the development of a diagnostic test for velvet antler using this antibody. Future work will assess the use of the antiserum in an enzyme immunoassay (for example, an ELISA) and its ability to detect velvet antler in mixtures with other components.

In summary, an antiserum that is able to discriminate velvet antler from other tissues, both from deer as well as other species, has been raised. Potentially this result might lead to a diagnostic test for velvet antler in extracts or mixed products, and provide the ability to prove that these do in fact contain velvet. This development would significantly enhance the development of non traditional markets for velvet antler products.

TLC Analysis of Glycolipid Fraction of Velvet Antler Extracts

Materials and Methods

Tissue Samples

The same tissues used for the GDD experiments were available for testing.

Tissue Extraction

10g of velvet antler or other tissue was homogenised with 10 volumes of chilled acetone, and was then filtered. The tissue was re-homogenised with a further 5 volumes of acetone, filtered, and the residue was air dried. The resulting acetone powder was subjected to a Folch-type extraction (Folch, 1957). Briefly, this involved extraction of the acetone powder by homogenisation with 2:1 chloroform/methanol. After filtration, the residue was re-extracted, this time with 1:2 with chloroform/methanol along with the addition of 5% (v/v) water. Chloroform was added to the combined filtrates in order to adjust them to a final 2:1 ratio of chloroform:methanol. The mixture was shaken with 0.88% potassium chloride solution, and then allowed to separate into two phases. Most of the glycolipids were contained in the upper phase, which was separated and was retained. The lower phase was re-extracted with chloroform/methanol/0.88% potassium chloride (3:48:47) and then with chloroform/methanol/water (3:48:47). The combined upper phases were concentrated under vacuum to remove the organic solvents, and were dialysed against multiple changes of Milli-Q water at 4°C. The contents of the dialysis bag were then lyophilised to give the glycolipid fraction as a light tan powder.

Anion Exchange Chromatography

Each crude glycolipid fraction was dissolved in 2:1 chloroform/methanol at a concentration of 4mg/ml. 250µl of this solution was applied to a HEMA DEAE mini column (Alltech), which was equilibrated in the acetate form of the anion exchange resin. The column was successively eluted with the following solvent mixtures, to give five fractions containing glycolipids separated on the basis of their ionic charges:

1. 8ml chloroform/methanol/water (30:60:8)
2. 2ml chloroform/methanol/water (30:60:8)
3. 5ml chloroform/methanol/0.05M ammonium hydroxide (30:60:8)
4. 4ml chloroform/methanol/0.2M ammonium hydroxide (30:60:8)
5. 4ml chloroform/methanol/0.5M ammonium hydroxide (30:60:8)

Fractions 1 and 2 contained polar lipids which were uncharged or weakly charged at the operating pH, while successively higher charged molecules (for example, containing more sialic acid residues) were eluted in the following three fractions. Solvent was removed from each of the five fractions by concentration in a vacuum centrifuge (Heto). A small volume of Milli-Q water was then added to each tube, and was frozen in a -40°C ethanol bath. The tubes were returned to the vacuum centrifuge and were evaporated to dryness with heating under reduced pressure. The residue from each fraction was then dissolved in 50µl of 2:1 chloroform/methanol and was stored in a -20°C freezer until required for analysis.

Thin Layer Chromatography

High performance thin layer chromatography (HPTLC) silica gel plates (Merck) were washed with chloroform/methanol/0.02% calcium chloride (60:40:9), and were air dried. Activation of the plates was performed by heating in an oven at 100°C for one hour, and plates were then stored in a desiccator until used. Plates were used within 2 days of activation.

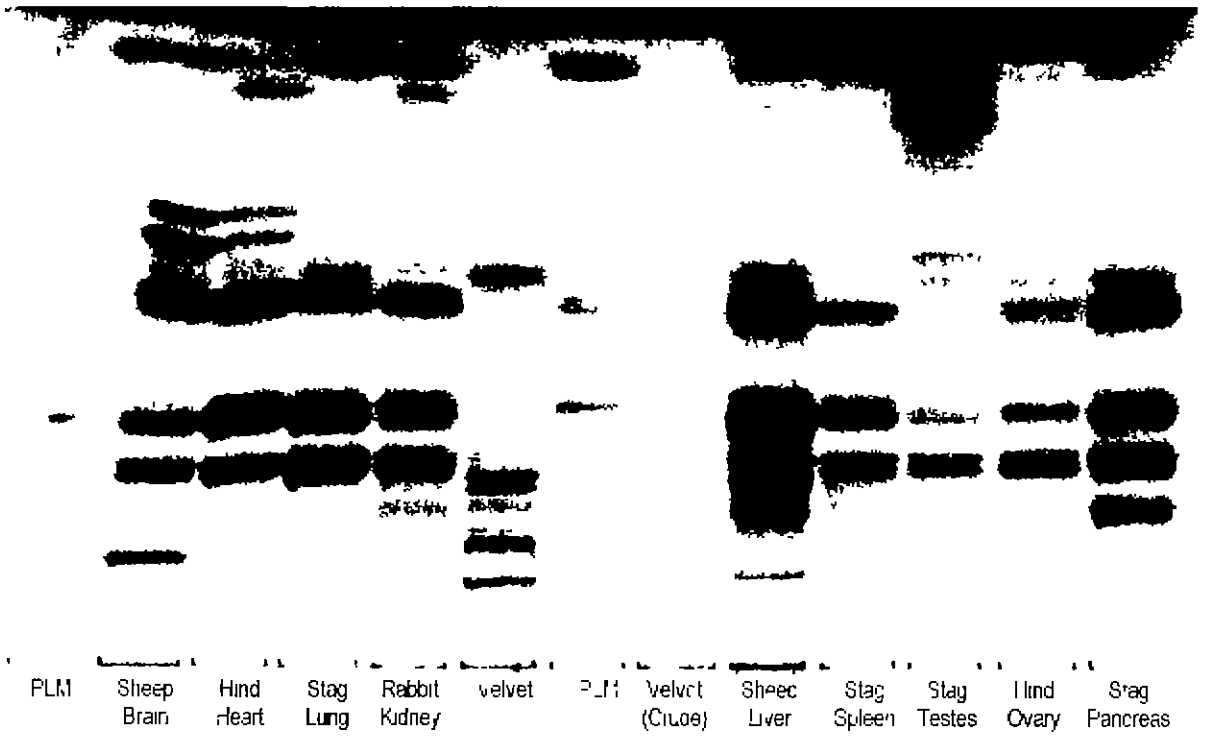
10-20µl of each glycolipid fraction was applied to an activated HPTLC plate, which was then dried in a vacuum desiccator for 15 minutes. Following development in chloroform/ methanol/1M ammonium hydroxide (60:35:8), the plate was air dried for 15-20 minutes. It was usually then visualised by use of a 10% copper (II) sulphate in phosphoric acid dip, followed by heating at 170°C for 30 minutes. Some plates were instead sprayed with the one of the following reagents (Kates, 1991) for detection of specific lipid components: resorcinol/copper (II) sulphate/hydrochloric acid (gangliosides); orcinol/sulphuric acid (glycolipids); bismuth nitrate/potassium iodide/acetic acid (Dragendorff reagent) (choline-containing lipids); sodium hypochlorite/benzidine (sphingolipids); concentrated sulphuric acid/glacial acetic acid (sterols and sterol esters). Plates were scanned using a laser densitometer (Molecular Dynamics) or a colour flat-bed scanner (Mustek) for storage of results.

Results

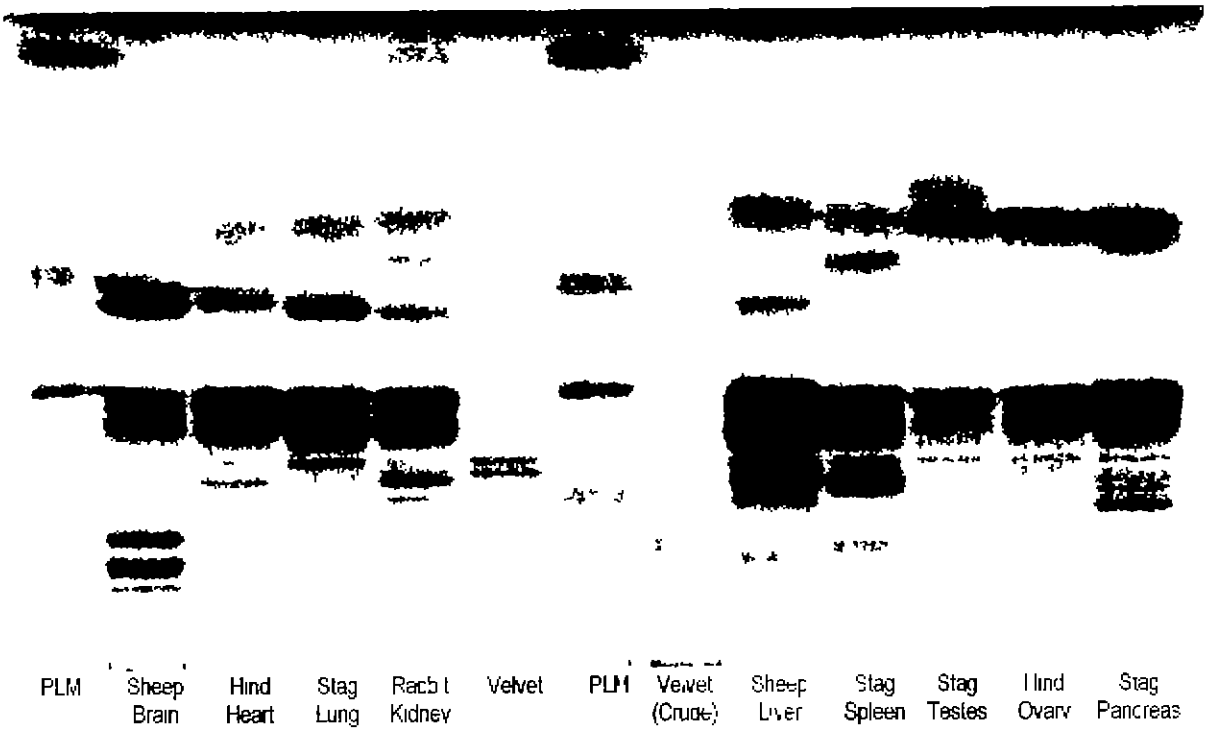
Initial work (not shown) centred on the separation of the whole glycolipid fractions prepared by Folch (1957) extraction of velvet antler and other tissues. The complexity of the extracts, however, soon made it apparent that further fractionation of the extracts would be necessary. They were therefore applied to anion exchange mini-columns and eluted with buffers of increasing ionic strength. This produced 5 fractions per extract which were then each analysed by TLC. Representative HPTLC plates for Fractions 1, 3 and 4 of a range of tissues are shown in Figures 12(a)-(c). The pattern of bands on HPTLC plates of each of the fractions was found to be distinctive for groups of tissues (Table 3). This was particularly true of fractions from velvet antler, which showed a much greater proportion of low mobility lipid bands as compared to other tissues. Low mobility of a band is associated with high polarity, which is typical of complex lipids such as glycolipids and some phospholipids. Most of the velvet antler bands were in fact demonstrated to be glycolipids by their positive reaction to an orcinol spray reagent (Figure 13). The HPTLC plate from Fraction 4 [Figure 12(c)] demonstrated some striking contrasts. In this fraction, velvet antler had just two significant bands, both of which were shown to be gangliosides when sprayed with resorcinol (a reagent which gives purple bands with components containing sialic acid) (data not shown). Sheep brain also contained low mobility bands, which were shown to be glycolipids by spraying with orcinol (Figure 13), but only the most polar of these gave a positive reaction with resorcinol. Deer testes and ovaries were both different to other tissues in that they had almost no significant bands in Fraction 4. In contrast, most other tissues displayed a number of strong bands, with the strongest ones generally being of higher mobility than those of velvet antler and of brain.

FIGURE 12. HPTLC plates of the glycolipid fractions of tissues following anion exchange chromatography on DEAE mini-columns. Plates were developed in chloroform:methanol:1M ammonium hydroxide (60:35:8) and were visualised by use of a 10% copper (II) sulphate in phosphoric acid dip followed by heating at 170°C. "Velvet (Crude)" is the crude glycolipid extract of velvet antler applied to the plate without further fractionation and "PLM" is a mixture of standards containing *lyso*-phosphatidyl choline, phosphatidyl choline, phosphatidyl ethanolamine and cholesterol, in order of increasing mobility on the HPTLC plate.

(a) Fraction I



(b) Fraction 3



(c) Fraction 4

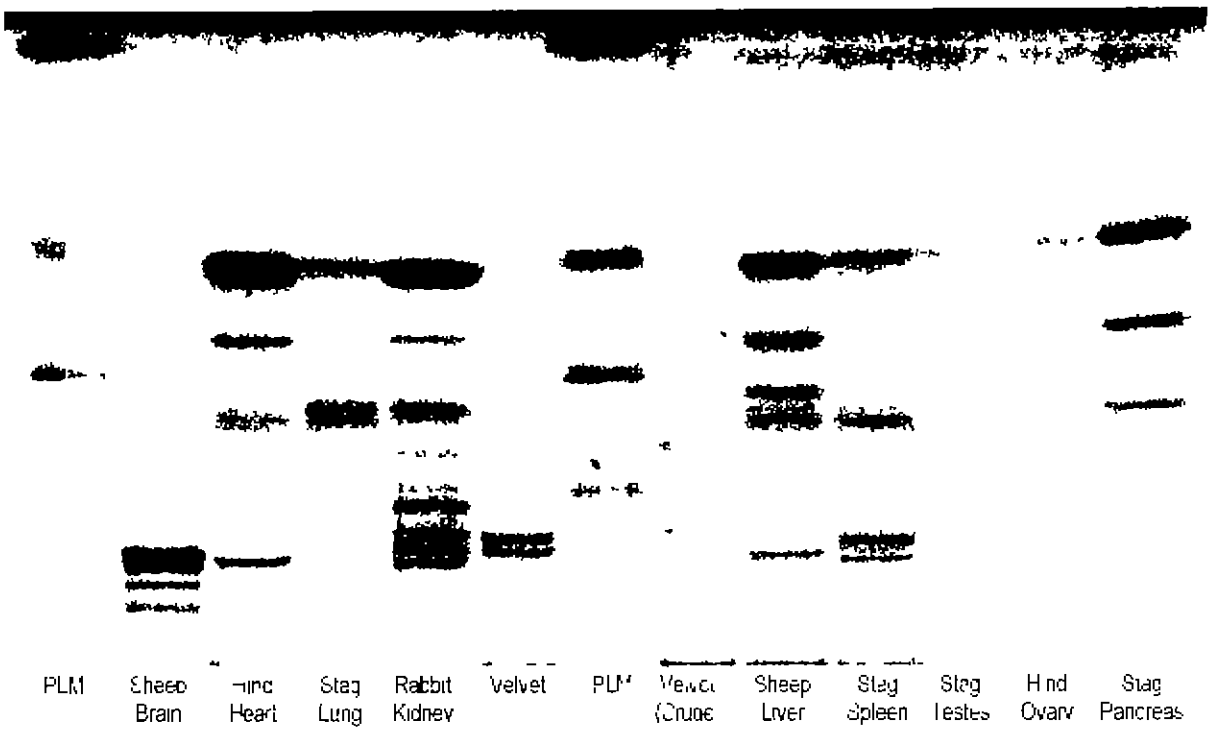
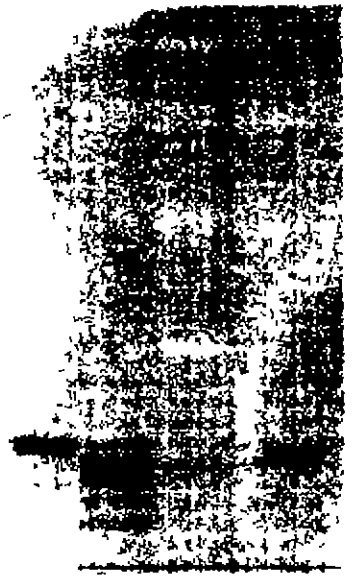


TABLE 3. Groups of glycolipid extracts which gave similar patterns of bands on HPTLC plates following fractionation by ion exchange chromatography on DEAE mini-columns. Data are given for Fractions 1, 3 and 4 from a range of tissues from deer, rabbits and sheep.

Fraction	Groups of Like TLC Patterns	
	Tissue	**Species**
1	Brain	All
	Heart	All
	Kidney	All
	Liver	Deer, sheep
	Liver	Rabbit
	Lung	All
	Spleen	Deer
	Spleen	Rabbit
	Spleen	Sheep
	Velvet antler	Deer
3	Brain	All
	Heart	Deer (hind), rabbit, sheep
	Heart	Deer (stag)
	Kidney	Deer (hind)
	Kidney	Deer (stag)
	Kidney	Sheep, rabbit
	Liver	All
	Lung	Deer (hind), sheep (2-tooth)
	Lung	Deer (stag), rabbit, sheep (hogget)
	Spleen	Deer, rabbit, sheep (hogget)
	Spleen	Sheep (2-tooth)
	Velvet antler	Deer
4	Brain	All
	Heart	Deer, rabbit
	Heart	Sheep
	Liver	Deer, rabbit
	Liver	Sheep
	Lung	Deer, sheep (hogget)
	Lung	Sheep (2-tooth)
	Spleen	Deer, rabbit, sheep (hogget)
	Spleen	Sheep (2-tooth)
	Velvet antler	Deer

FIGURE 13. HPTLC plate of Fraction 4 of velvet antler, sheep brain and sheep lung. The plate was developed in chloroform/methanol/1M ammonium hydroxide (60 35 8), and was visualised by use of an orcinol spray reagent for the identification of glycolipids. Ganglioside G_{D3} was included as a positive control for the orcinol detection reagent.



Discussion

The glycolipid fraction of extracts of velvet antler, has in the present study, been shown to be of different composition to that of other tissues. Following fractionation by ion exchange chromatography using convenient commercially available mini-columns, distinctive patterns have been obtained on HPTLC plates and these permit the identification of groups of tissues. Significantly, the pattern exhibited by fractions from velvet antler extracts have been shown to be completely unlike those of a wide range of other tissues from deer and other species (sheep, rabbits). Considering all the fractions together, a pattern emerges which permits the discrimination of the various tissues. In particular, the adulteration of samples of velvet antler by other tissues is very easy to establish. Biometric analytical methods are being developed which it is believed will lead to a very powerful diagnostic test for velvet antler in mixtures with other components. Preliminary work using TLC sprays which react selectively with particular types of lipids has permitted the nature of a number of the individual components in the fractions to be deduced, and this information is expected to add additional power to the diagnostic method.

In conclusion, a powerful diagnostic tool for velvet antler is expected to be available in the near future. This could be of critical importance to the velvet industry as it moves towards the establishment of non traditional markets. The work may also provide some clues as to the basis of some of the wide ranging therapeutic properties of velvet antler, given that its glycolipid fraction (which contains many complex lipids like gangliosides and phospholipids of known bioactivity) is markedly different to that of other tissues. This aspect of velvet antler composition is highly deserving of further research in the future.

GENERAL DISCUSSION

The studies described in this paper are a preliminary demonstration of the efficacy of New Zealand velvet antler. New Zealand velvet antler is thought of as a new addition to the market and it is perhaps understandable that it must establish itself against the benchmarks of traditional products. The present studies set out to position New Zealand velvet antler by testing it to determine whether it was effective under experimental conditions which were carefully selected to cover a range of the known therapeutic functions of velvet antler.

While direct comparisons of therapeutic activity among New Zealand, Russian and Chinese velvet were not made in the present studies, it can be stated that velvet antler of New Zealand origin was shown to be effective in the tests used. Much of the Russian literature concerns the use of pantocrine which is an alcohol/water extract. It is therefore likely that the proportions of active ingredients will differ given the different extracts used in the present studies.

The research described in this paper is broad based and depends on our ability, in New Zealand, to be able to vary certain velvet antler parameters, for example, stage of growth at removal and processing technique. It is also crucial to be able to study fresh antler very quickly after removal. These studies were based on the philosophy that we should study all aspects of velvet antler growth and processing and thereby complement the pharmacological studies which had taken place in Russia, China, Korea and Japan. We also have used our ability to trace the efficacy of extracts back to the stag of origin, the timing of removal of the velvet antler and the way the velvet antler was processed. This ability is crucial and, to the best of our knowledge, makes our approach unique world-wide. Taken together the results shed new light on the application of antler processing techniques for specific activity and the role of timing of velvet antler removal in determining efficacy. Future extracts and applications of velvet antler will call for greater quality control and more precisely defined product specifications. Clearly the diagnostic studies will become of paramount importance.

In conclusion New Zealand velvet antler has been shown to be effective in a wide range of tests. New Zealand velvet antler can be used with confidence.

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