The clastogenic effect of adult *Spirocerca lupi* secretory products on murine fibroblasts

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By

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OUR DEEPEST FEAR

Mariane Williams

Our deepest fear is not that we are inadequate.
Our deepest fear is that we are powerful beyond measure.
It is our light, not our darkness that most frightens us.
We ask ourselves,
Who am I to be brilliant, gorgeous, talented, and fabulous?
Actually, who are you not to be?
You are a child of GOD.
You playing small does not serve the world.
There is nothing enlightened about shrinking
so that other people won't feel insecure around you.
We are all meant to shine, as children do.
We were born to make manifest the glory of GOD that is within us.
It is not just in some of us, it is in everyone.
And as we let our own light shine we unconsciously
Give other people permission to do the same.
As we are liberated from our own fear,
Our presence automatically liberates others.

DECLARATION

The laboratory work described in this dissertation was conducted in the department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, unless stated otherwise in the dissertation.

This dissertation has not previously been submitted by me or any other person for a degree at this or any other university. These are my own results.

I, Ms K. B. Sako, declare the above-mentioned statements to be truthful.

Ms K. B. Sako

Prof. V. Naidoo (Supervisor)

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ABSTRACT

Spirocerca lupi (S. lupi) is a nematode that parasitizes vertebrates, particularly canids. In 25% of *Spirocerca* infections in dogs, nodules progress from inflammatory to pre-neoplastic and eventually to sarcomatous neoplasia within a few months. Researchers have postulated that the parasite itself induces the sarcomatous transformation through the excretory / secretory products (ESPs) thought to contain growth factor-like substances, possibly proteins and/or chemicals. With the mechanism of the sarcomatous neoplastic transformation being incompletely understood, the objective of this study was to investigate whether adult *S. lupi* parasite ESPs could induce proliferation (carcinogenicity) in primary mammalian fibroblast cell cultures. The adult *S. lupi* parasite ESPs were also investigated by chromatography for presence of potential clastogens.

The mammalian fibroblasts were harvested from Balb/c mice pinnae, prepared and maintained *in-vitro* using Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10 % foetal bovine serum (FBS). Live adult *S. lupi* parasites were obtained from dogs at necropsy. The parasites were subsequently cultured in various media (RPMI 1640 Medium, Iscove's Modified Dulbecco's Medium, Dulbecco's Modified Eagle's Medium, Ham's F12 Medium and saline) and maintained at 37 °C in an incubator in order to obtain worm ESPs. The adult *S. lupi* parasite ESPs obtained from the culture media were extracted and dissolved in organic solvents (Ethylacetate, Methanol, Acetone and Hexane) at different dilutions (10, 20 and 30 µl) and exposed to the cultured fibroblasts. The ESPs extracted from media did not induce an increase in mitosis compared to the controls.

The ESPs were further analysed using thin layer chromatography (TLC) and liquid chromatography-coupled mass-spectroscopy (LC-MS/MS). Chromatography revealed the Iscove's media to be richest in worm ESPs. LC-MS/MS revealed nine compounds (301.3625 m/z, 400.2112 m/z, 450.8195 m/z, 464.8737 m/z, 538.1112 m/z, 580.2783 m/z, 594.2576 m/z, 660.5320 m/z, 682.5770 m/z) in adult *S. lupi* parasite ESPs, for

which library comparison revealed to be proteins similar to those isolated from *Nematostella vectensis, Caenorhabditis brenneri* and *Sus scrofa.* The protein Caebren was also identified.

We conclude that the essential media (Iscove's, DMEM, RPMI and Ham's F12) do not contain the necessary nutrients required for the survival of the parasites. The media in which the parasites were incubated, whilst rich in compounds, were also unable to induce a direct clastogenic effect in cultured murine fibroblasts. As a result, it would appear that the neoplastic transformation induced by the parasite is not due to the excretion of simple clastogenic proteins or chemicals and more importantly, may actually be related to the parasite actively feeding. Further work is therefore required to ascertain the nutrient requirements of the *S. lupi* parasite, in order to study its clastogenic effect which seems likely to be of protein origin.

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Abbreviations

ESPs:	Excretory / secretory products
TLC:	Thin layer chromatography
U-HPLC:	Ultra-high pressure liquid chromatography
L1:	Stage 1 larvae
L3:	Stage 3 larvae
L4:	Stage 4 larvae
CT:	Computed tomography
DRM:	Doramectin
IVM:	Ivermectin
MO:	Milbemycin oxime
MXD:	Moxidectin
S.C.	Subcutaneous
PGP:	Permeability glycoprotein
CRP:	Concentration reactive protein
VEGF:	Vascular endothelial growth factor
FGF:	Fibroblast growth factor
PDGF:	Platelet derived growth factor
EGF:	Epidermal growth factor
IGF:	Insulin-like growth factor
TGF-β:	transforming growth factor-β
IL:	Interleukin
G_0:	Resting phase
G_1:	Gap 1 phase
G_2:	Gap 2 phase
S:	Synthesis phase
M:	Mitosis
CDK's:	Cyclic dependent kinases
PBS:	Phosphate buffered saline
RPMI:	Roswell Park Memorial Institute

FSC:	Foetal calf serum
FBS:	Foetal bovine serum
RO:	Reverse osmosis
PH:	Potential hydrogen
BA:	Blood Agar
CNA:	Colistin-nalidixic acid
MUG:	Methyl-umbellinferyl-β-glucuronide
LC-MS/MS:	Liquid chromatography mass spectrometry
MS:	Mass spectrophotometer
EMW:	Ethylacetate, methanol, water
BEA:	Benzene, ethylacetate, ammonia
CEF:	Chloroform, ethylacetate, formic acid
UV:	Ultra-violet
RF:	Retardation factor
DMEM:	Dulbecco's modified eagle's medium
UPBRC:	University of Pretoria biomedical research centre
HI:	Heat-inactivated
S.D:	Standard deviation
Ace:	Acetone
Hex:	Hexane
TC:	Tissue culture
CO_2:	Carbon dioxide
HE:	Haematoxylin eosin
Tregs:	T-regulatory cells
MSc:	Master of Science
SEM:	Scanning electron microscopy
OVAH:	Onderstepoort Veterinary Academic Hospital
Cat:	Catalogue
PABA:	para-amino benzoic acid
Spp:	Species

1 INTRODUCTION

1.1 BACKGROUND

Spirocerca lupi (S. lupi) is a parasitic nematode that enjoys a worldwide distribution, with particularly high incidence in tropical and subtropical regions of the world (Bailey, 1972). The parasite induces a disease known as spirocercosis in mainly the domestic dog (*Canis domesticus*), which is characterized by caudal thoracic aortic aneurysms with mineralization, caudal thoracic ventral vertebral body spondylitis and the formation of nodules within the oesophageal submucosa (Dvir et al., 2001; Mazaki-Tovi et al., 2002). These nodules usually contain three to six *S. lupi* adult worms and are characterized histologically by necrosis, inflammation and fibrous repair caused by the presence of the worms. With time, however, in a percentage (± 25%) of these *Spirocerca*-cases, the oesophageal nodules progress to a pre-neoplastic stage (characterized histologically by significant fibroblastic activity whereby a significant proportion of the fibroblasts appear atypical) and finally into osteosarcomas, fibrosarcomas or undifferentiated sarcomas (Dvir et al., 2001).

While the pathophysiology of this neoplastic transformation remains elusive, several researchers have postulated that the *S. lupi* adult worms either directly or indirectly induce the sarcomatous differentiation via the production and secretion of growth factor-like substances or perhaps direct carcinogens (Dvir et al., 2010). With the mechanism of neoplastic formation being unknown, this study aimed to investigate whether worm excretory/secretory products (ESPs) could induced carcinogenic changes in cultures of mammalian fibroblastic cells.

1.1.1 *Hypothesis*

Spirocerca lupi excretory/secretory products (ESPs) have carcinogenic effects on cultured fibroblasts.

1.1.2 *Benefits arising from the project*

This study will contribute to the further understanding of the mechanisms underlying *S. lupi* induced neoplastic transformation of oesophageal nodules in dogs. The study will also contribute towards a Master of Science degree (MSc).

1.1.3 *Aims and Objectives*

Aims

- To establish an *in-vitro* culture method for *S. lupi* adult nematode.
- To study the effects of *S. lupi* ESPs on *in-vitro* cultured murine fibroblasts.

Objectives

- To maintain *S. lupi* adults artificially in a culture system using a variety of culture media.
- To ascertain the most appropriate culture medium that could sustain the adult worms for the longest period of time.
- To separate and visualise the ESPs within the parasite culture media using thin layer chromatography (TLC) and various visualisation reagents.
- To separate and detect ESPs within parasite culture media using the ultra-high pressure liquid chromatography with mass spectrometry (U-HPLC/MS) method.
- To evaluate *in-vitro* cultured fibroblasts for any evidence indicative of possible neoplastic transformation following exposure to ESPs extracted in organic solvents.

2 LITERATURE REVIEW

2.1 SPIROCERCA LUPI

2.1.1 *Morphology*

Spirocerca lupi of the class Secernentea, order Spirurida and family Thelaziidae, is a typical nematode parasite, characterised by being non-segmented, cylindrical in shape, tapering at both ends and covered by a tough protective covering or cuticle (Markell, 1986). *S. lupi* worms are spiralled, pinkish in colour with a cuticle characterised by tiny oblique striations (Figure 2.1.1-1) (Naem, 2004).

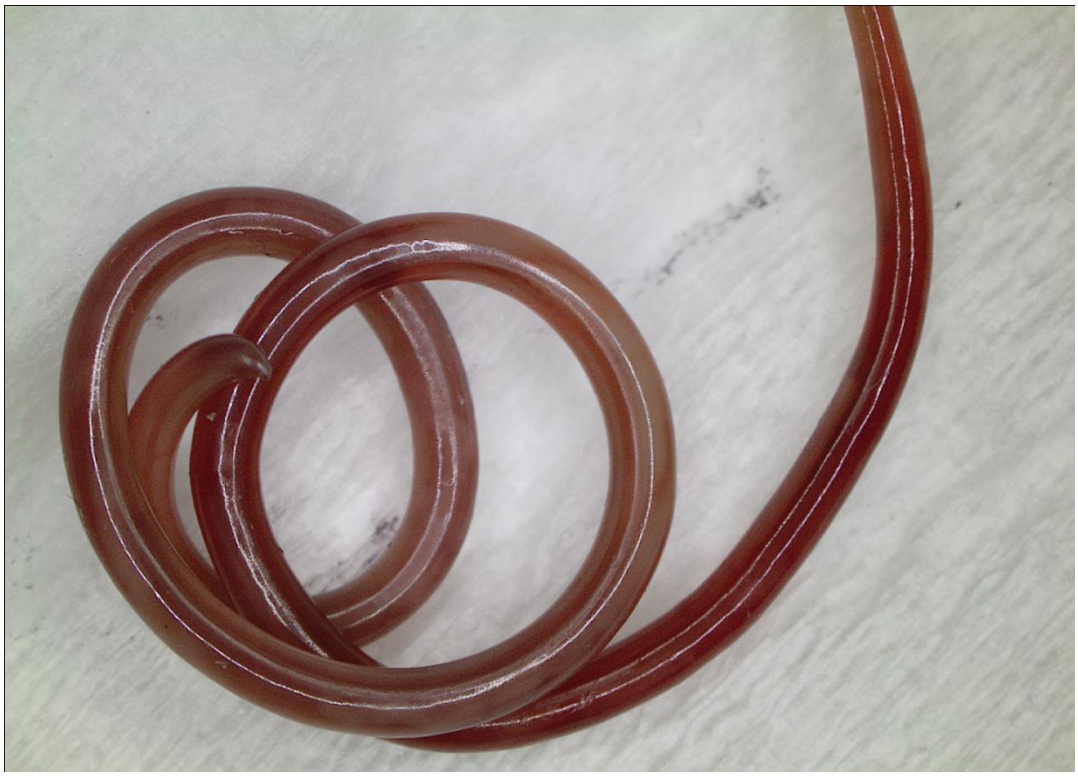


Figure 2.1.1-1: *Spirocerca lupi* adult female worm removed from an oesophageal nodule at necropsy. The picture shows the general nematode shape of the parasite, and its characteristic pink, glossy appearance.

The adult males and females show sexual dimorphism whereby the males grow up to 54 mm and the females up to 80 mm in length within the final host (Soulsby, 1982). Furthermore, the tail of a male *S. lupi* adult worm is spiralled while that of a female tends to be blunt. These tails both possess a well-developed pair of subterminal phasmids (sensory structure) (Naem, 2004). The parasite has been further described by scanning electron microscopy (SEM), by Naem (2004), who identified the worm's body surface structures, namely, the excretory pore on the anterior end, the cephalic region (mouth) composed of two pairs of submedian cephalic papillae and the two lateral amphids, and lastly a pair of cervical papillae with cilia (Figure 2.1.1-2). The cervical papillae are believed to enable the adult worms to migrate in small passages within the viscera of the definitive host and the subterminal phasmids assist in the survival of the worm by adapting to any encountered stimulus in the body of the host (McLaren, 1976; Naem, 2004).

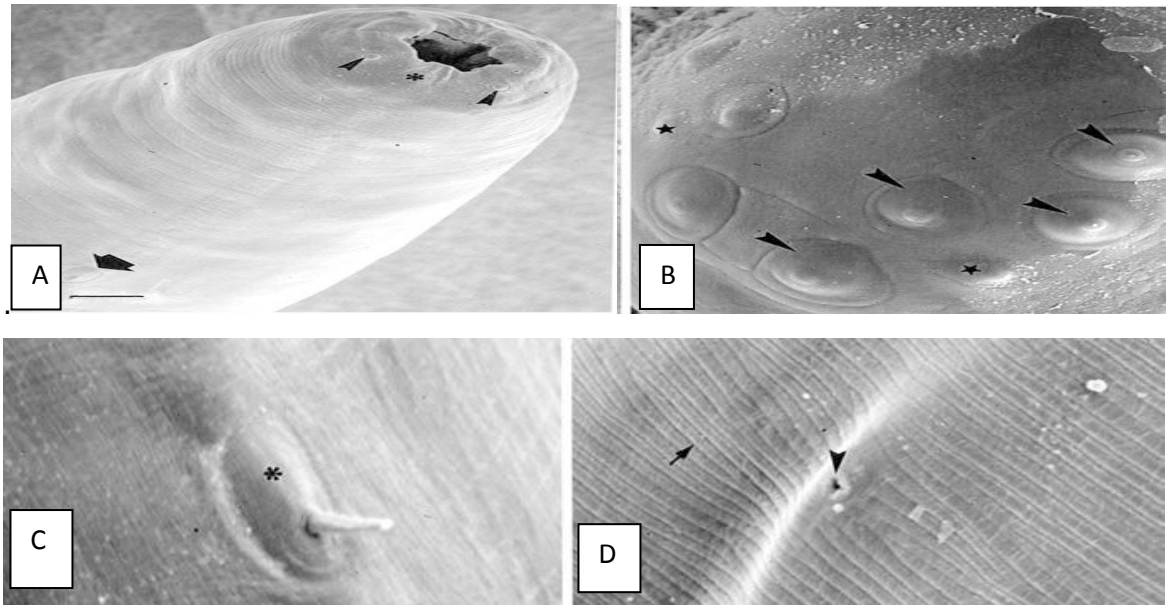


Figure 2.1.1-2: Scanning electron micrographs of an *S. lupi* adult worm (Naem, 2004). A: Shows cephalic papillae (arrowheads), amphid (star) and cervical papilla (arrow); B: Shows the nipple shaped papillae (arrowheads) and two subterminal phasmids (stars); C: Illustrates the cervical papilla with cilia (star); D: Shows the excretory pore (arrowhead) and transverse striations (arrow).

2.1.2 *Distribution*

S. lupi parasites have a worldwide distribution with particularly high incidence in the tropical and subtropical regions of the world (Bailey, 1972). The majority of reports on the incidence of spirocercosis (the disease caused by *S. lupi* in dogs) emanate from Israel (Mazaki-Tovi et al., 2002), Greece (Mylonakis et al., 2001), Turkey (Ramachandran et al., 1984), India (Ramachandran et al., 1984), Pakistan (Anataraman and Sen, 1966), Southern United States (Dixon and McCue, 1967), Brazil (Oliveira-Sequeira et al., 2002), Kenya (Brodey et al., 1977) and South Africa (Lobetti, 2000). These nematodes have a clear seasonal distribution with greater numbers occurring in warm climates (van der Merwe et al., 2008). However, contradictory results have also been reported, whereby no obvious seasonality observed was seen in dogs in South Africa. Nonetheless, regional differences are present, as Israel was found to have a 62% higher incidence when compared with the 46% incidence of spirocercosis found in South Africa (Gauteng) (Mazaki-Tovi et al., 2002; Lobetti, 2000). A study conducted in Boksburg (Gauteng Province, South Africa) showed a 14.5% prevalence of spirocercosis amongst 69 necropsied dogs (Minnaar and Krecek, 2001).

Figure 2.1.2-3: World map showing the distribution of *S. lupi*. Blocks on the map indicate the different areas of occurrence of the parasite.

2.1.3 *Life Cycle*

The life cycle of *S. lupi* includes intermediate hosts (beetles), paratenic hosts (wild birds, lizards, rodents, hedgehogs, rabbits and poultry) and the definitive host (mainly dogs) (Fox et al., 1988) (Figure 2.1.3-4). *Spirocerca lupi* eggs containing stage 1 larvae (L1) are shed in the faeces or with the vomit of the canine definitive host. Coprophagous beetles of the Scarabaeidae family feed on the vomit or faeces, ingesting the larvated eggs. The emerging larvae (L1) then encyst in the tissues of the beetles and develop to stage 3 larvae (L3) within approximately two months. Paratenic hosts or the final host feeds on the coprophagous beetles, ingesting the L3 larvae. Following the ingestion of L3 larvae by the paratenic host, the larvae excyst and then re-encyst in tissues of the paratenic host. This part of the life-cycle can be prolonged as the larvae (L3) can be transferred from one paratenic host to another, as predator paratenic hosts feed on prey ones (Fox et al., 1988).

The definitive host gets infested when they feed on either the infected paratenic host or intermediate host (van der Merwe et al., 2008), of which ingestion of the paratenic host is thought to be the most common route (Sharpilo, 1983). After ingestion, the (L3) larvae are released into the lumen of the stomach and within two hours, actively migrate through the gastric mucosa (Hu and Hoeppli, 1936). By two days post-infestation-, the migrating larvae reach the gastric serosal surface, the gastric and coeliac arteries by four days post-infection, and the caudal thoracic aorta by approximately 7 to 109 days after infestation. The larvae moult within the caudal thoracic aorta into L4 larvae and immature adults (Hu and Hoeppli, 1936), before finally migrating to the subadjacent caudal thoracic oesophagus by 93 to 227 days post-infestation, where they settle and form characteristic nodules within the serosa and submucosa (Bailey, 1963; Bailey, 1972; van der Merwe et al., 2008). During this extensive process of migration, the parasites induce haemorrhagic, necrotic and inflammatory lesions which can be life-threatening (e.g. aortic aneurysm rupture with resultant haemothorax and acute death). Despite the parasite using the circulatory system to disperse, they never actually enter

the bloodstream, with the routes of migration being restricted to only the blood vessel walls. No information could be found on mating and the mechanisms of egg production in adult *S. lupi* parasite.

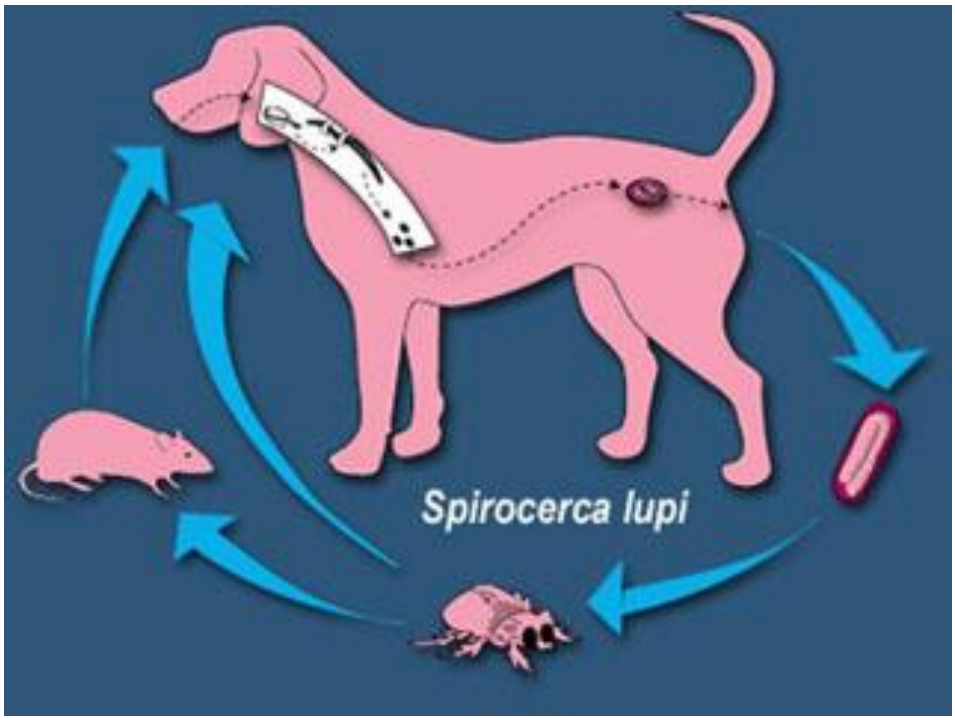


Figure 2.1.3-4: Life cycle of *S. lupi* (Morrison 2011)

This figure illustrates the transfer of excreted eggs from the definitive host (in this case a domestic dog), the consumption of the eggs by the intermediate beetle host and the subsequent consumption of the beetle by a paratenic host (in this case a rodent) prior to re-infection or initial infection of the final host.

2.1.4 *Clinical Disease in Dogs*

Once a dog is infected, the clinical signs most often seen include regurgitation, vomiting, weight loss and/or dysphagia (Mazaki-Tovi et al., 2002; Ranen et al., 2004; Dvir et al., 2010) due to the formation of one or more oesophageal nodule(s) which may become neoplastic (van der Merwe et al., 2008). The infestation may be so extensive that a dog hosts up to 143 adult worms in various nodules (Minnaar and Krecek, 2001).

Reliable (especially early) diagnosis of the oesophageal nodule(s) is through endoscopy and/or computed tomography (CT). Endoscopy allows for direct visualization of the intra-luminal oesophageal nodule(s) while CT detects the small intra-luminal, intra-mural, extra-mural and extra-luminal nodule(s), atypically located nodule(s) and early dystrophic aortic mineralization (Dvir et al., 2001; Mazaki-Tovi et al., 2002). Thoracic radiography is another less sensitive/reliable diagnostic technique used to detect *S. lupi* lesions. This technique has the ability to detect the caudal thoracic vertebral spondylitis, sizable caudodorsal mediastinal mass(es) and aortic undulation caused by aneurysm formation. However, radiography has significant drawbacks; for example, small or atypically located nodules and aortic mineralization are rarely visualized using this technique (Dvir et al., 2001). Direct faecal examination or centrifugal floatation/sedimentation techniques are used to detect the small (35 X 15 µm), thick-shelled larvated eggs shed in the faeces. However, these techniques are the least sensitive among the above-mentioned diagnostic techniques as the *S. lupi* infested dogs shed eggs intermittently or animals could be infected with non-shedding immature worms (Christie et al., 2011).

2.1.5 *Pathology induced by the parasite*

The pathognomonic lesions for spirocercosis include, scarring of the distal/caudal thoracic aorta within which may occur osseous metaplasia with or without haematopoiesis and/or dystrophic calcification with aneurysm formation; caudal thoracic vertebral spondylitis (affecting the ventral vertebral bodies) and lastly, the formation of nodule(s) in the caudal thoracic oesophagus (Figure 2.1.5-5) that may progress to oesophageal sarcoma (Dvir et al., 2001; Mazaki-Tovi et al., 2002).

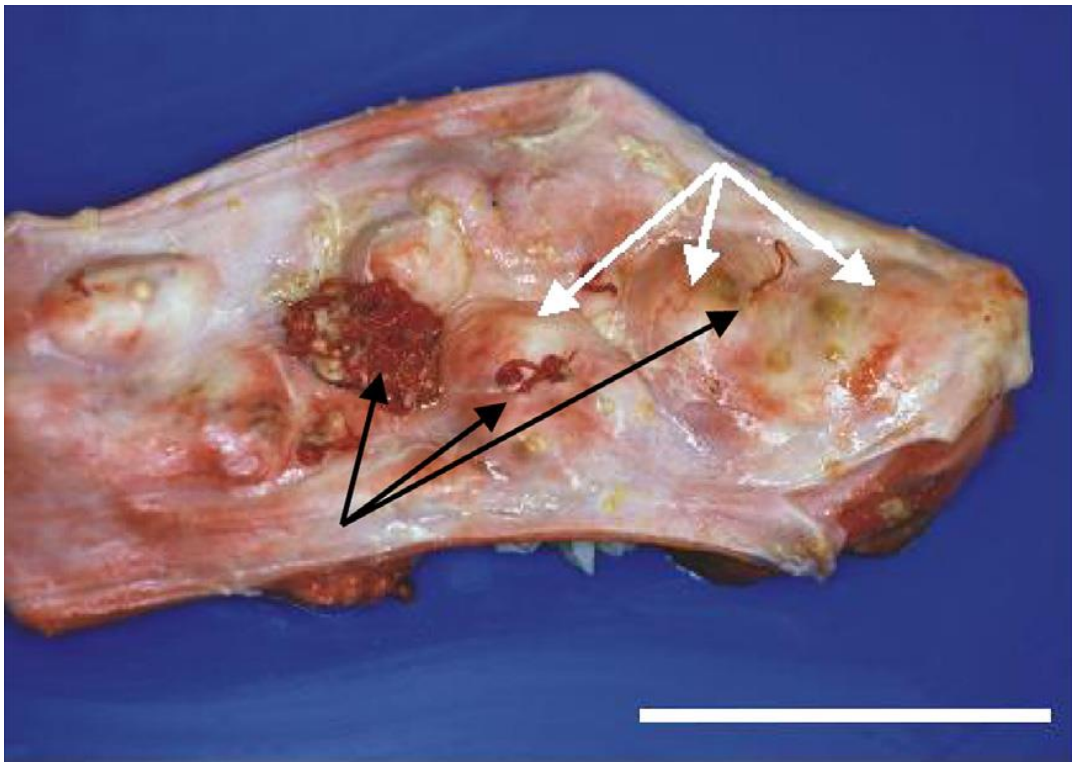


Figure 2.1.5-5: Nodules induced by *S. lupi* within the wall of a dog's oesophagus (van der Merwe et al., 2008). The black arrows indicate pink worms emerging from the nodules and white arrows indicate multi-nodular transformation of the oesophageal wall.

In histological sections, early oesophageal nodules contain central worm(s) immediately surrounded by necrotic cell debris and degenerate neutrophils. These worm migratory tracts are in turn surrounded by fewer neutrophils, variable numbers of eosinophils, occasional plump fibroblasts and numerous fibrocytes with prominent intervening collagen. Even more, peripherally located within the nodules are isolated clusters of plasma cells, fewer lymphocytes and haemosiderin-containing macrophages (Dvir et al., 2008). The presence of numerous neutrophils in benign oesophageal nodules might signify the presence of bacteria (Bailey, 1963), although thus far, scant information is available on the presence of bacteria within these oesophageal nodules.

In up to 25% of *S. lupi*-infested dogs, the oesophageal nodule progresses (as observed at the histological level) from inflammatory nodule, to pre-neoplastic fibroblastic nodule to sarcomatous neoplasia (Dvir et al., 2001). The “pre-neoplastic” nodule contains most of the above-mentioned elements, but is mainly characterized by significant fibroblastic activity (with numerous atypical fibroblasts) with a relatively small amount of collagen and mature fibrocytes. In these mature/older nodules the fibroblasts appear more embryonic with numerous mitosis, prominent (usually multiple) and vesicular nucleoli, chromatin (resembling neoplastic fibroblasts in canine fibrosarcoma) (Bailey, 1963; Hu and Hoeppli, 1936).

Once neoplastic, the oesophageal sarcoma has been further categorized histologically as osteosarcoma, fibrosarcoma or anaplastic sarcoma (van der Merwe et al., 2008) (Dvir et al., 2008). The fibrosarcoma category includes interwoven spindle-shaped cells with numerous mitoses and an intervening collagenous matrix (Dvir et al., 2010). The osteosarcoma category is much more diverse and includes interwoven bundles of plump, polygonal osteoblasts, variable numbers of multinucleated osteoclasts and osteoid matrix and/or mature bony trabeculae / spicules, foci of chondroid differentiation may also be found. The anaplastic sarcoma group is characterized by interlacing malignant spindle-shaped cells with no identifiable intercellular matrix (Dvir et al., 2008). While the adult *S. lupi* worms are usually not present within neoplastic nodules, occasionally adult worms have been identified within the neoplastic oesophageal nodules (Bailey, 1963).

2.1.6 *Treatment*

2.1.6.1 Drugs available

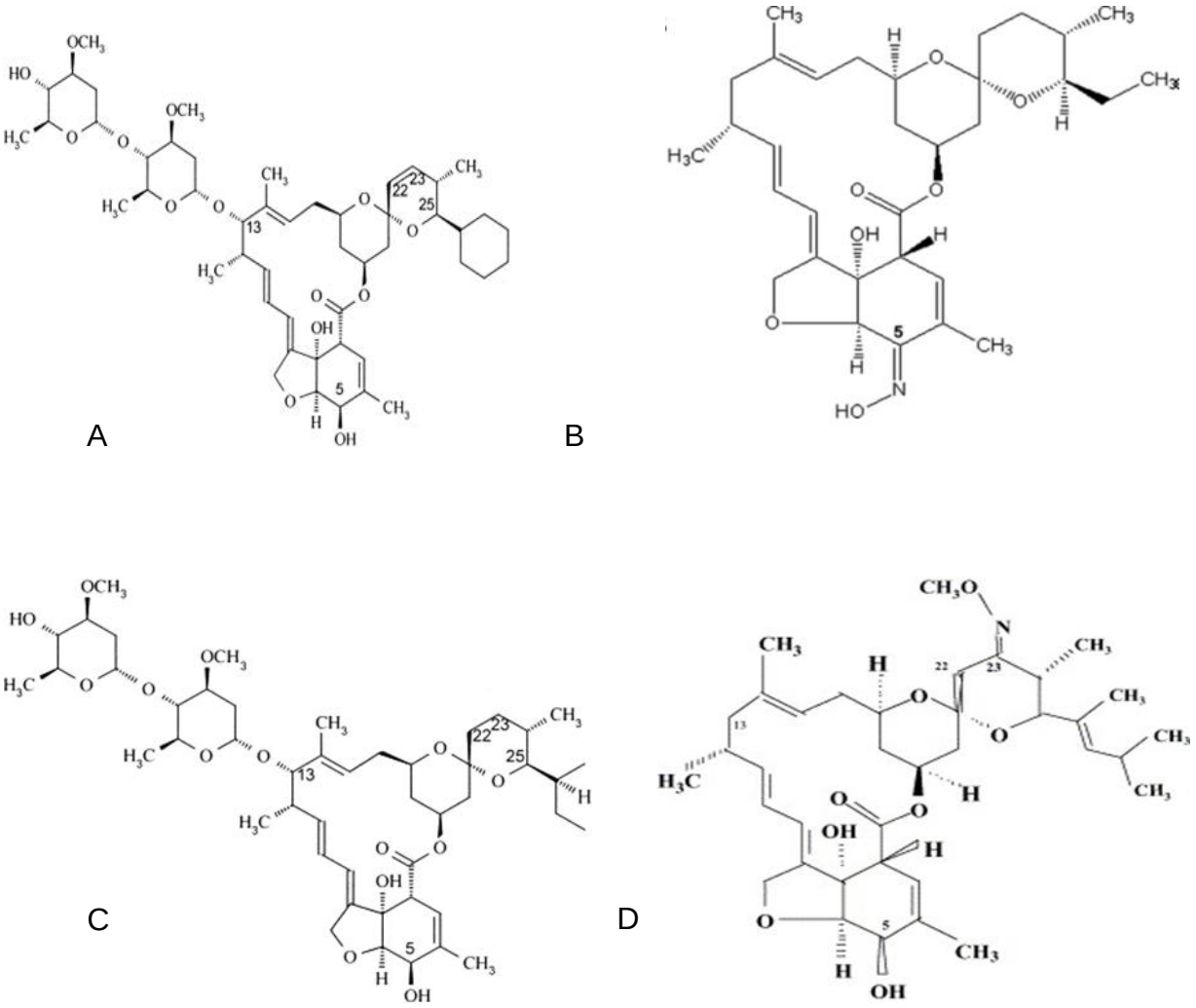


Figure 2.1.6.1-6: Chemical structures of the macrocyclic lactones commonly used for the treatment of spirocercosis, showing their common macrocyclic ring. A: Doramectin, an Avermectin which constitutes the macrocyclic ring attached to the disaccharide group at carbon 13 and a cyclohexyl group at carbon 25; B : Milbemyin oxime, a Milbemycin derivative; C: Ivermectin, a compound of 22,23-dihydroavermectin B 1a and 22,23-dihydroavermectin B 1b; D: Moxidectin, a Milbemycin which is a synthetic derivative of Nemadectin.

The macrocyclic lactones (Doramectin (DRM), Ivermectin (IVM), Milbemycin oxime (MO) and Moxidectin (MXD)) have been found to be effective treatment agents in dogs with non-neoplastic *S. lupi*-induced oesophageal nodules. Naturally derived macrocyclic lactones are formed by soil dwelling actinomycetes of the *Streptomycetes* spp. and are classified as members of the avermectin or milbemycin chemical groups. Members of these groups are structurally related, hence, possess the same physicochemical properties. The avermectins, DRM and IVM are characterized by the presence of the disaccharide group at the carbon 13 position of the macrocylic ring (Gokbulut et al., 2001). Doramectin as compared to the rest of the drugs has the cyclohexyl group at the carbon 25, while the milbemycin, moxidectin lacks a disaccharide group at carbon 13 but possesses a methoxime at carbon 23 and a dimethyl-butenyl side-chain at carbon 25 (Figure 2.1.6.1-6) (Wagner and Wendlberger, 2000; Xiang et al., 2010).

The macrocylic lactones (IVM, DRM, MO and MXD) employ a peculiar mechanism for inducing fatality in parasites. The drugs seem to cause starvation and/or paralysis by interfering with chloride channels. The channels in question are suspected to be inhibited when the drug binds to the α-subunits of the glutamate-gated ions channels in muscles of the pharynx and of the entire body (Geerts and Gryseels, 2000).

2.1.6.2 Response to treatment

Despite numerous drugs being used to manage the disease, treatment of spirocercosis is not always reliable. For the Avermectins:

- Berry (2000) investigated the efficacy of DRM at a dose of 200 µg/kg via subcutaneous (s.c.) injection at 14 day intervals for three consecutive treatments in seven dogs diagnosed with *Spirocerca*-induced oesophageal nodules. By the end of the experimental period (six weeks post-treatment), the oesophageal nodules had completely regressed in only four out of the seven dogs. One dog

only showed complete regression of oesophageal nodules 22 months after the last treatment. The two remaining dogs received further treatment with 500 µg/kg DRM daily administered orally for an additional six weeks. The treatment was successful in the two dogs.

- Lavy et al. (2002) used DRM (Dectomax, Pfizer, Brazil) at a higher dose of 400 µg/kg via s.c. injections at two week intervals for six treatments and continued treating monthly with 400 µg/kg s.c. injections until 768 days post-inoculation, in seven beagle dogs experimentally infested with 40 *S. lupi* larvae (L3). By six days after the second treatment, the regimen showed suppression of egg production in faecal flotation in all seven dogs, and by 35-544 days after the last treatment, the oesophageal nodules had disappeared in six dogs, albeit the seventh dog still showed evidence of regressed nodules.

- Mylonakis et al. (2004) used IVM (Valaneq, Merck, Holland) at an even higher dose of 600 µg/kg, injected s.c., twice at 14 day intervals followed by oral administration of 0.5 mg/kg prednisolone (Presolone, Nycomed, Greece) every twelve hours at two week-intervals (reduced to once daily for an extra week) on eight dogs with oesophageal nodules and *S. lupi* eggs in their faeces. Suppression of egg shedding resulted in all eight dogs by 60-80 days post-treatment. At 180 days post treatment (the end of the treatment period), the oesophageal nodules had disappeared in all dogs and it is believed that the simultaneous use of prednisolone contributed to the faster nodular regression through the management of the immuno-inflammatory response induced by the parasite.

- Most recently, Lobetti (2012) evaluated the time required for resolution of *Spirocerca*-induced oesophageal nodules using DRM at 500 µg/kg given orally

once daily for 42 day to dogs (n=20) naturally infested with *S. lupi* worms. By the end of the 42 day treatment period, the oesophageal nodules had completely regressed in only 13 dogs. Five dogs showed resolution of oesophageal nodules after treatment was extended to 84 days in total. The two remaining dogs received further treatment for a further 42 days (126 days in total) prior to nodule resolution.

For the Milbermycins:

- Le Sueur et al. (2010) evaluated the efficacy of a combination of imidacloprid 10 % /moxidectin 2.5 % spot-on (Advocate® for dogs, Bayer Animal Health). The study included puppies (n=112) (aged three months on average) naturally infested with *S. lupi* worms on Rèunion Island. The puppies were divided into two groups, group A which included the treated puppies (n=58) and group B which included the untreated puppies control group (n=54). Group A puppies were treated with imidacloprid 10 % / moxidectin 2.5% spot-on at 2.5 mg/kg, monthly, for a period of nine months. At 10 months post-inclusion (end of the treatment period), endoscopy (done at 4 weekly intervals) showed presence of oesophageal nodules in one group A dog (n =1) and also in group B dogs (n=19). Drug efficacy of 98.3% was achieved in group A (treated) dogs.

- The efficacy of MO against pre-adult *S. lupi* worms was tested by (Kok et al. (2011). For this study, dogs (n=21) were purposefully infested with 40 stage 3 larvae (L3). The presence of oesophageal nodules was confirmed endoscopically in all dogs post-infection prior to treatment. This study was subdivided into study 1 and 2. For study 1, dogs (n=7) received a single oral treatment of MO tablets (12.5 mg MO and 125 mg praziquantel; Milbemax®, Novartis Animal Health Inc.) at day 30 post-infection and dogs (n=7) used as a control group were untreated. For study 2, dogs received the same treatment at day 28 post-infection (n=4) as

above, and thereafter 4 additional treatments at 28-day intervals (n=2) or 9 additional treatments at 14-day intervals also from day 28 post-infection (n=2). The remaining dogs (n=3) were used for control purposes. Dogs for both studies were euthanized and necropsied on day 168 or 169 post-infection. Study 1 was able to demonstrate an efficacy of 79.8 %, while study 2 demonstrated 100% efficacy.

- Most recently, the efficacy of imidacloprid 10 % / moxidectin 2.5 % spot-on (topically applied endectocide) formulation (Advocate®, Advantage® Multi, Bayer) was evaluated against immature and mature stages of *S. lupi*. Dogs (n=24) used for the study were divided into 3 equal groups to group 1 were allocated the dogs that were untreated (control groups) and groups 2 and 3 comprised of dogs that were treated with 10 mg/kg Advocate® (Bayer). Group 2 dogs received monthly treatments whereas group 3 dogs received weekly treatments. In group 3 dogs (n=8), the number of oesophageal nodules decreased from approximately a range 4 to 6 to a range of 1 to 2 in only 4 dogs (conclusion of treatment period) while oesophageal nodule numbers remained unchanged in 3 other dogs. One dog had no evidence of oesophageal nodules throughout the study period. In group 2 dogs (n=8), oesophageal nodules had completely regressed on day 169 or 176 post-treatment. Reduction in egg shedding (using the sugar flotation method) was observed from day 208 post-treatment in group 3 dogs. Aortic lesions (confirmed at necropsy) ranged from 5 to 7 in group 1 dogs and 6 to 16 in group 3 dogs. The drug efficacy in group 2 dogs was 100 % and 98.5 % in group 3 dogs (Austin et al., 2013).

2.1.6.3 Drug resistance

No information is available on the resistance profile of *S. lupi*. However, based on the mechanism of resistance reported in other parasites, resistance by *S. lupi* probably

involves the P-glycoprotein pump (Pgp). *Haemonchus contortus* (*H. contortus)* was found to possess four genes of Pgp (Sangster, 1994)(Geerts and Gryseels, 2000), while *Onchocerca spp.,* specifically, *O. volvulus, O. ochengi* and *O. gibson* also tested positive for genes of Pgp (Kwa et al., 1998). At the cellular level, the Pgp enhances drug efflux from human cells thereby protecting the parasite from the effects of the drug (Kwa et al., 1998; Bourguinat et al., 2008).

2.2 PATHOGENESIS OF THE NEOPLASTIC TRANSFORMATION

While the ability of the parasite to induce neoplastic transformation is well recognised, the mechanisms behind these transformations remain elusive. One of the major theories that have been proposed links the neoplastic transformation with chronic inflammation. It has been suggested that the worm induces chronic irritation within the oesophagus with resultant chronic inflammation. Due to constant irritation, a few cells within the affected area may undergo metaplasia whilst the majority ultimately become neoplastic. To this extent, different studies have looked at specific markers of inflammation in an attempt to link inflammation with neoplastic transformation in spirocercosis:

- C-reactive proteins (CRP): In a study by Mukorera et al. (2011), it was demonstrated that spirocercosis was associated with an increase in CRP concentrations in comparison to healthy controls (13.4 ± 17.9 mg/L), albeit with no significant difference between animals with neoplastic (76.5 ± 44.8 mg/L) and non-neoplastic lesions (60.4 ± 48.0 mg/L). The latter was not a surprising finding as CRP is a non-specific indicator of acute inflammation, whose role is linked to the activation of the complement cascade and phagocytosis. As described above, the *Spirocerca* nodule is associated with leucocytic infiltration, predominantly lymphoplasmacytic in non-neoplastic nodules and neutrophillic in neoplastic nodules (mainly associated with extensive necrosis of the malignant neoplastic

masses). While this study was able to demonstrate a clear link between inflammation and spirocercosis, the link between inflammation and neoplastic transformation remained tenuous.

- Regulatory T-cells: Dvir et al. (2011) proposed that the neoplastic transformation may be linked to changes in regulatory T-cell (Tregs) function. Their theory was based on the finding of increased expression of CD4+ regulatory T cells in patients with various types of cancer, whereby increased Tregs have been associated with inhibition of anti-tumour immune responses. A similar increase in Tregs has also been reported in dogs with malignant melanoma. Despite strong support for the role of Tregs in neoplasia, Dvir et al. were unable to demonstrate an increase in Tregs within the *Spirocerca*-induced nodules or draining lymph nodes, using immunohistochemical techniques.

- Vascular endothelial growth factor (VEGF): In a study by Dvir et al. (2010), the effect of VEGF and spirocercosis related neoplasia was evaluated. As a growth factor, VEGF is responsible for the neovascularization that results within the neoplastic nodule. For this study, they were able to demonstrate that both *Spirocerca*-induced and non-*Spirocera*-induced neoplastic lesions in dogs were associated with an increased expression of VEGF. Unfortunately, since the increase was therefore non-specific it was surmised that the increased VEGF was due to the neoplastic transformation and not necessarily the driving factor behind said transformation in spirocercosis.

- Fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF): Both FGF and PDGF have been associated with active wound healing and neoplastic changes in people and animals. As a result is has been suggested that an up-regulation of these mediators could be associated with an increase in tumour incidence. As for VEGF, Dvir et al. (2010) were able to demonstrate increased expression of both these factors within the *Spirocerca*-induced neoplastic nodules.

Unfortunately, this increase was similar to that seen in non-*Spirocerca*-induced neoplastic lesions. As a result it could only be concluded that these factors were a general indicator of neoplastic change and not necessarily an inciting cause.

- Interleukins: With cytokines being important in the inflammatory cascade, Dvir et al. (2012) proposed that an increase in cytokine expression may be positively correlated with the occurrence of neoplastic change. For this study IL-2, IL-4, IL-6, IL-8, IL-10, IL-18 were measured in the plasma of dogs with both neoplastic and non-neoplastic oesophageal nodules. Only IL-8 and IL-18 showed significant differences in their plasma concentration among the three groups (healthy controls (n=25), dogs with neoplastic lesions (n=29) and dogs with non-neoplastic lesions (n=49)), with IL-8 increasing from 150 pg/ml in the control group to 429 pg/ml for non-neoplastic oesophageal lesions and to 634 pg/ml in the case of neoplastic oesophageal lesions. In contrast, IL-18 showed highest concentrations in the non-neoplastic group (53 pg/ml), followed by the control group (46 pg/ml) and finally the neoplastic group (33 pg/ml). It was surmised that the increase in IL-8 was responsible for the infiltration of inflammatory cells into the nodules. The authors also speculated that the increase in IL-8 could be as a result of the parasite being associated with a bacterial infection as seen with *Wolbachia* during dirofilariasis. Interestingly, *Wolbachia* has been associated with tumour formation in *O. volvulus* infections. The decrease in IL-18 concentrations, however, could not be properly explained as other helminth infections have been associated with increased IL-18 concentrations.

- *Wolbachia*: As mentioned above, *Wolbachia* species have been associated with neoplastic transformation in *Onchocerca* infections. In the most recent study conducted by Gottlieb et al. (2012), they showed that *Spirocerca* larvae and adults were negative for the presence of *Wolbachia*, *Cardinium* and *Rickettsia* bacterial symbiont species. They were, however, positive for the presence of *Comamonas*

spp. Furthermore, this symbiont was localised to the gut epithelium of the worm and was suspected to play a role in digestion.

As can be seen from numerous current studies on the pathogenesis of neoplastic transformation in spirocercosis, inflammation plays a role in the neoplastic transformation of oesophageal nodules. However, this being said, the above-mentioned studies failed to conclusively demonstrate that the inflammation in any way causes neoplastic transformation. Whilst inflammation and carcinogenicity have been linked, very little attention has been given to the possibility that the worm could be secreting a clastogenic substance that might be responsible for nodule formation and progression to neoplasia. Medical literature, to date, has reported numerous cases of infectious agents inciting neoplastic changes using far more direct mechanisms.

2.2.1 *Clastogenicity*

A substance that induces breakage, deletion, gain or loss of chromosomes and/or rearrangement of chromosomes is known as a clastogen. Clastogenic substances induce a clastogenic mutation which may be capable of causing tumors (von Recum, 1998; Schwab, 2011). In the latter, the clastogen is categorized as a mutagenic agent and can either be of chemical or physical origin. Clastogens are also capable of inducing non-tumorous chromosomal lesions that include chromosome irregularities and sister chromatid exchange (Berman, 2009). Under normal conditions, dividing cells undergo different phases which are regulated by cyclin-dependent kinases (CDK's) throughout the cell cycle process (Vermeulen et al., 2003).

- G0 or resting phase: cells are in a non-proliferating stage.
- G1 or gap 1 phase: cells get ready for the synthesis of DNA.
- S or synthesis phase: replication of the DNA.
- G2 or gap 2 phase: cell growth in preparation for mitosis.
- M or Mitosis phases: procedure of nuclear division.

When a clastogenic substance interferes with the processes above, cell cycle dysregulation (Figure 2.2.1-7) results and is evident in neoplastic cells (for example multinuclei or micronuclei).

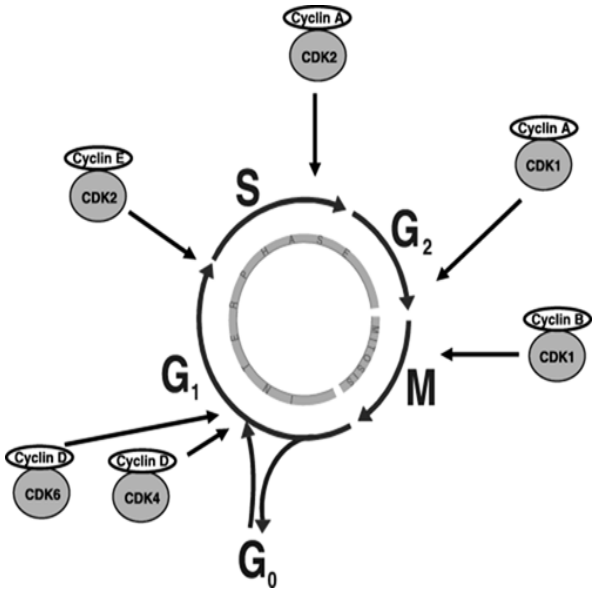


Figure 2.2.1-7: Stages of cell cycle (Vermeulen et al., 2003)

The figure above illustrates the cell cycle phases which include the resting phase (G_0), Gap phase 1 (G_1), Synthesis phase (S), Gap phase 2 (G_2) and Mitosis (M) phase. The site of activity of different regulatory CDK (cyclin-dependent kinase)/cyclin complexes are also indicated. The CDK's (1, 2, 4 and 6) are regulatory proteins that form complexes with cyclins (A, B, C and D) (stimulating proteins) to permit progression from one phase to the next.

2.2.2 *Pathogenic organisms as clastogens*

The carcinogenic effect of worms and/or bacteria is not novel and has been previously reported in the medical literature. The following parasites were classified as carcinogens as they release factors that induce neoplasia (Thuwajit et al., 2006):

- *Helicobacter pylori (H. pylori)* is classified as a group I carcinogen, inducing gastric adenocarcinoma in humans (Wong et al., 2004). While the pathogenesis is incompletely understood, it is believed to be related to an increase in IL-8. The latter could be demonstrated *in-vitro* as human gastric cell lines (MKN45, cultured in Roswell Park Memorial Institute (RPMI) 1640) exposed to recombinant human IL-1β (10 ng/ml) and viable *H. pylori* cells (1.5x107 CFU/ml), showed increased secretion of IL-8 (Aihara et al., 1997).

- *Clonorchis sinensis* (*C. sinensis*) is a liver fluke that induces clonorchiasis (hepatobiliary disease) and cholangiocarcinoma (neoplasia of the bile duct) in humans. Using cholangiocarcinoma cell lines (HuCCT1) findings have suggested that *C. sinensis* ESPs, harvested by incubating parasites in sterile PBS, were responsible for the carcinogenic effect. Following cell counts, the growth of cells increased from 20-40 %. This increase in cell growth was associated with an increase in cyclins E and B, both of which play an important role in cell cycle progression. Also evident was that the cells progressed from G2 to the M phase. Other proteins that were increased were the kinases (CDK2 and CDK4) (Kim et al., 2009) and the proteases, namely, cysteine proteases, serine proteases and cytosol aminopeptidase. The latter proteases are common to other worms ESPs e.g. *H. contortus* (Ju et al., 2009).

- *Opisthorchis viverrini* (*O. viverrini*), is another liver fluke, that is known to induce cholangiocarcinoma in humans, once again via ESPs. This was demonstrated in NIH-3T3 fibroblasts *in-vitro.* The said fibroblasts were cultured in a double well chamber with the fibroblast confluent in the lower well and the adult *O. viverrini* parasite in upper well. After 3 days, fibroblast proliferation was shown to have increased (Thuwajit et al., 2004). Also of importance, this study was able to demonstrate that gene expression doubled, with the genes controlling metabolism and biosynthesis being most affected. Other genes expressed included those for kinesins and the cytokines. While the specific ESPs were not identified it was

suspected that the CDK's and cyclin proteins expressed in high concentration in the *O. viverrini* ESPs played a major role in cell proliferation (Thuwajit et al., 2006).

2.2.3 *Worm culturing*

As is evident from the above-mentioned data, the harvesting of parasite ESPs requires a degree of culturing. However, the nutrient media required to sustain *S. lupi* is yet to be described. However, with various parasites having been established in a variety of cell models, for example, *Schistosoma mansoni (S. mansoni)* has been maintained in RPMI 1640 Medium with 10 % bovine foetal serum, *O. volvulus* in tissue culture medium 199 supplemented with 10 % fetal calf serum (FSC) and *S. mansoni* worms (from cercaria to adult stage) in 0.5 % lactalbumin-hydrolyzate (salt), 0.1 % glucose, 10 % inactivated rabbit serum, 1 % rabbit red cells, Earle's saline, 100 µg/ml penicillin and 100 µg/ml streptomycin (Clegg, 1965) and *C. sinensis* in sterile saline, it is plausible that the *S. lupi* parasite could potentially survive in similar media in order to allow for harvesting of the ESPs for further study.

2.3 CONCLUSION

S. lupi induces a disease known as spirocercosis in mainly in the domestic dog. In *Spirocerca* cases, the induction of an inflammatory response purposely plays a role in neoplasia. However, inflammatory mechanisms may only explain part of the pathogenesis underpinning neoplastic transformation in spirocercosis. With other parasites such as *O viverrini* and *C.sinensis* have been shown to cause cancer via their ESPs, this study will evaluate the effects of *S. lupi* organic solvent-extracted ESPs on fibroblast cultures since the fibroblast is the predominant cell type associated with *S. lupi*-induced oesophageal nodules in carnivores.

3 MATERIALS AND METHODS

3.1 ESTABLISHMENT OF AN *IN-VITRO* SPIROCERCA CULTURE SYSTEM

3.1.1 *Collection of worms*

Adult worms were collected from domestic dogs (n=4) as soon as possible post-mortem. Three dogs were euthanized (due to spirocercosis) at the Onderstepoort Veterinary Academic Hospital (OVAH) of the University of Pretoria and the other at the Sinoville Animal Clinic in Pretoria. Euthanasia was always on recommendation of the treating veterinarian and was in no way dictated by the project i.e. worms were sampled completely opportunistically. Carcasses were transported to the post-mortem hall (Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria) where oesophageal nodules were carefully dissected for live worms. In total, 45 adult worms were harvested from two dogs, 36 worms from the dog from Sinoville Animal Clinic and nine worms from the rest. The harvesting of worms post-mortem was approved by the Animal Use and Care Committee of the University of Pretoria (V063/12). In all cases, the owners had agreed to a post-mortem evaluation of their pets. The worms were collected in sample collection bottles containing pre-warmed culture media (RPMI 1640 Medium, Iscove's Modified Dulbecco's Medium, Dulbecco's Modified Eagle's Medium and Ham's F12 Medium) prior to transportation to the culture laboratory.

3.1.2 *Culture method*

The harvested worms were immediately transported to the laboratory for culture. Prior to plating, the parasites were rinsed three times using sterile PBS (phosphate buffered saline) to remove the detritus on the parasite's cuticle. Using sterile forceps the parasites were transferred individually into wells of 24-well microtitre plates (Nunc) within a laminar flow cabinet (SA LABS). The parasites were then completely immersed in 2 ml of one of four media, which are commonly used in parasite cultures; RPMI 1640 Medium (Highveld Biologicals, Cat. No. 3709 – 1), Iscove's Modified Dulbecco's

Medium (Highveld Biologicals, Cat. No. 3779), Dulbecco's Modified Eagle's Medium (Highveld Biologicals, Cat. No. 3687) or Ham's F12 Medium (Highveld Biologicals, Cat. No. 3474-1). The control was pre-warmed saline (Adcock Ingram critical care). All exposures were undertaken in triplicate.

Plates were subsequently transferred into a 2 L air-tight container humidified with sterile reverse osmosis (RO) water in a 500 ml glass beaker. The container was saturated with carbogen (5 % CO_2 in oxygen, Afrox Scientific) for 2 min each time prior to placement in an incubator at 37 °C (Queue, Bio-Rad). The culture media and saline control were replaced at 48 hr intervals. Cultures were monitored at 24 hr intervals for bacterial contamination and replacement of the gas environment. Following every media change the supernatant was transferred to 1.5 ml sterile eppendorf micro-tubes frozen at -80 °C for future use.

3.2 PROCESSING OF CULTURE MEDIA

3.2.1 *Measuring pH of culture media and saline*

The potential hydrogen (pH) of thawed culture media and saline used for worm cultivation was measured using a pH meter (Hach, Cole-Parmer), and the values obtained were compared to pH values of sterile media and saline.

3.2.2 *Bacterial culture*

The samples (culture media and saline) used for worm culture were extracted in 1 ml quantities using sterile syringes (B. Braun Medical Inc., Omnifix®) and needles (SurGuard2™, Terumo®). Samples droplets were plated on MacConkey agar, sheep's blood agar (BA), colistin-nalidixic acid agar (CNA) and broth containing methyl-umbellinferyl-β-glucuronide (MUG) and incubated at 35 °C in aerobic atmosphere in an incubator for 24 hrs. Thereafter, growth of bacterial colonies and lactose consumption in samples plated on MacConkey agar were monitored. The recovered colonies of BA and

CNA were tested for biochemical reactions using catalase (-), lancefield group D (+) and pyrrolidonyl arylamidase (Kinzelman et al., 2003).

3.3 VIABILITY OF CULTURED HELMINTHES

Worm survival (viability) period was measured by the presence of normal motility and macro-morphology in culture media and saline (control). Normal motility included worm movement and macro-morphology referred specifically to intact cuticle, changes in the cuticle and in other parts i.e. oesophagus of the worm. When movement ceased, the intact state of the cuticle was used to measure worm viability. Worms with an intact cuticle and no movement were considered to be alive. Worms were monitored at 24 hr intervals.

3.4 EXTRACTION AND VISUALISATION OF EXCRETORY PRODUCTS FROM MEDIA

3.4.1 *Extraction*

Frozen media was thawed and mixed in a 1:1 ration with either acetone, hexane or methanol. Following mixing in a vortex (Biochemical, Medical Scientific CC), the samples were centrifuged at 3500 rpm for 3 min at 9 g (Allegra®, Beckman Coulter), at room temperature. Samples were rapidly frozen in dry ice/methanol mixture to potentially remove the aqueous phase. After the ice-bath, the supernatant was dried using pressurized nitrogen (15 psi) at 60 °C for 60 min. The dried samples were preserved at 4 °C in a fridge until used. Processed samples were stored for ± 4 months in total.

3.4.2 *Visualization using Thin Layer Chromatography*

Dried samples were reconstituted in methanol, hexane, acetone or ethylacetate in 1 ml volumes. The samples (10 µl) or sterile culture media (control) were spotted on TLC

plates. Separation of individual bands was done on aluminium-backed TLC plates (10 cm x 20 cm) layered with silica gel 60 (Merck, Germany). The plates were subsequently placed into glass tanks saturated in one of three elution systems. These included EMW (Ethylacetate, Methanol, Water) (50:6:5, v/v/v) – polar/neutral, BEA (Benzene, Ethanol, Ammonia) (90:10:1, v/v/v) – non-polar/basic and CEF (Chloroform, Ethylacetate, Formic acid) (50:40:10, v/v/v) – intermediate polarity/acidic. Developed plates were visualized under UV light (254 and 360 nm, Camac universal UV lamp TL-600) prior to spraying with a mixture of vanillin (Sigma Aldrich), methanol and sulphuric acid (0.2: 56: 2, v/v/v); p-Anisaldehyde (Sigma), Ethanol, Sulphuric acid (2: 36: 2, v/v/v) or ninhydrin (Tritech Forensics), deionized water, butanol (200: 200: 200, v/v/v) to visualize the bands (Skipski et al., 1962; Kotze and Eloff, 2002). They were then heated in an oven at 110 °C and immediately scanned. The retardation factor (RF) value (which equals the length travelled by the element from initial point divided by total length travelled by the solvent system) was calculated for each band.

3.5 FINGERPRINT OF EXCRETORY/SECRETORY PRODUCTS USING U-HPLC/MS AND PROTEIN(S) IDENTIFICATION

The samples were sent to Mintek™ (a commercial laboratory) for identification of possible proteins. The separation of ESPs was done using ultra-high performance liquid chromatography (U-HPLC) (method provided below). Peptide hydrolysis was done using liquid chromatography mass spectrometry (LC-MS/MS) and protein identification using the MicroTOF-QII (Bruker™) (the method was not made available for proprietary reasons). The twenty amino acids coding for proteins used by the lab are provided in Table 3.5-1.

The supernatant (culture media and saline used for culture) were concentrated by removing the water using a Lyoquest freeze dryer (Telstar®) at a temperature of -77 °C and vacuum of 0.046milli-bar. Thereafter, samples were diluted in a mixture of water and acetonitrile (1:1). At-least 20 µl of samples were injected into the ultimate 3000 ultra-high performance liquid chromatography (U-HPLC) (Thermo Scientific and Dionex)

fitted with an Acclaim™ 120 C18 column (Dionex) with a particle size of 3 µm, 2.1 mm x 100 mm diameter and average pore diameter of 120 Å. The mobile phase (0.1 % formic acid and water, 0.1 % formic acid and acetonitrile) containing formic acid as an ion pairing agent was added to the column at a flow-rate of 0.3 ml/min. The U-HPLC was connected to a MicroTOF-QII (Bruker™) high resolution mass spectrophotometer (MS). Peaks were identified by use of an attached library.

Table 3.5-1: The twenty amino acids used for protein coding.

Amino-acid names	Abbreviations
Alanine	Ala, a
Arginine	Arg, r
Asparagine	Asn, n
Aspartic acid	Asp, d
Cysteine	Cys, c
Glutamic acid	Glu, e
Glutamine	Gln, q
Glycine	Gly, g
Histidine	His, h
Isoleucine	Ile, i
Leucine	Leu, l
Lysine	Lys, k
Methionine	Met, m
Phenylalanine	Phe, f
Proline	Pro, p
Serine	Ser, s
Threonine	Thr, t
Tryptophan	Trp, w
Tyrosine	Tyr, y
Valine	Val, v

3.6 *IN-VITRO* CARCINOGENICITY ASSAY

3.6.1 *Consumables*

Dulbecco's Modified Eagle's Medium (DMEM) AQ media (Sigma Aldrich), D0819, Lot no. RNBC 1433; Foetal Bovine Serum (FBS) (Scientific group), BC/SO613/HI; Trypsin/Versene (125 % Trypsin, 0.1 % Versene EDTA) (Highveld Biologicals), Cat. No. 3768; Collagenase crude type x1 (Sigma Aldrich), Lot no. 039K8628, P-code 1000748554; Hyaluronidase type I-S (Sigma Aldrich), Lot no. 029K7-001, P-code 1000689699 and penicillin-streptomycin-neomycin (Sigma Aldrich), 051M0852, SL11205, cell culture treated flasks (25-175 cm^2) with filter caps (Thermo Scientific™, Nunc™), 50 ml centrifuge tubes (Corning®, Sigma Aldrich) and 8-well chamber slides (Lab-Tek, Nunc™) were used in this study.

3.6.2 *Skin Fibroblast preparation*

Skin samples were harvested from the pinnae of Balb/c mice (either sex ± 3 months old) supplied by the UPBRC (University of Pretoria, Biomedical Research Centre). Sample collection was purely opportunistic following on scheduled terminations from another approved study. All animals were deemed healthy prior to sample harvesting. Once samples were sectioned, they were temporarily kept in PBS. Once in the laboratory, the pinnae were cleaned by scrubbing them with a tooth-brush using Hibiscrub (Bioscrub™) as a disinfectant, rinsed with sterile Milli-Q water (Millipore corp.), prior to final spraying with 70 % ethanol and rinsing with sterile Milli-Q water. After cleaning, the pinnae were transferred to a laminar flow cabinet in a sterile 145 mm glass Petri dish covered with foil. The tissues were thereafter minced with a sterile surgical blade into pieces of approximately 5X5 mm in 100 µl of DMEM (47.5 ml) and 2.5 ml of Penicillin-Streptomycin-Neomycin.

The pieces of skin were digested using pre-warmed collagenase and hyaluronidase in DMEM (20: 20.3: 20.3; v/v/v) (8 ml for 10 pinnae) in centrifuge tubes with caps that were slightly loosened, to allow for gaseous exchange, at 37 °C in a humidified 5 % CO_2 incubator (Bio-Pharma, Thermo-electron Corp.) for 24 hrs. The tubes were agitated by

being shaken gently to disperse the pieces at 5 hr intervals, over the 24 hr period. After digestion, samples were centrifuged (Scientific, Taiwan, ROC) at 42 g, at 500 rpm for 5 min, pooled, washed by shaking gently in pre-warmed trypsin-versene solution suspension and pooled again. Pellets were finally suspended in 12 ml DMEM and 40 % FBS heat-inactivated (HI), and transferred in 2 ml aliquots into 50 ml flasks (Nunc, Thermo Scientific™). Penicillin-Streptomycin-Neomycin stock solution (50 µl) was also added to the flasks. Cultures were maintained at 37 °C in 5 % CO_2 for 24 hrs. The 10 % FBS in DMEM (culture medium) in the flasks was thereafter increased to 5 ml and cultures were incubated for an additional 7 days. Culture media were changed twice a week. Culture medium in flasks was poured-off, and then replaced with fresh, pre-warmed culture medium.

Once cultures reached confluence of approximately 80–90%, they were twice rinsed with trypsin-versene solution and trypsinized. The monolayers were incubated in 1 ml (for 50 ml TC flasks) and 2 ml (for 250 ml TC flasks) of trypsin-versene solution for approximately 4 min at 37 °C. Dispersion into single cells was accelerated by gentle scrubbing with cell-scrapers under a laminar flow cabinet. Culture medium (12 ml) was added to the flasks to inhibit the trypsin-versene solution. The fibroblasts in suspension were sedimented via centrifugation, the supernatant was discarded and cell pellets re-suspended with 3 ml of culture medium prior to cell counting. Cells were quantified using the trypan blue exclusion method. Fifty microliters of cell suspension was mixed 1:1 with a 0.4 % trypan-blue solution. The suspension was thoroughly mixed and 20 µl of stained suspension was transferred onto a haemocytometer. Non-stained cells were counted under the inverted microscope (at 10x magnification) in duplicate. Cell clumps were counted as one cell. The average cell count per ml of cell suspension was calculated using the standard haemocytometer equation: Cell count per square x 10 000 (haemocytometer volume) x 2 (dilution factor).

Cells were either used for exposure or, when in excess, frozen for future use. The fibroblasts were sedimented via centrifugation and the culture medium was discarded. Freezing medium (DMEM medium (3.5 ml), 500 µl of Glycerol and 1 ml of FBS) (5 ml)

added drop by drop into fibroblast cell suspension (400 000 cells/ml) was deposited in 1 ml aliquots into cryovials. The vials were placed in a freezer-boy container (Biocision^{TM}) and frozen at - 80 °C in a freezer (Snijders Scientific). When required, cells were rapidly thawed by gentle agitation in a 37 °C water-bath for 5 min. Pre-warmed culture medium (5.5 ml) was added to the cell suspensions (1 ml). The fibroblasts were sedimented via centrifugation, the supernatant was discarded and the pellets were re-suspended with 10 ml of culture medium prior to transferring into 50 ml flasks. Cells were then incubated at 37 °C in 5 % CO_2.

3.6.3 *Exposure to excretory/secretory products*

The dried extracts were reconstituted in organic solvents: acetone, methanol, hexane or ethylacetate to 1 ml. Product(s) were filter-sterilized using 0.25 µm filters (Millex, Millipore corp.) prior to making dilutions of 750 µl of product(s) and 250 µl of DMEM, 500 µl of product(s) and 500 µl of DMEM and 250 µl of product(s) and 750 µl of DMEM. The final concentration of the solvent extracts was therefore 10, 20 and 30 µl. Fibroblasts (480 000 per ml in suspension) were collected by trypsinization and cell suspension (400 µl) was dispensed into each well of 8-well chambered slides (Lab-Tek, USA). After incubating for 24 hrs, culture medium was changed and fibroblasts were treated with 40 µl of one of the three concentrations of ESPs and/or above-mentioned organic solvents for a total exposure period of 48 hrs. The experiment was carried out in triplicates for exposures of ESPs and/or organic solvents and untreated fibroblasts. The untreated (control) fibroblasts and test fibroblast pellets were always deposited on separate 8-well chambered slides to prevent interference. The control wells were exposed to 40 ul of the relevant organic solvent diluted in the same manner as the for the test solvents viz. 750 µl of solvent and 250 µl of DMEM, 500 µl of solvent and 500 µl of DMEM and 250 µl of solvent and 750 µl of DMEM. The final concentrations of the solvents were therefore 10, 20 and 30 µl.

3.6.4 *Evaluation of cells*

Culture medium in wells was discarded and slides were removed. The slides were subsequently air dried and fixed for 3 min with 100 % methanol. Slides were stained

with Lily Mayer's haematoxylin for 1 min and rinsed in tap water for 7 min. The cytoplasm was stained with eosin for thirty sec and cells were rinsed with 100 % methanol. Slides were then rinsed in xylene and cover-slips were mounted onto slides using entellan.

Photographs of fibroblasts exposed to ESPs or organic solvents and / or untreated controls at 10 x magnifications (viz. photos of 5 non –over lapping fields per slides) were taken. Cytological analysis was undertaken on digital photographs (4140 x 3096 pixel) obtained using an Olympus DP72 digital camera attached to an Olympus light microscope (Olympus, Japan). Each digital photograph covered a surface area of 1 x 10^4 $\mu m^{2.}$ The photographs were downloaded onto a computer and adjusted to 50 % (1360 x 1024 pixels) of their initial size in Paint (Microsoft Windows 7). A grid was superimposed over the photographs. Fibroblasts (n=100), both normal and mitotic, were counted by placing markings in an anticlockwise manner for each photograph. The fibroblasts that were in mitosis or had just undergone mitosis (cell replication) were used as marker of cell proliferation.

3.6.5 *Statistical analysis*

The mitotic indices were calculated in percentages for all concentrations. The mean and standard deviation (S.D.) were determined for the five mitotic indices for each organic solvent per concentration and also for mitotic indices per concentration for all four organic solvents used. The data was plotted in a standard Cartesian plane (mitotic indices versus ESPs and/or organic solvent exposures) using Microsoft Excel 2010 and compared visually with the solvent controls, for overlapping standard deviations.

4 RESULTS

4.1 HARVESTING OF WORMS

When worms were isolated from nodules, they were mostly curled-up and showed resistance to being pulled or stretched out. In pre-warmed culture medium, worms harvested from the dogs received from OVAH showed slight movement while those received from the Sinoville Animal Clinic were curled-up. The sample collection bottles were completely sealed. When rinsing in pre-warmed PBS, no movement was observed. At all times, worms still retained the pink-coloured cuticle. In ± 30 min of culture, worms showed movement. All the worms isolated from nodules were therefore deemed viable following post-mortem nodule dissection prior to establishment of the cultures.

4.2 WORM CULTIVATION

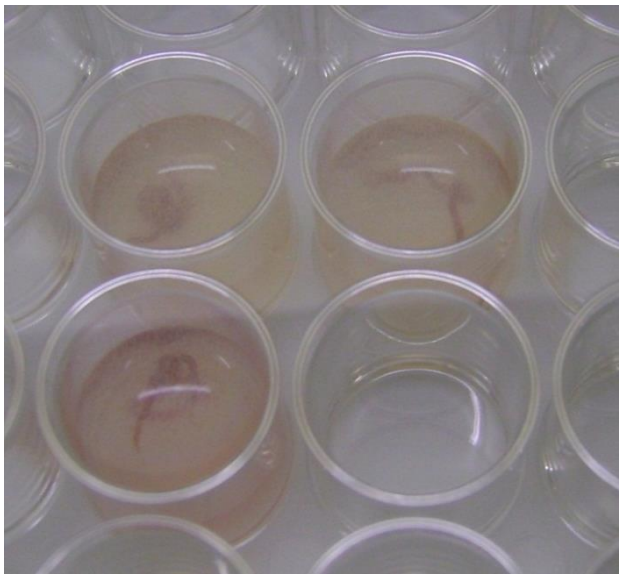


Figure 4.2-8: *Spirocerca lupi* adult worms visible in wells in a microtitre plate, incubated at 37 °C. Culture medium was replaced at 48 hr intervals. Worms were moving at the time of taking the picture.

Worms were plated at different times, as the worms became available. As a result, experiments did not follow a linear pattern. In most cases, the worms survived well, showing normal motility and macro-morphology for the first two days within culture media. For the succeeding three days, the worms were mostly curled-up; showing slight movement and the cuticle was still intact. Thereafter, the worms started dying. For the culture media groups (Iscove's, Dulbecco's and Ham's F12), all parasites were dead by 72 hrs post-harvest (Table 4.2-4). For the control group, five parasites survived for 96 hrs in 0.9 % saline. Survival was better in the RPMI 1640 culture medium (Table 4.2-2) (Figure 4.2-8) with the worms only dying by 120 hrs. Two worms in the saline group survived to 144 hrs (Table 4.2-3). The dead worms showed damaged cuticles and were transparent versus pink (Figure 4.2-9). Within 24 hrs of culture in sterile culture media, the culture media underwent a colour change (from red to yellow). However, by 48 hrs (day 3) the RPMI 1640 culture medium returned to its original colour. The culture medium also had a strong and unpleasant odour at 24 hrs.

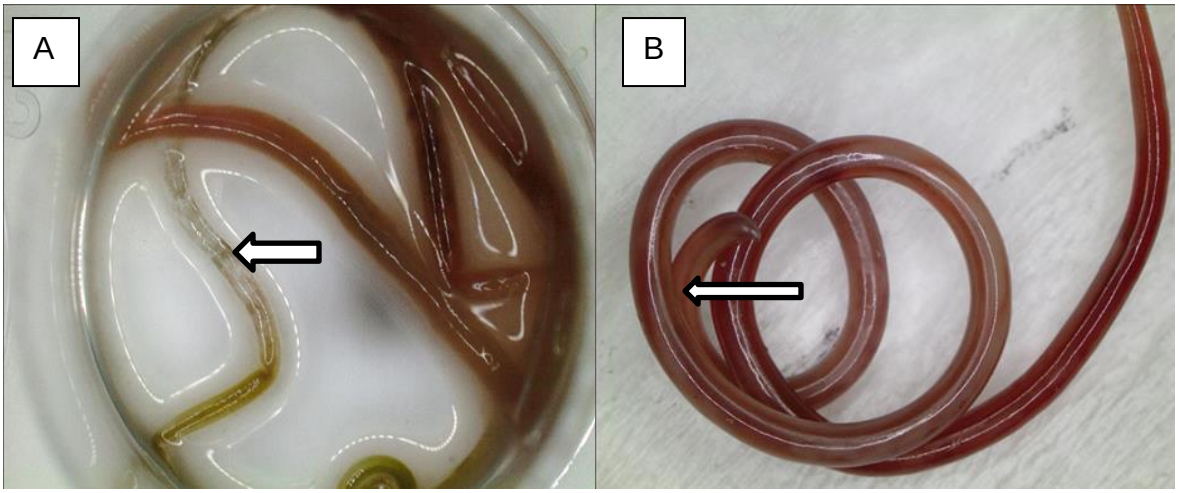


Figure 4.2-9: A: *Spirocerca lupi* adult worm dead *in-vitro* in culture media. Note the transparent cuticle and oesophagus with mottled grey accumulations indicated (arrow). B: A healthy worm with its normal pink colour and intact darker pink oesophagus (arrow).

Table 4.2-2: Viability scores for adult *S. lupi* worms (n=3) placed in RPMI 1640 culture medium. RPMI 1640 culture medium was changed every 48 hrs, for 120 hrs.

Time (h)	Replicates	Worm viability	Comments
0	3	Alive	All 3 worms were moving. No changes observed.
24	3	Alive	All 3 worms were moving. Colour change (from red to yellow) in medium was observed. Wells had a strong unpleasant odour.
48	3	Alive	There was a reduction in worm activity. Medium turned back to its original colour.
72	3	Alive	Worms were not moving, mostly curled up. Sterile medium turned from red to yellow but with a less strong odour.
96	3	Alive	All the 3 worms were not moving. The yellow colour change was still retained in medium.
120	3	Dead	The 3 worms died. The worm cuticle turned white, the visible oesophagus had obtained segmental (greyish) accumulations.

Table 4.2-3: Viability scores for adult *S. lupi* worms (n=6) placed in RPMI 1640 culture medium (n=3) or Saline (n=3). Saline and RPMI 1640 culture medium was changed every 48 hrs, for 144 hrs.

Time (h)	Worm replicates	Worm viability	Comments
0	3 in RPMI 3 in Saline	Alive	All worms were moving. No changes observed.
24	3 in RPMI 3 in Saline	Alive	All worms were active. Colour change in medium (from red to yellow) was observed. No colour change observed in Saline. Wells had unpleasant odour.
48	3 in RPMI 3 in Saline	Alive	All worms were still moving except for 1 in saline. Medium regained its original colour.
72	3 in RPMI 3 in Saline	Alive	Worms in medium and saline were not moving. Colour change in sterile medium (from red to yellow) was observed.
96	3 in RPMI 3 in Saline	Alive	Worms were curled- up. The cuticle of all worms in medium and 1 in saline was turning white. Medium retained its original colour.
120	3 in RPMI 3 in Saline	Dead	All worms in medium and 1 in saline died. 2 worms in saline were curled-up. Oesophagus of dead worms obtained segmental (greyish) accumulations.
144	2 in Saline	Dead	Worms in saline were not moving. The cuticle of worms in saline was changing from pink to a whitish colour.

Table 4.2-4: Viability scores for adult *S. lupi* worms (n=36) in Iscove's Modified Dulbecco's Medium (n=9), Dulbecco's Modified Eagles Medium (n=9), Ham's F12 Medium (n=9) and Saline (n=9). Saline and culture media were changed every 48 hrs in all cases, for 144 hrs.

Time (h)	Culture media	Worms replicates	Worm viability	Comments
0	Iscove's medium Dulbecco's medium Ham's F12 medium	9 per medium and Saline.	Alive	Worms were curled-up. After 5 minutes, 1 worm was identified to have secreted a yellow/mustard substance that formed a precipitate in the Ham's F12 medium.
24	Iscove's medium Dulbecco's medium Ham's F12 medium	9 per medium and saline.	Alive	Worms were not moving in media and saline. Iscove's and Dulbecco's medium turned from red to yellow. The Ham's F12 turned from light pink to white. No colour change was observed in Saline. All wells were malodorous.
48	Iscove's medium Dulbecco's medium Ham's F12 medium	9 per medium and saline.	Alive	Worms were not moving in media and saline. The Ham's F12 medium also turned blue. No colour change was observed in other media.
72	Iscove's medium Dulbecco's medium Ham's F12 medium	9 per medium and saline.	Alive	Worms were curled-up. The colour change (seen on day 2) re-occurred. The cuticle of some worms in media obtained a white colour.
96	Iscove's medium Dulbecco's medium Ham's F12 medium	9 per medium and saline.	Dead	All worms in media and 4 in saline died. 5 worms in saline were curled-up. The cuticle of worms in the saline was still pinkish.
120		5 in saline	Dead	The worms were curled-up. The worm's cuticle obtained a whitish colour. Oesophagus obtained greyish accumulations.
144		5 in saline	Dead	The worms died. The saline obtained a yellowish colour.

4.2.1 *Measuring pH of culture media and saline*

For all culture media and saline examined after worm culture, a slight increase in pH was observed. All replicates of culture media used in worm culture, with the exception of Ham's F12 medium remained alkaline, whereas one sample of Ham's F12 medium used for worm culture was found to be acidic.

Table 4.2-5: The measured pH of culture media (experiment) and saline (control) used in worm culture. Measured pH was done in replicates of three and compared to pH of sterile culture media and saline.

Culture medium	Sterile pH	pH after worm culture			Av. pH
Iscove's Modified Dulbecco's Medium	7.37	7.01	7.25	7.89	7.38
Dulbecco's Modified Eagle's Medium	7.71	7.51	7.74	7.75	7.67
Ham's F12 Medium	7.70	6.66	7.47	7.60	7.24
RPMI 1640 Medium	7.73	7.31	7.46	7.81	7.53
Saline	5.93	5.26	5.50	7.25	6.00

4.2.2 *Isolation and identification of bacteria in culture media and saline*

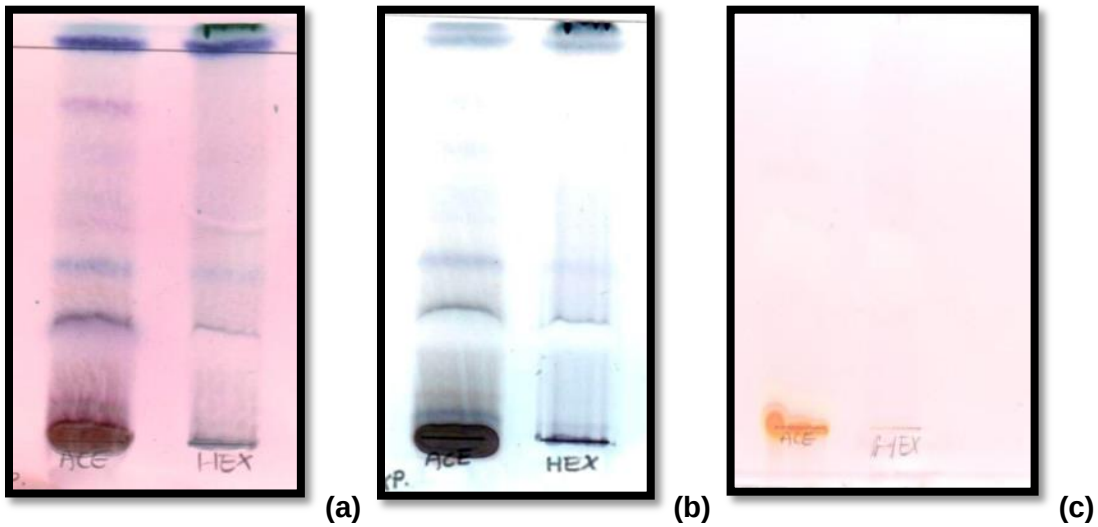
Culture media and saline were positive for bacterial contamination. The following bacteria: *Escherichia coli (E. coli), Enterococcus faecalis (E. faecalis), Pseudomonas aeruginosa (P. aeruginosa)* and *Serratia marcescens (S. marcescens)* were identified in aerobic culture.

Table 4.2-6: Bacterial isolation from culture media (experiment) and saline (control) used for worm culture. The experiment was undertaken in three replicates.

Culture medium	**Bacteria identified per replicate**		
	Replicate 1	Replicate 2	Replicate 3
Iscove's Modified Dulbecco's Medium	*E. coli* *E. faecalis*	–	–
Dulbecco's Modified Eagle's Medium	*E. coli* *E. faecalis*	*P. aeruginosa*	–
Ham's F12 Medium	*E. coli* *E. faecalis* *P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*
RPMI 1640 Medium	*S. marcescens*	*S. marcescens*	*S. marcescens*
Saline	*E. coli* *E. faecalis* *P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*

4.3 SEPARATION OF BANDS USING TLC

The Iscove's culture medium was selected for separation using TLC. Following elution, TLC plates (Figure 4.3) revealed several prominent bands. For all elution systems, no bands were separated when ethylacetate and methanol extracts were used. Acetone was the best extract with most number of bands being seen on TLC plates, followed by hexane. For the BEA elution system, the following bands were visualised; seven (RF values = 0.086, 0.257, 0.400, 0.700, 0.786, 0.943, 0.986) for acetone (p-anisaldehyde); five (RF values = 0.257, 0.414, 0.514, 0.943, 0.986) for hexane (p-anisaldehyde); six (RF values = 0.079, 0.105, 0.303, 0.434, 0.947, 0.973) for acetone (vanillin) and five (RF value = 0.079, 0.303, 0.434, 0.947, 0.973) for hexane (vanillin). For the EMW elution system, the following bands were visualized; four (RF values = 0.082, 0.301, 0.507, 0.795) for acetone (p-anisaldehyde); five (RF values = 0.083, 0.111, 0.319, 0.611, 0.750) for acetone (vanillin) and one (RF value = 0.301) for acetone (ninhydrin). For the CEF elution system, the following bands were visualized; four (RF values = 0.078, 0.234, 0.571, 0.844) for acetone (p-anisaldehyde) and three (RF values = 0.506, 0.671, 0.911) for acetone (vanillin).



(a) **(b)** **(c)**

Figure 4.3-10: TLC of two fractions (ace - acetone; hex - hexane) of Iscove's Modified Dulbecco's Medium used in *S. lupi* culture developed in BEA solvent system; sprayed with p-anisaldehyde-sulphuric acid (a), vanillin-sulphuric acid (b) and ninhydrin (c).

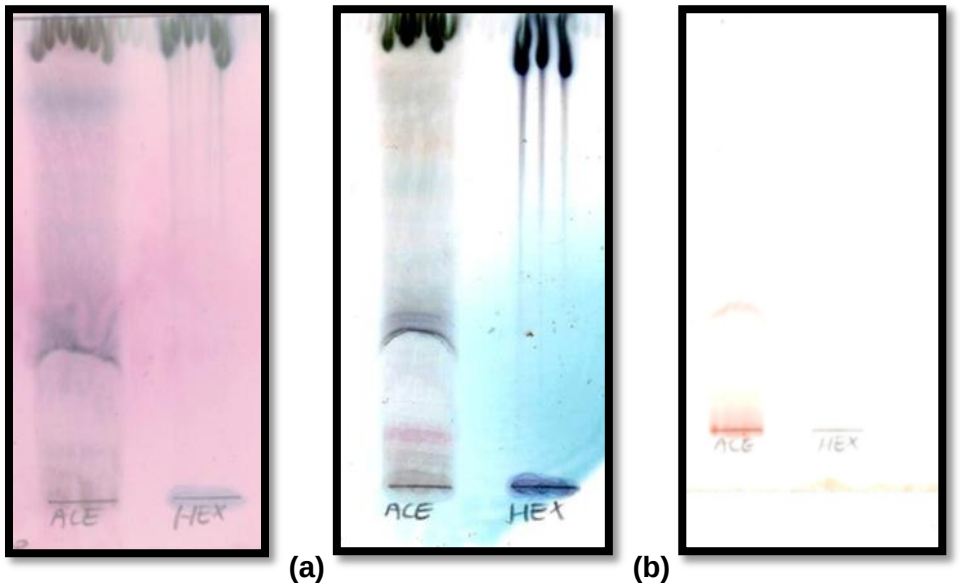


Figure 4.3-11: TLC fingerprinting of two fractions (ace - acetone; hex - hexane) of Iscove's Modified Dulbecco's Medium used *in S. lupi* culture developed in EMW solvent system; sprayed with p-anisaldehyde-sulphuric acid (a), vanillin-sulphuric acid (b) and ninhydrin (c).

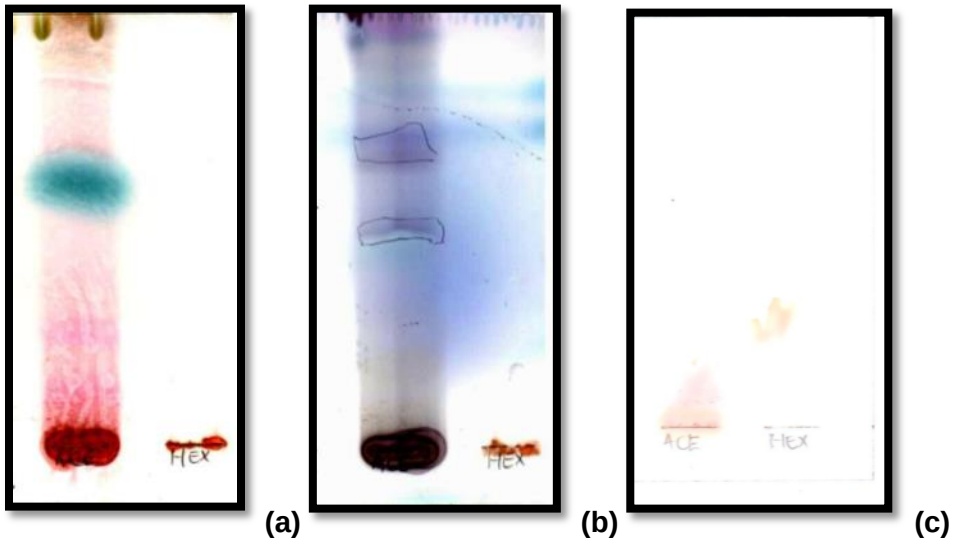


Figure 4.3-12: TLC fingerprinting of two fractions (ace - acetone; hex - hexane) of Iscove's Modified Dulbecco's Medium used in *S. lupi* culture developed in CEF solvent system; sprayed with p-anisaldehyde-sulphuric acid (a), vanillin-sulphuric acid (b) and ninhydrin (c).

4.4 SEPARATION OF EXCRETORY PRODUCTS USING U-HPLC/MS

All culture media (Iscove's, Dulbecco's, Ham's F12, RPMI 1640) and saline used in worm culture were tested for presence of biological substances i.e. proteins using U-HPLC/MS. Positive results were obtained for Iscove's and RPMI 1640 culture media and are provided in Figure 4.4. The identified peaks represent the separated amino acid peptides. Proteins with fragment ions of 795.97 m/z and 879.64 m/z were separated from the Iscove's culture media.

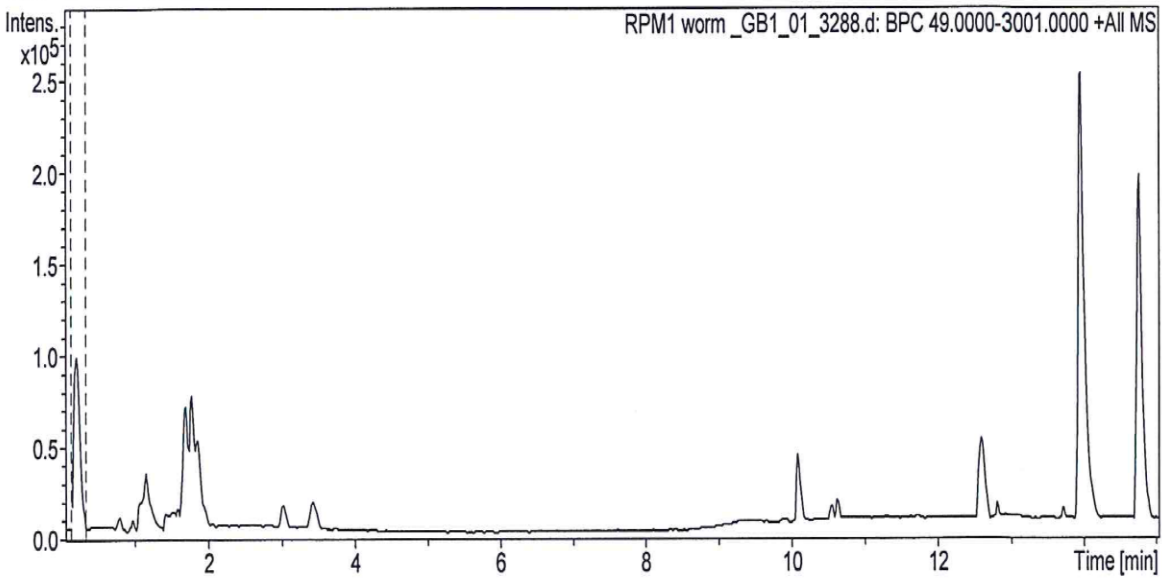


Figure 4.4-13: Chromatogram of RPMI 1640 culture medium used for culturing adult *S. lupi* parasite following U-HPLC/MS. The gradient spectrometry showed separation of numerous peaks.

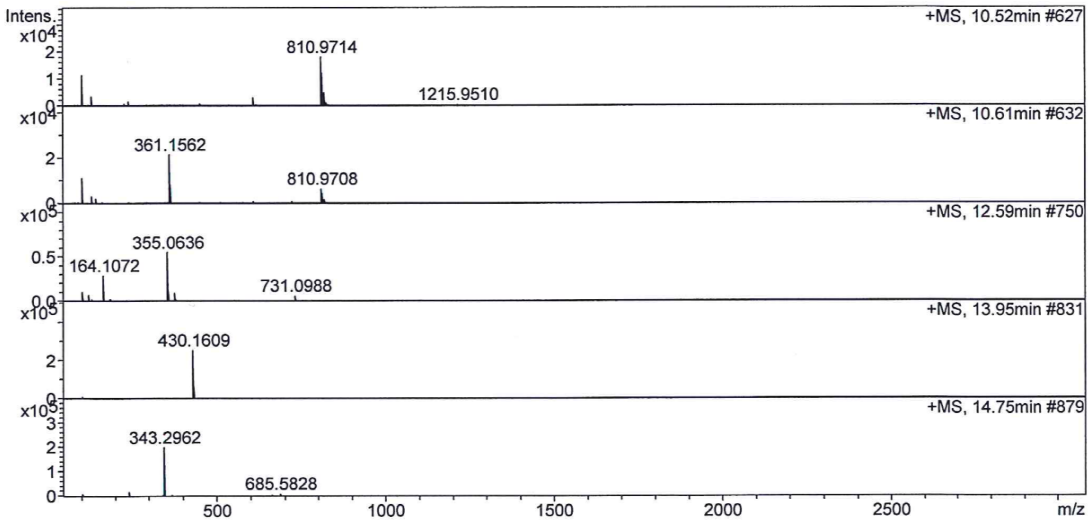


Figure 4.4-14: Mass spectrometry of U-HPLC separated RPMI 1640 culture medium used for culturing adult *S. lupi* parasites. The multiple mass spectrometry shows amino acid peptides with 164.1072 m/z, 343.2962 m/z, 355.0636 m/z, 361.1562 m/z, 430.1609 m/z, 685.5828 m/z, 731.0988 m/z, 810.9708 m/z, 810. 9714 and 1215.9510 m/z molecular ion masses.

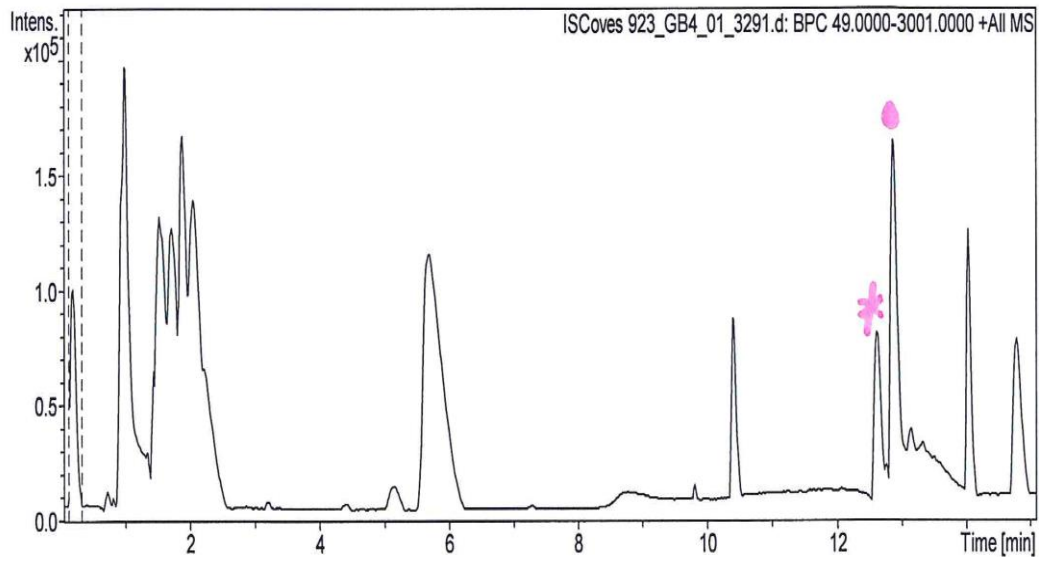


Figure 4.4-15: Chromatogram of Iscove's culture medium used for culturing adult *S. lupi* parasite following U-HPLC/MS. The pink dot and star represents the separated proteins.

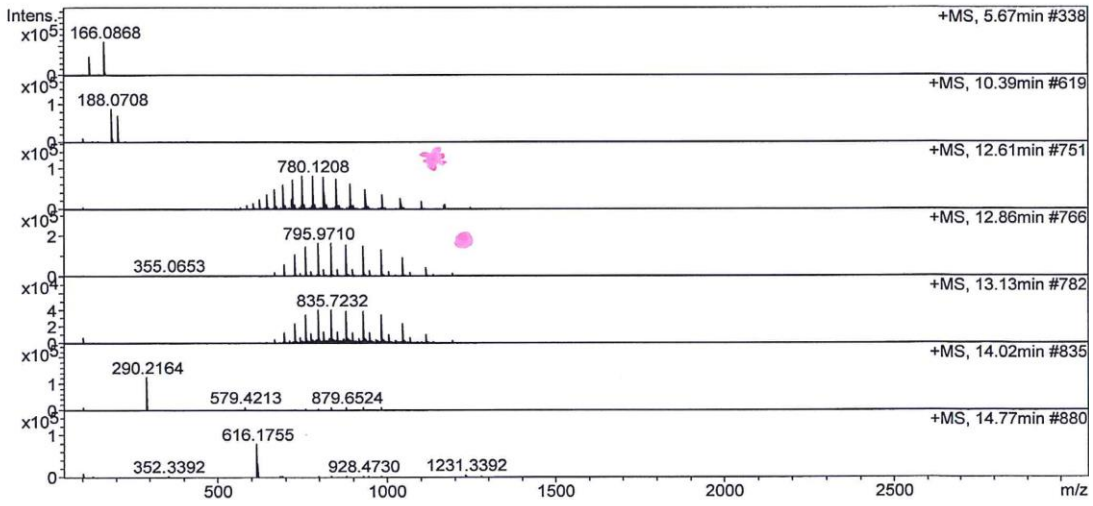


Figure 4.4-16: U-HPLC/MS of Iscove's culture medium used for culturing adult *S. lupi* parasites. Multiple chromatograms show separation of amino acid peptides with 166.0668 m/z, 188.0706 m/z, 290.2164 m/z, 352.3392 m/z, 355.0653 m/z, 579.4213 m/z, 616.1755 m/z, 780.1208 m/z, 795.9710 m/z, 835.7232 m/z, 879.6524 m/z, 928.4730 m/z and 1231.3392 m/z molecular ion masses. The pink dot and star represent the separated proteins.

4.5 VERIFICATION OF LC-MS/MS AND IDENTIFICATION OF PROTEIN(S)

The LC-MS/MS revealed nine amino acid peptides (Fig. 4.5) in adult *S. lupi* parasite ESPs.

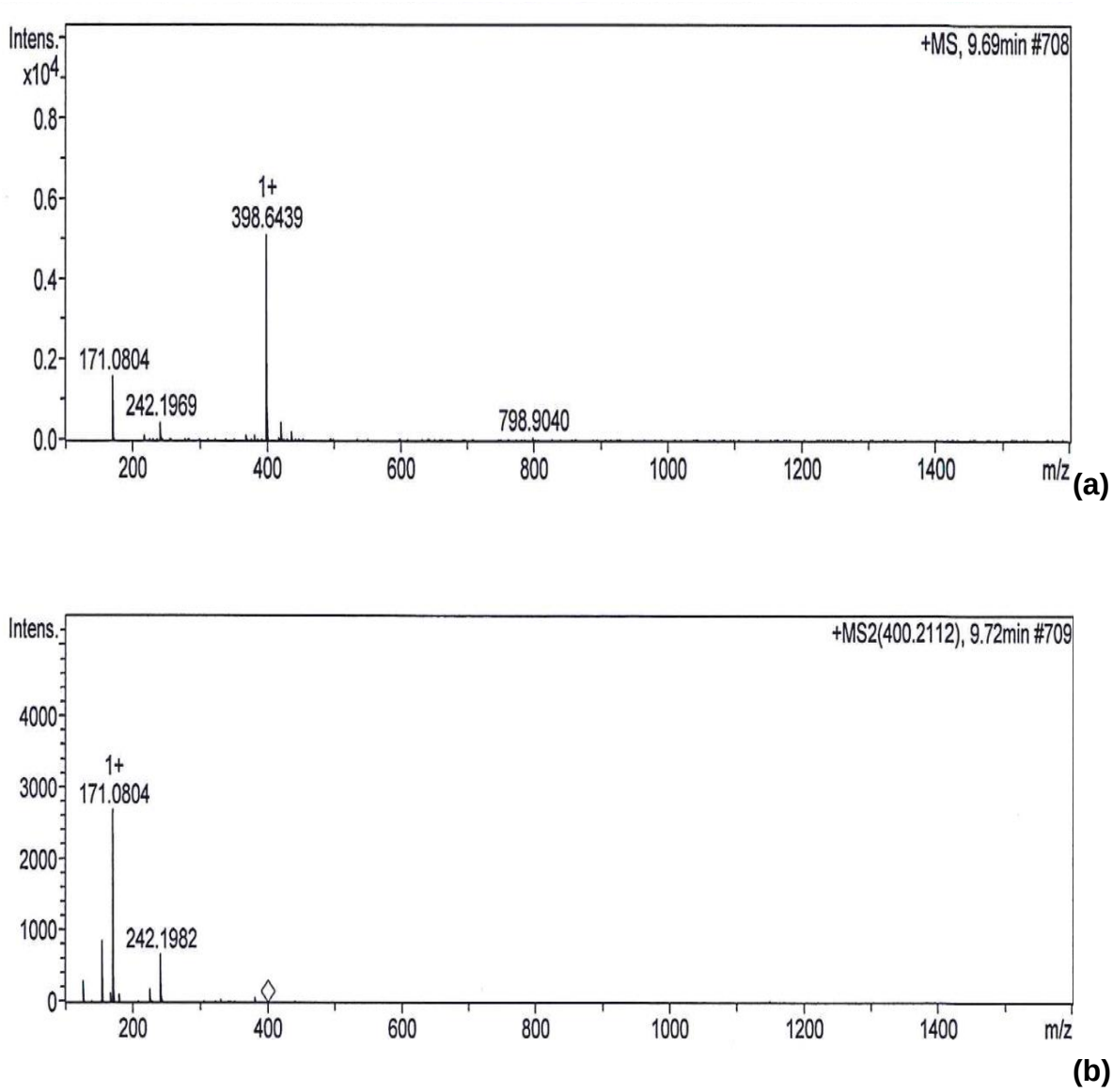
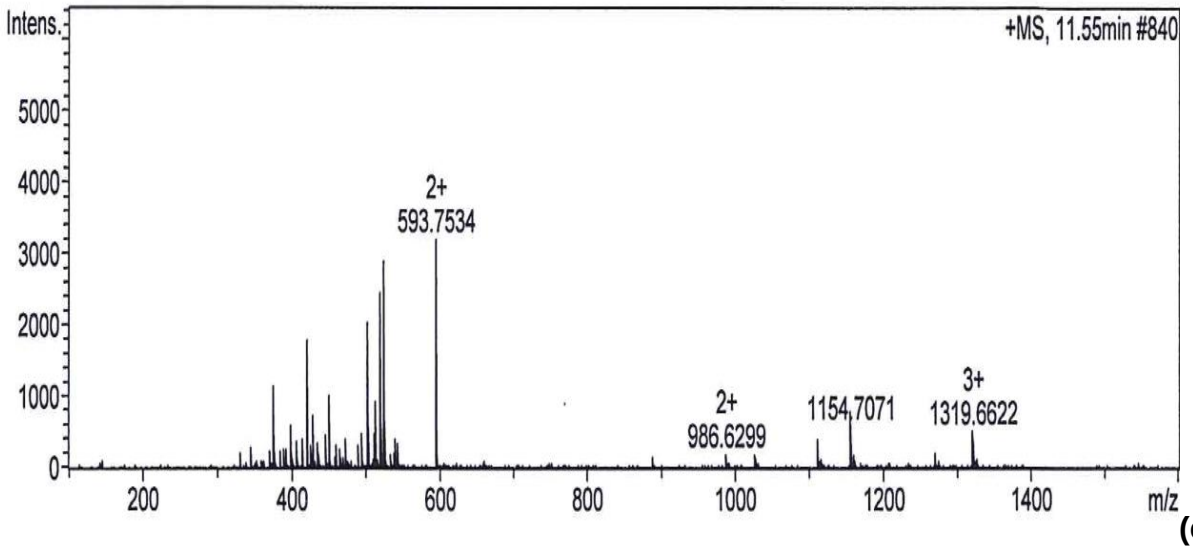
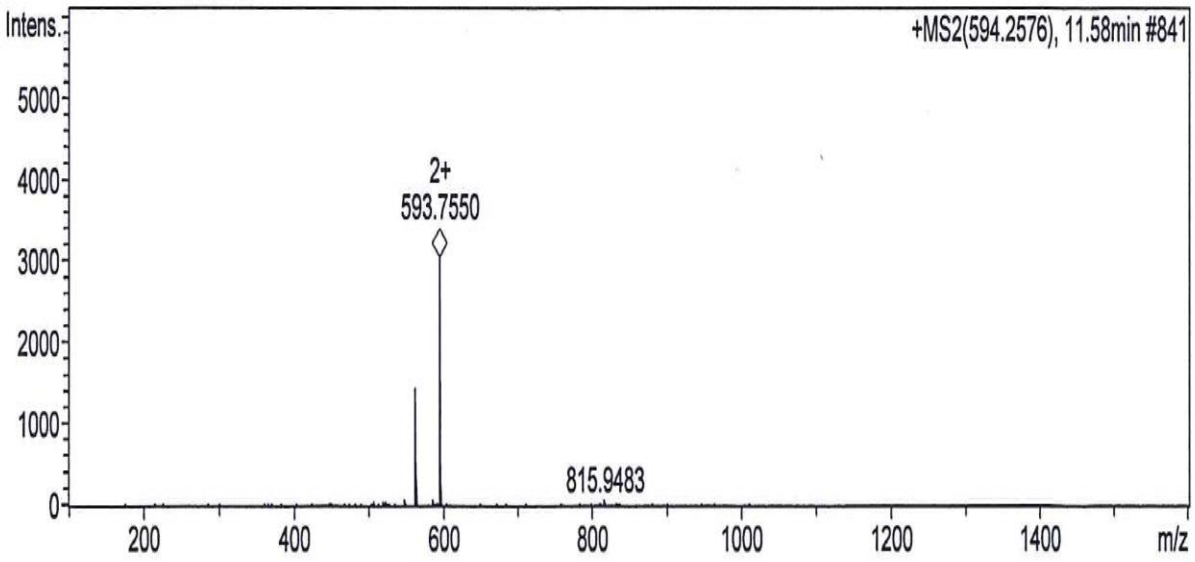


Figure 4.5-17: LC-MS/MS scan (a and b) at time 9.69 min and 9.72 min. The amino acid peptide (400.2112 m/z) was eluted.

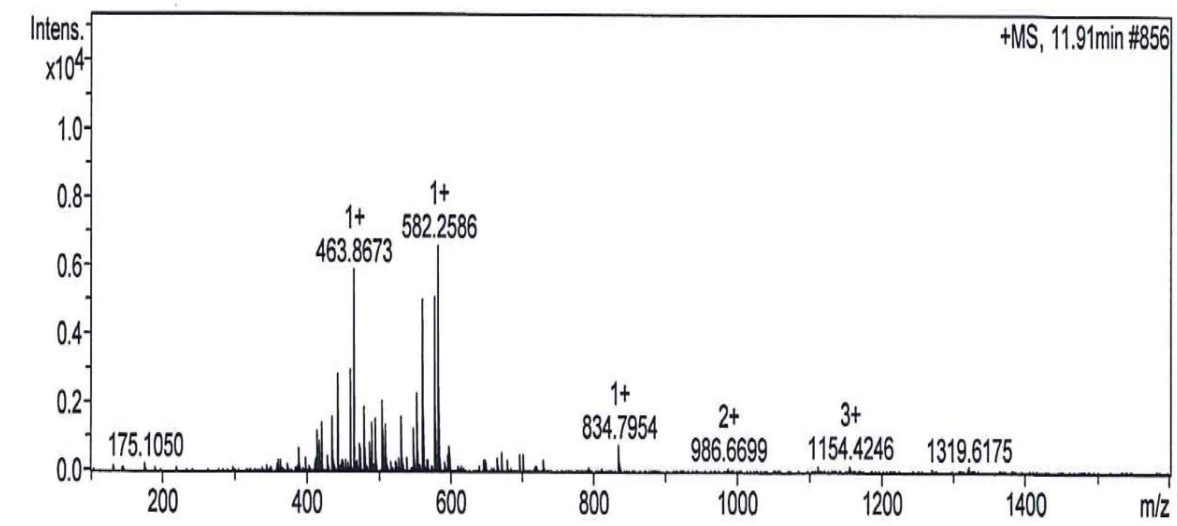


(c)

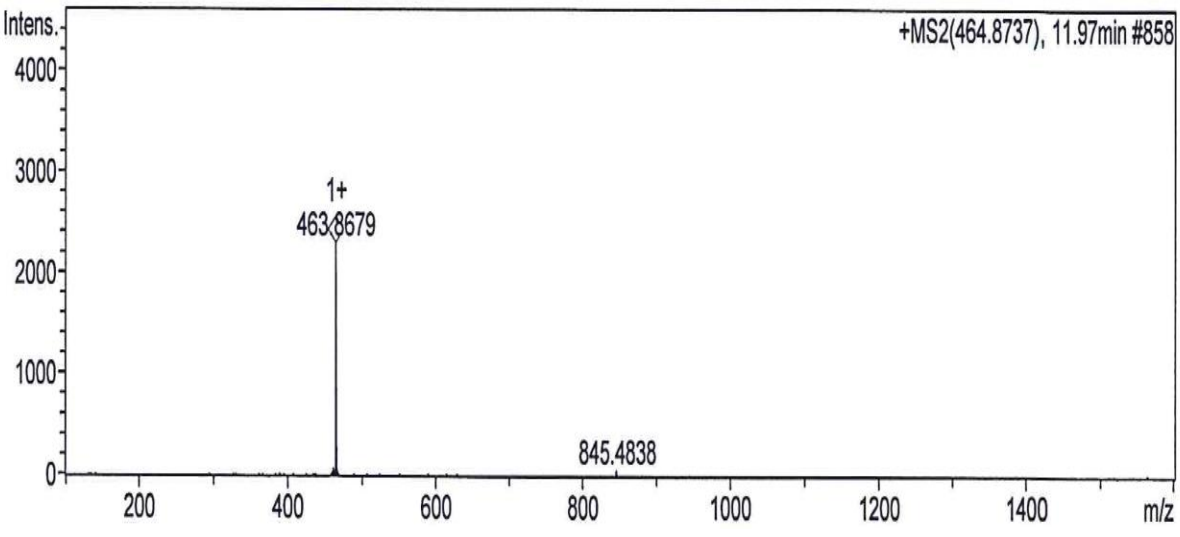


(d)

Figure 4.5-18: LC-MS/MS scan (c and d) at time 11.55 min and 11.58 min. The amino acid peptide (594.2576 m/z) was eluted.

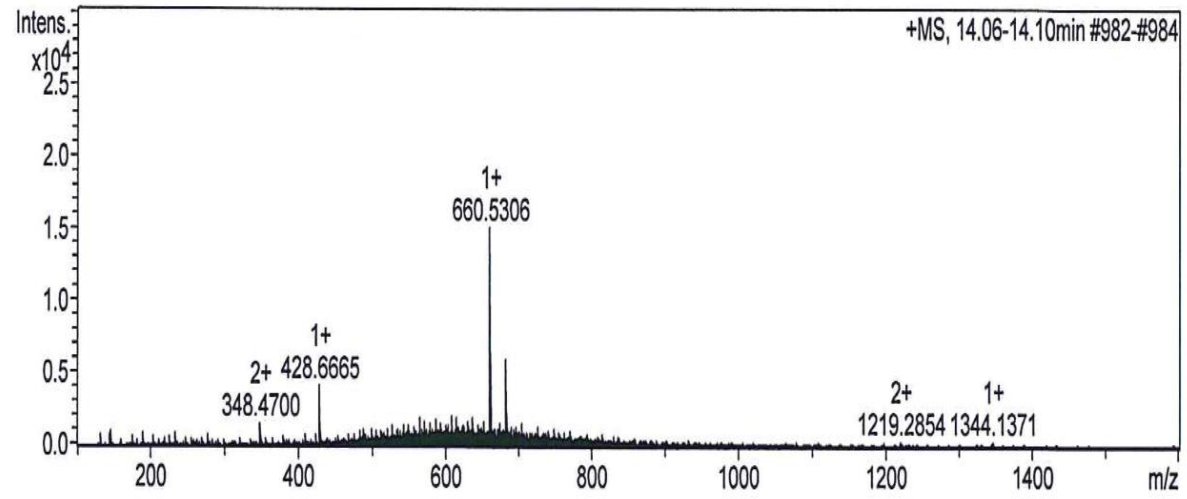


(e)

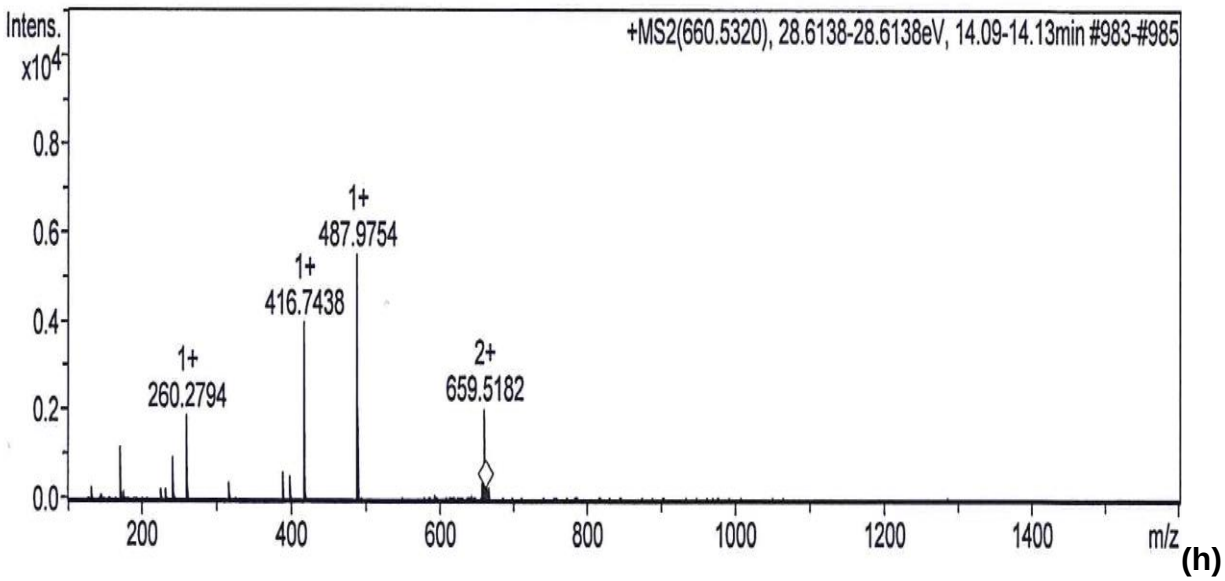


(f)

Figure 4.5-19: LC-MS/MS scan (e and f) at time 11.91 min and 11.97 min. The amino acid peptide (464.8737 m/z) was eluted.

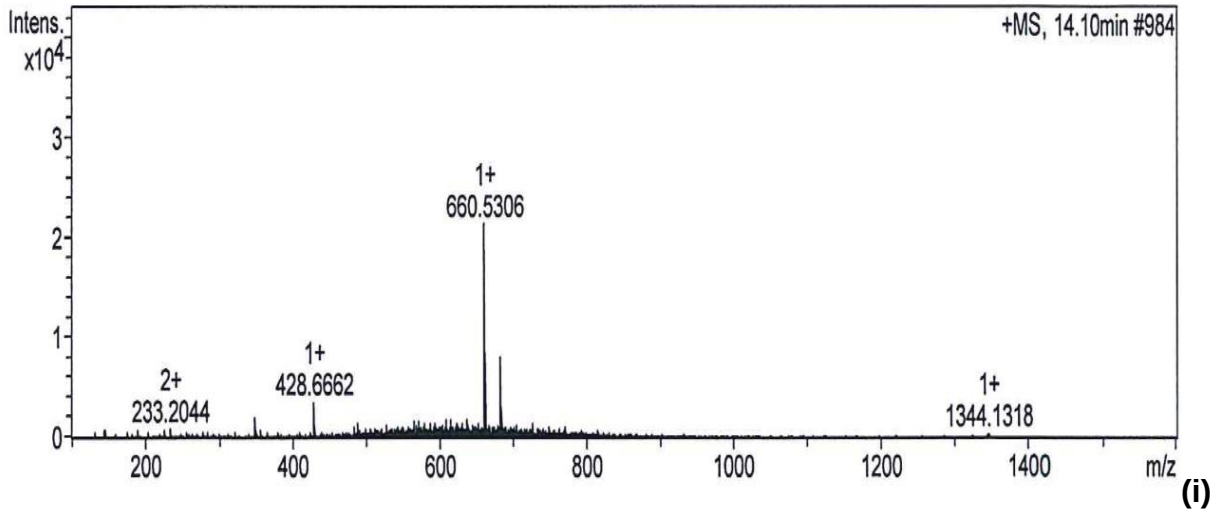


(g)

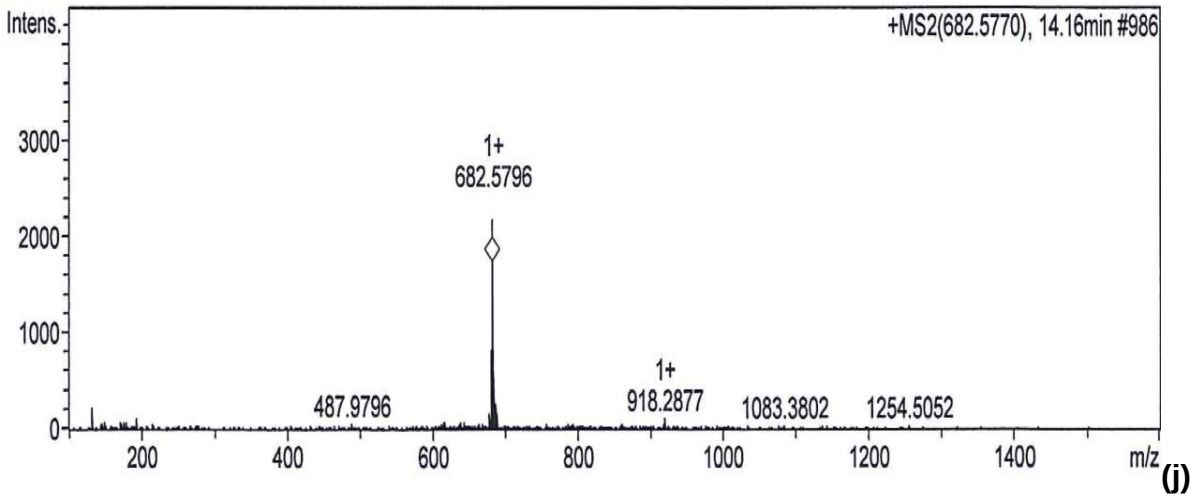


(h)

Figure 4.5-20: LC-MS/MS scan (g and h) at time 14.10 min and 14.13 min. The amino acid peptide (660. 5320 m/z) was eluted.

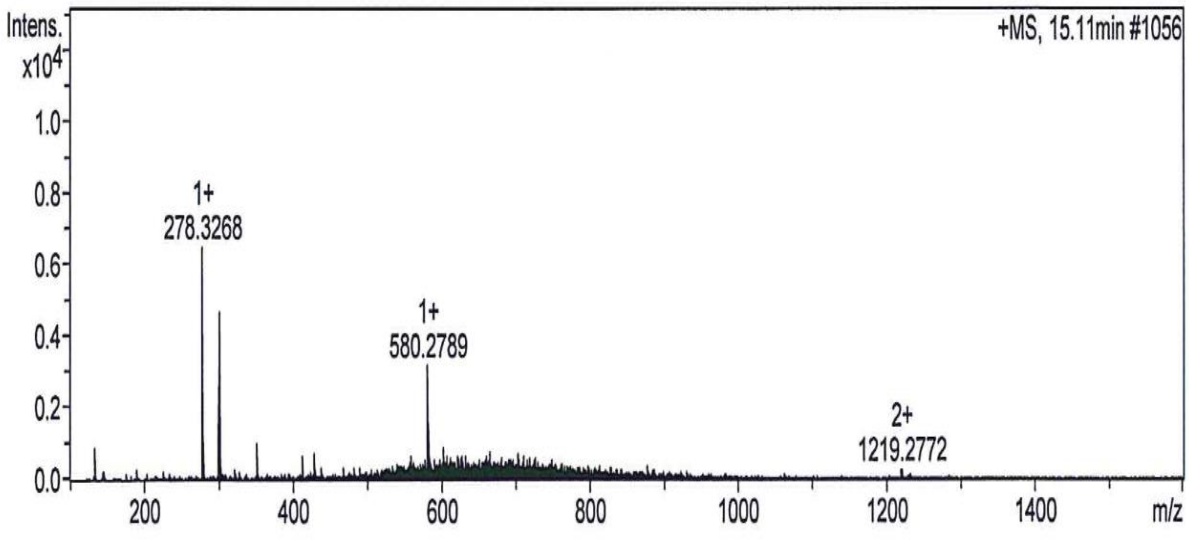


(i)

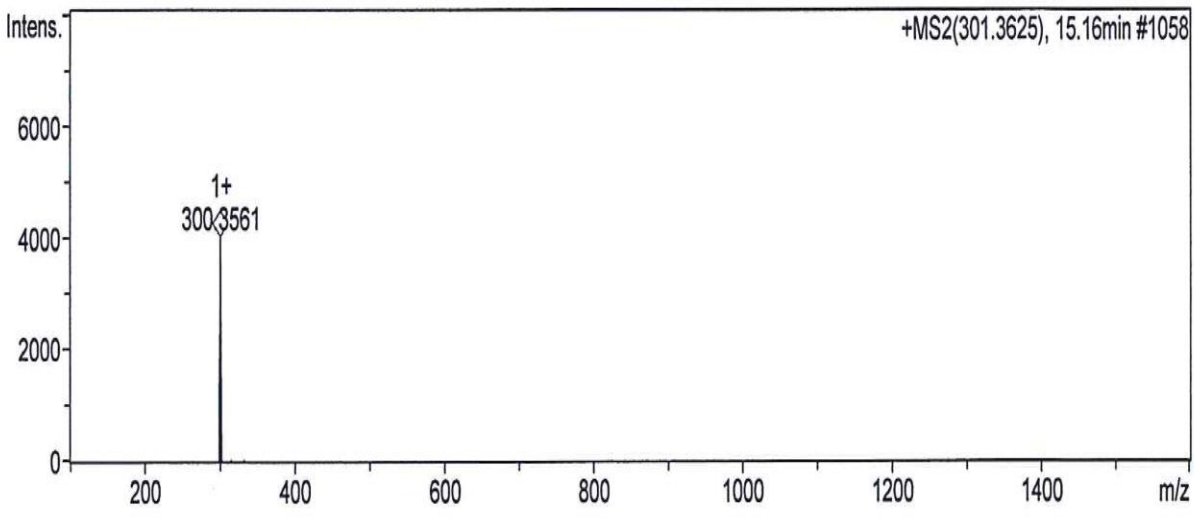


(j)

Figure 4.5-21: LC-MS/MS scan (i and j) at time 14.10 min and 14.16 min. The amino acid peptide (682.5770 m/z) was eluted.

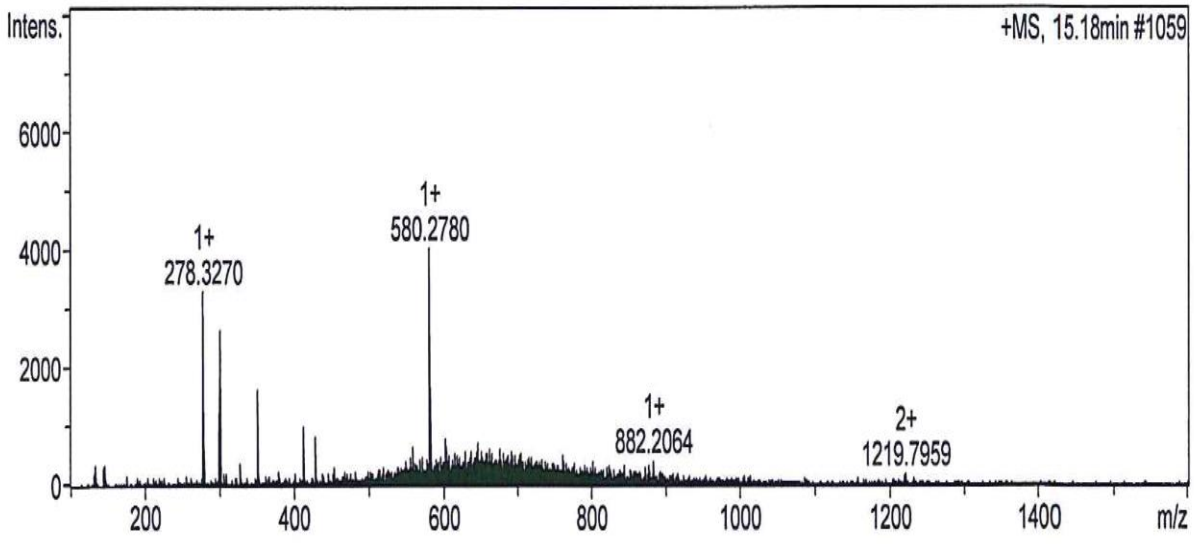


(k)

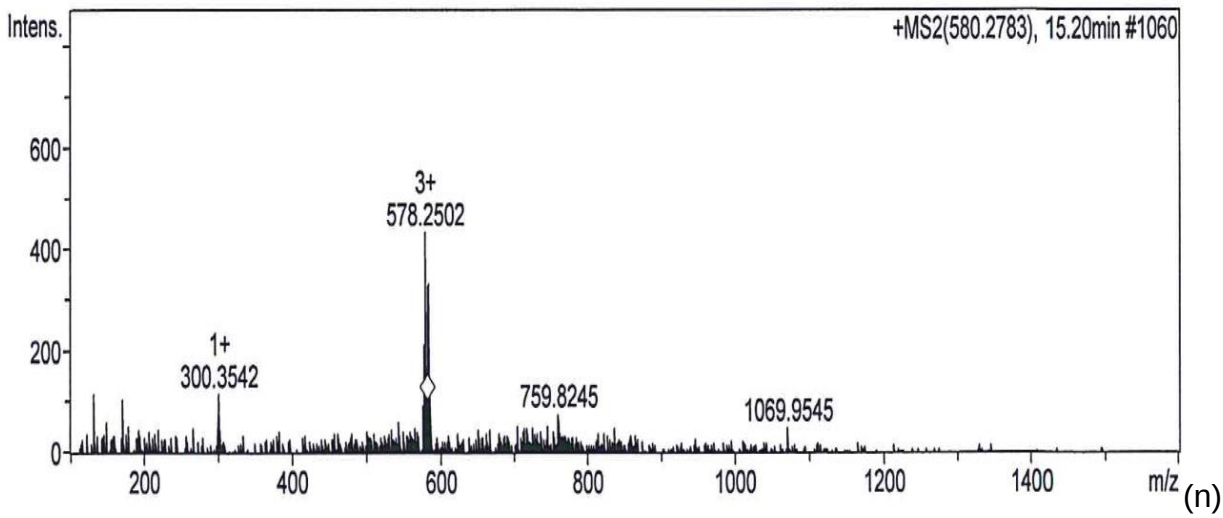


(l)

Figure 4.5-22: LC-MS/MS scan (k and l) at time 15.11 min and 15.16 min. The amino acid peptide (301.3625 m/z) was eluted.

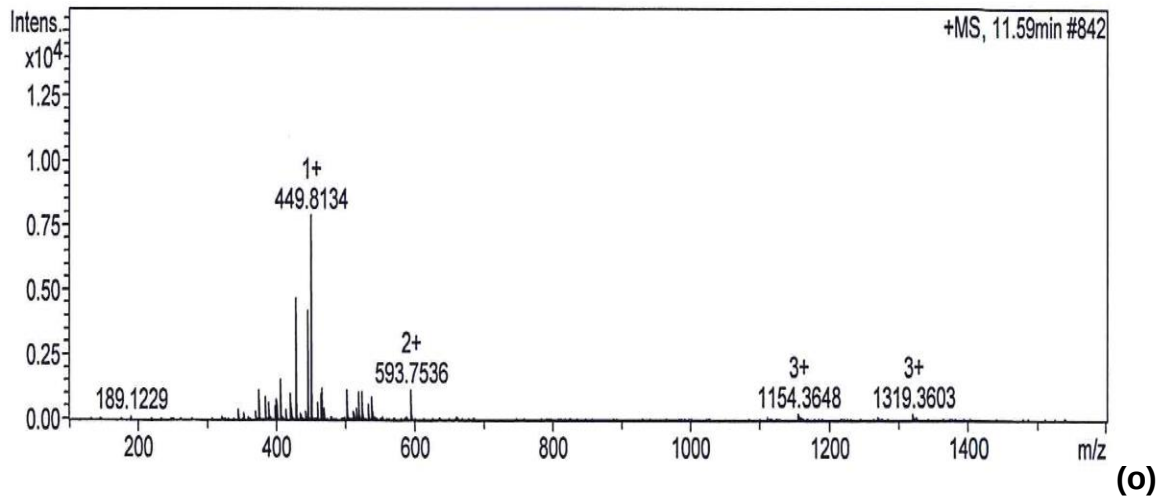


(m)

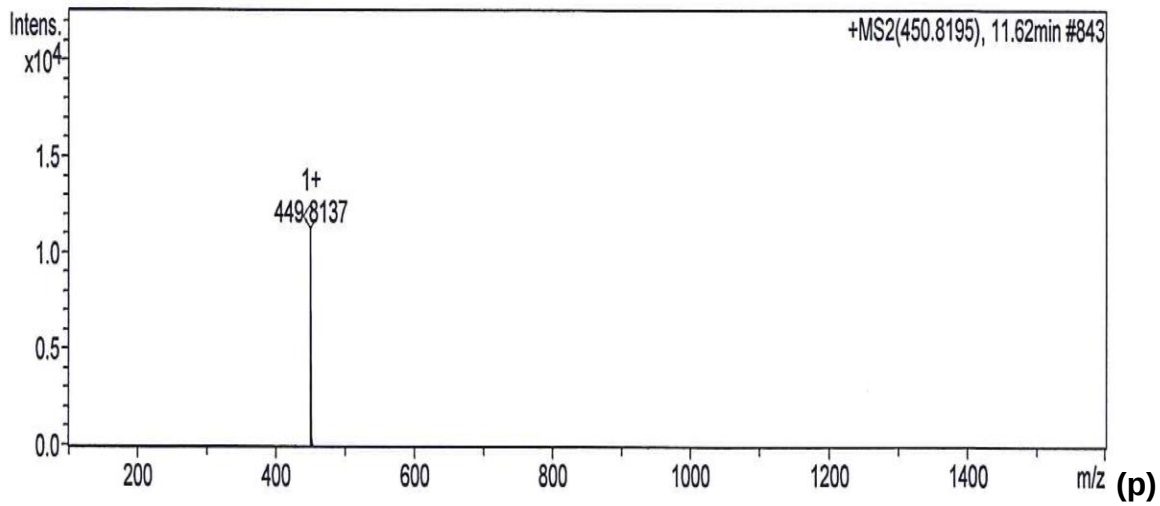


(n)

Figure 4.5-23: LC-MS/MS scan (m and n) at time 15.18 min and 15.20 min. The amino acid peptide (580.2783 m/z) was eluted.

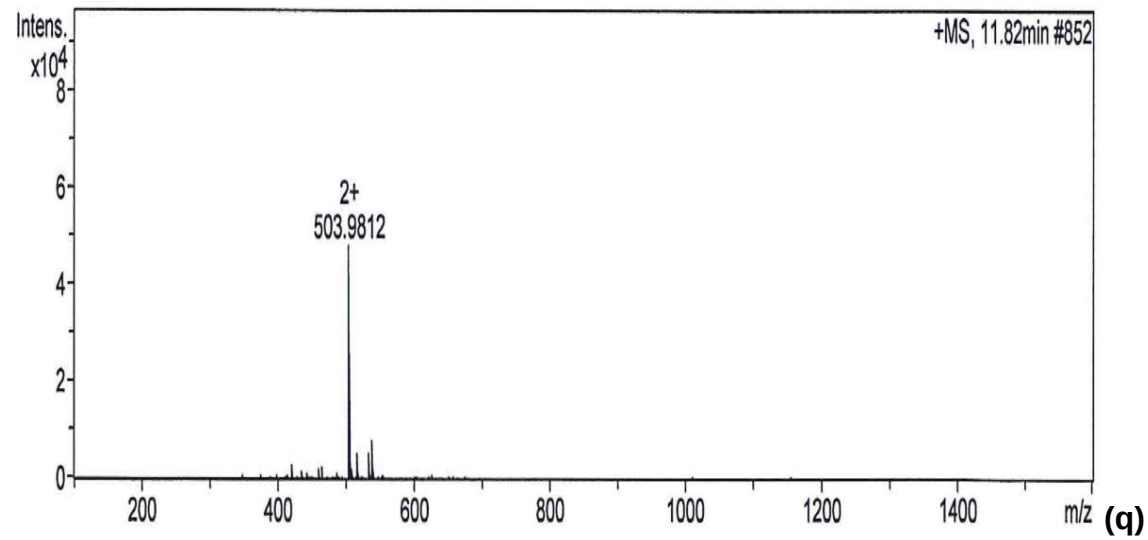


(o)

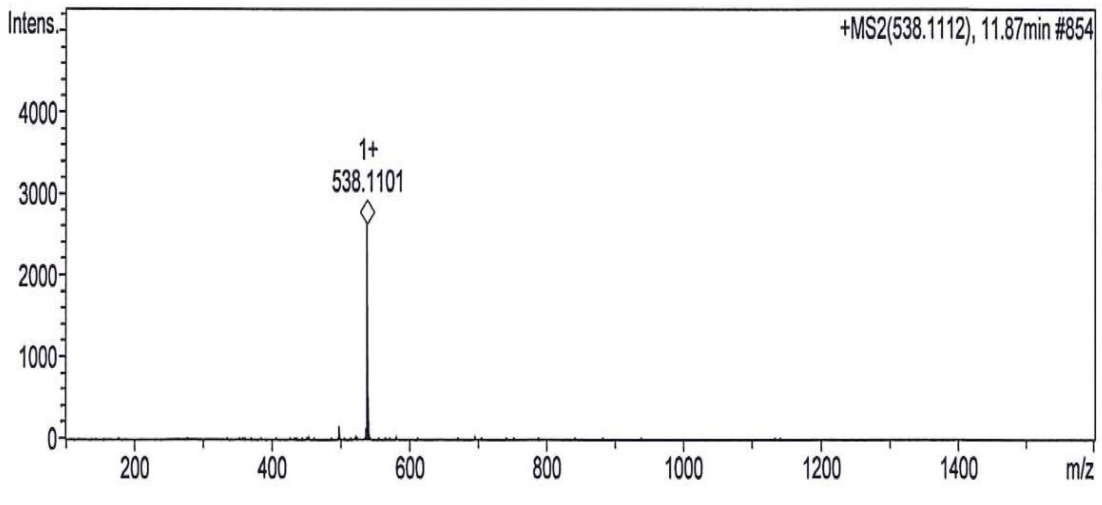


(p)

Figure 4.5-24: LC-MS/MS scan (o and p) at time 11.59 min and 11.62 min. The amino acid peptide (450.8195 m/z) was eluted.



(q)



(r)

Figure 4.5-25: LC-MS/MS scan (q and r) at time 11.82 min and 11.87 min. The amino acid peptide (538.1112 m/z) was eluted.

Despite nine proteins being identified, comparison with the molecular ion masses published from the protein library allowed for the characterization of only three of these proteins (same as the proteins produced by *Nematostella vectensis,* Caebren (*Caenorhabditis brenneri*) and *Sus scrofa)*. While the identification of these proteins was possible, no information was available on their potential carcinogenic effects:

1. *Nematostella vectensis (N. vectensis)* Protein

The adult *S. lupi* culture media and saline tested positive for *N. vectensis* (starlet sea anemone) with v1g241481 the gene. The BLAST analysis of supernatant showed similarities to the identified protein (http://www.ncbi.nlm.nih.gov/protein/156391163) with accession number: XP_001635638. The following is the sequence of the protein:

1	malndqtfsl	nelpiapvga	qpqtfqpsll	dptsmilyns	lgrkhmssds	llsttrlagd
61	qlnmtinqqf	vslkgtmsys	ellqgllyqg	eklgqygtfk	alqfsyltis	dennvtmttr
121	psgvcmltdk	rllflssqas	hsssmqevgd	akklpggysl	naslgdstfy	lpiplrlfrs
181	vemngksgvs	gevmisgkep	acggfcgicg	mlkewsagpv	tisqlnemcv	niglllppwe
241	rrgylhifvt	pdtpnsvvrd	fvallqqhah	glh		

2. Caebren

The protein caebren is produced by *Caenorhabditis brenneri (C. brenneri)* (a small roundworm or nematode) and was identified in supernatant (culture media and saline). The protein showed similarities to BLAST analysis (http://www.ncbi.nlm.nih.gov/protein/341884530) and the accession number: EGT40465. The following is the sequence of the protein:

1	myhmlpyayr	eqpncsrdqh	gefqnlvedl	vnkvsretdl	vdadnpngrk	lfnsmdsffs
61	yglltadray	whciktflpr	aeqkmliaec	gdandrflsi	gwlkasfnkg	tlhfmllamn
121	nevnkrwlpk	hyhvnaclrn	agllekitef	igrlqpvqfa	fystrqmrpv	vvpaavveta
181	tvqissrqaa	rqrkitekee	sqevvpnivp	eeippgiadn	fsqgvvlddl	mrqrnyrmid
241	qsnaqnqagt	sqqnpgpsqp	vieeekledv	mtkkmscvhl	esmdpdgidq	ilasvgpddt
301	nipeikvedg	eyhmsqgdil	nlainifers	sekivecyqv	lenfhtdamq	lrflvvtnfn
361	lyvfkhvqip	ngtaemitss	egmfmplirm	phdriqtfrv	sidnlsfcvv	atekgfpyfv
421	enneqddqsl	fmyvaamagl	esgarvvnti	inavdhsahk	lpsrllvddh	vgymtflqpn
481	lekelrrkfd	vqasllcmhy	eqsememirs	lgtkagylfr	asvgtwmknt	adtqqnycva
541	iggeflmftd	stckiegskr	lklcdttfes	kephfqlkgq	egifefects	amdfkewcki

601	ldmhtknptp	tpyyasclai	ftehsialvq	egerfwsdgf	lrllsqidrr	aisqavivhp
661	pedkesyfkt	rspalclvtr	ddtihyifir	yskeldrvaa	avqsvygvqv	lkfseemlqt
721	svgmainnvv	ctankiwpl				

3. *Sus scrofa*

The bicaudal D-related protein 1 isoform X1 from *Sus scrofa* (wild boar) was identified in culture media and saline. BLAST analysis (http://www.ncbi.nlm.nih.gov/protein/194043060) showed similarities and the accession number: XP_001928078 was recorded in GenBank. The following is sequence of the protein:

1	msafclglag	rtsapaepds	accmelpaaa	pdavgspaaa	aalisfpggp	gdlelaleee
61	lallaagerp	sdpgehpqae	sgpptegael	qpppaqdpel	lsvirqkekd	lvlaarlgka
121	llernqdmsr	qyeqmhkelt	dklehleqek	helrrrfenr	egewegrvse	lesdvkqlqd
181	elerqqvhlr	eadrektrav	qelseqnqrl	ldqlsrasev	erklsmqvha	lredfrekns
241	stnqhiirle	slqaeikmls	drkrelehrl	satleendll	qgtveelqdr	vlilekqghd
301	kdlqlhqsqm	elqevrlsyr	qlqvkveelt	eerslqssaa	tsasllseie	qsmeaeeleq
361	ereqlrlqlw	eaycqvrylc	shlrgndsad	savstdssmd	essetssakd	vpagslrtal
421	selkrliqsi	vdgmeptvtl	lsvemtalke	erdrlrvtse	dkepkeqlqk	airdrdeaia
481	kknavevela	kckmdvmsln	sqlldaiqqk	lnlsqqleaw	qddmhrvidr	qlmdthlkeq
541	srpaaalsra	hgsgrtdeps	taegkrlfsf	frki		

4.6 CULTURING OF FIBROBLASTS

The fibroblasts were plated at different times, depending on the availability of Balb/c mice. The pinnae (n=30) of Balb/c mice were harvested and sliced to allow for cellular dissociation from the extra-cellular matrix. The slicing method was successful as cells were able to dissociate from tissue. The prepared enzyme (collagenase-hyaluronidase - DMEM) kept at -40 °C also allowed cellular dissociation at each period used. Both growth media (DMEM- 40 % FBS (HI)) and culture media (DMEM- 10 % FBS (HI)) were, however, prepared more frequently due to changes in pH. Following plating, in all cases, the fibroblasts were sub-cultured (split) at least twice. In nine days post-culture (including the seven day cell-attachment period) (Figure 4.6-37), fibroblast colonies were observed at varying degrees of confluence (term used as an estimate of the number of adherent cells in TC flasks). The 80-90 % confluence was observed from day 12 to 14 post-culture in 50 ml TC flasks and day 12 to 16 post-culture in 250 ml TC flasks.

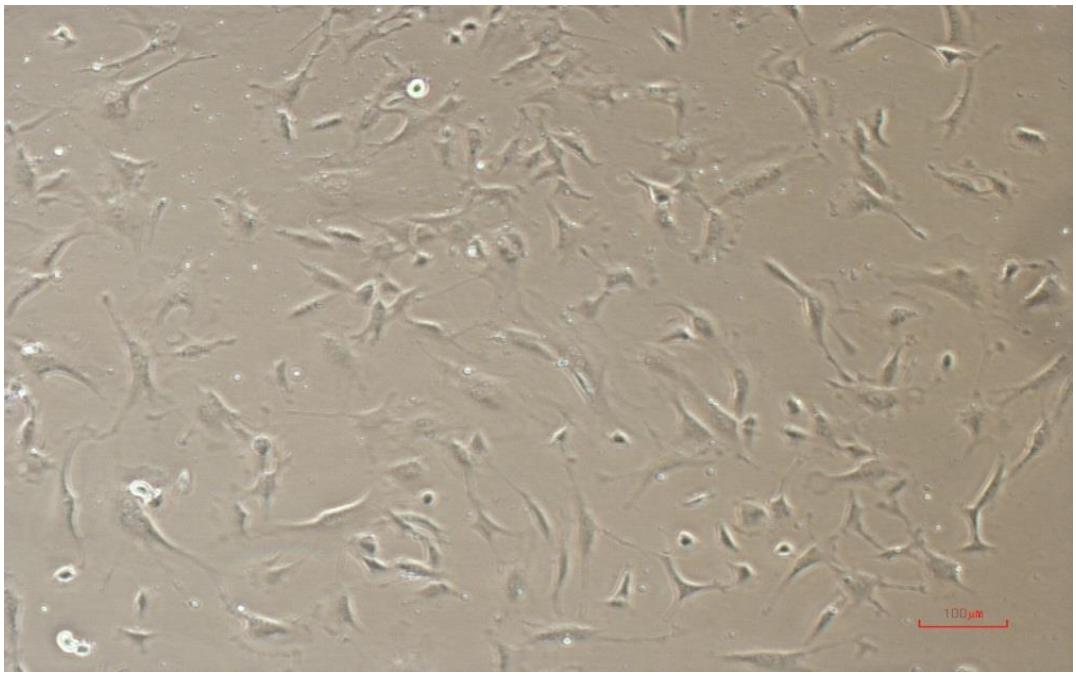


Figure 4.6-26: Photograph (10 x magnification) of fibroblasts harvested from Balb/c mice on day nine post-culture. The slide represents a confluence of 70 %.

4.7 IN-VITRO CARCINOGENICITY ASSAY

The fibroblasts in both the experiment and control groups survived for the entire 48 hr duration of culture with good attachment in the 8-well chambered slides. In comparison to the control groups (fibroblasts treated with organic solvents and the untreated fibroblasts), fibroblasts exposed to ESPs displayed narrowed and stretched shape (Figure 4.7-27).

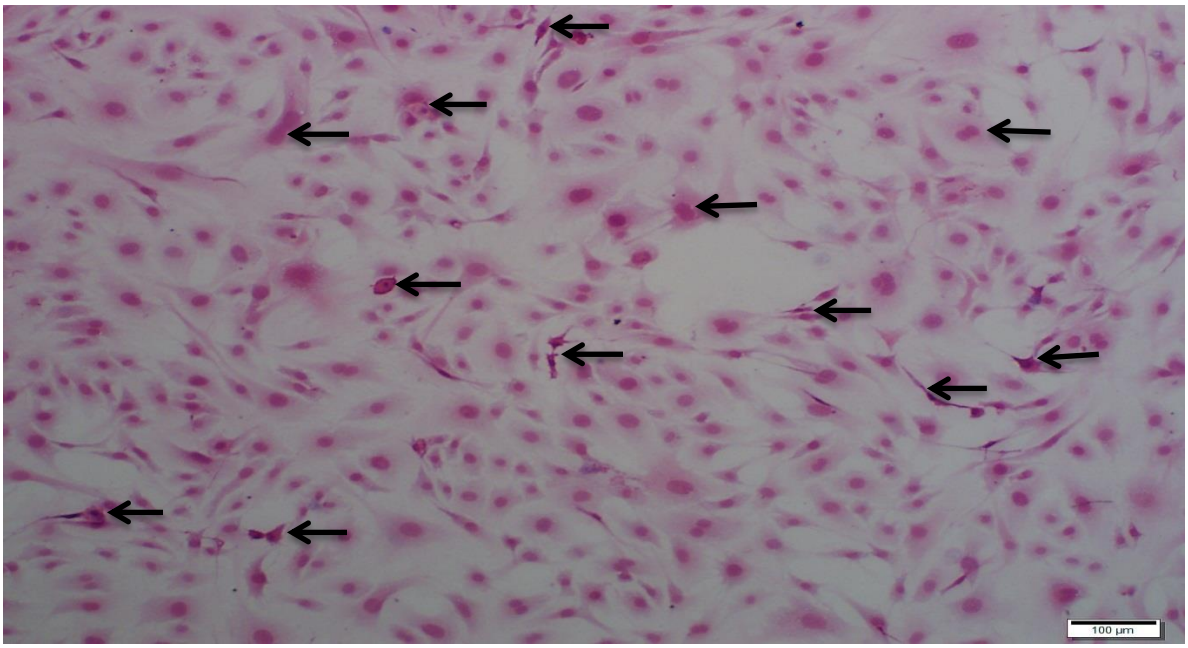


Figure 4.7-27: Photograph of fibroblasts harvested from Balb/c mice, treated with *S. lupi* ESPs (dissolved in methanol). Fibroblasts were stained with haematoxylin and eosin (HE) (10 x magnification). The arrows indicate cells in mitosis.

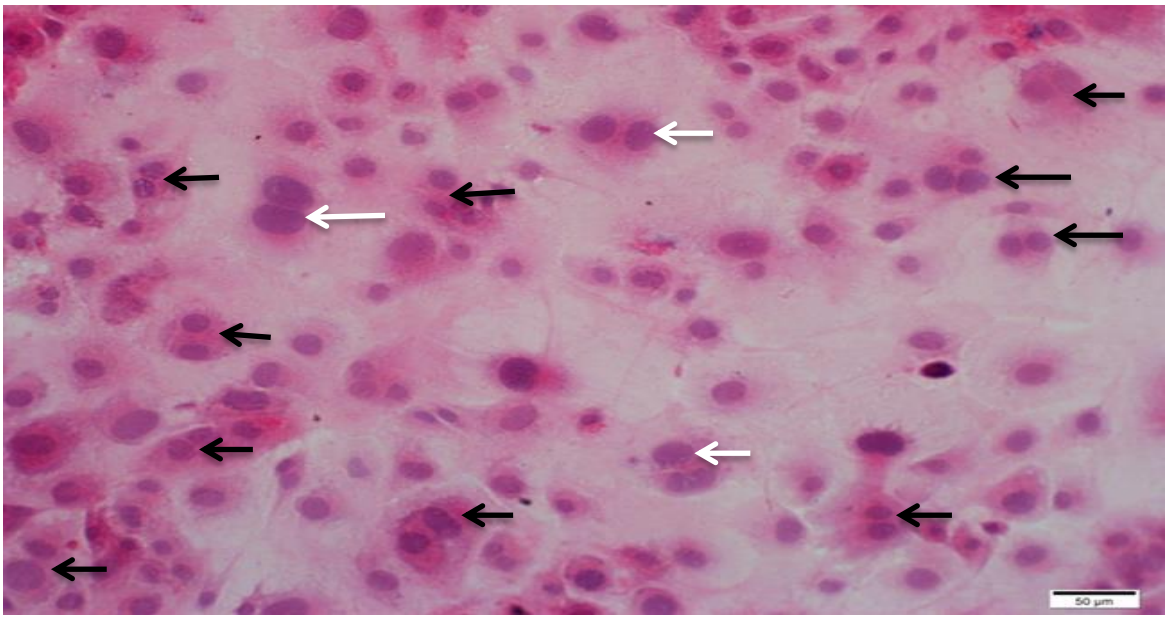


Figure 4.7-28: Photograph of fibroblasts (untreated control) harvested from Balb/c mice, stained with HE. There was evidence for nuclear pleomorphism (bi-nucleates and karyomegaly) (white arrows).

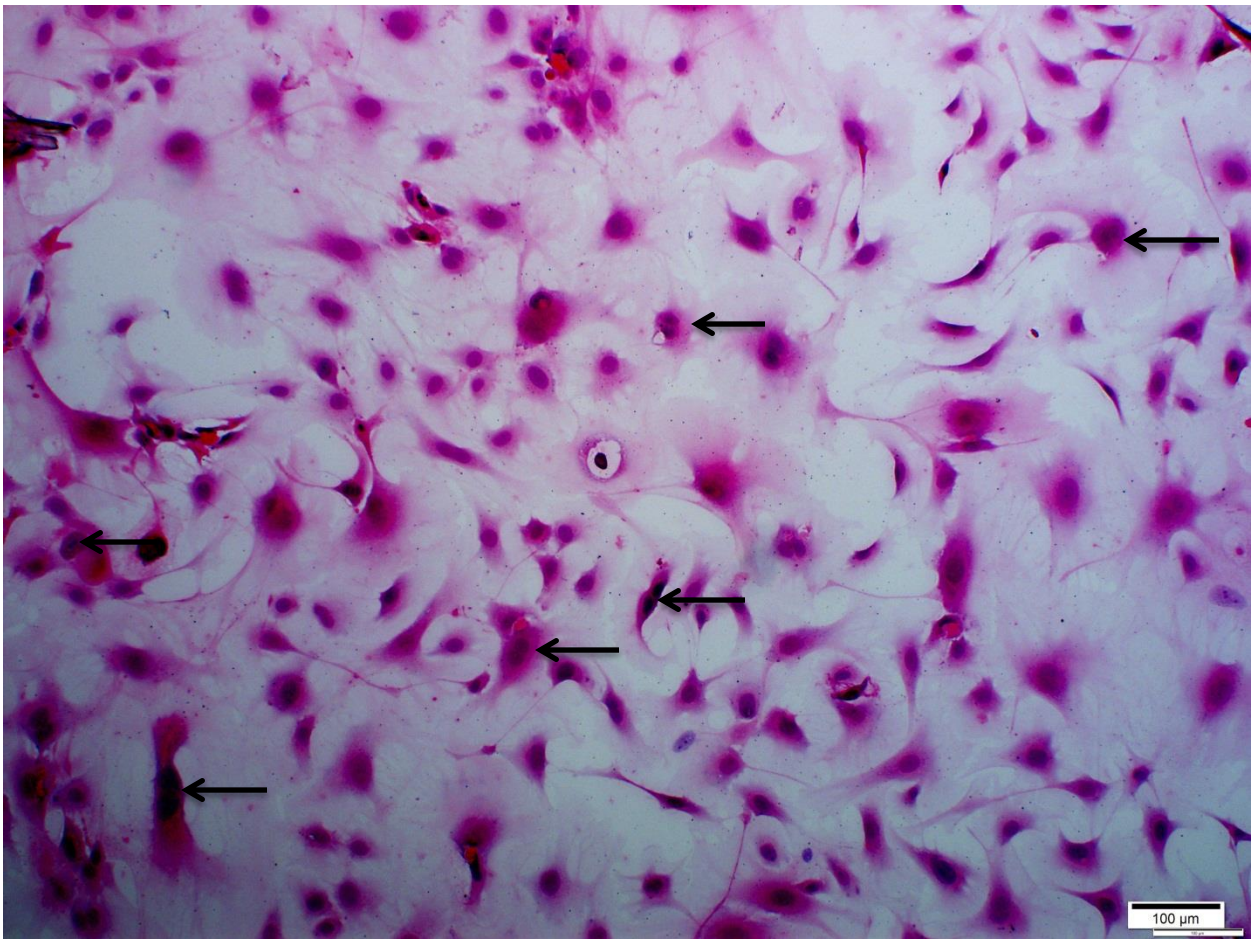


Figure 4.7-29: Photograph of fibroblasts harvested from Balb/c mice, treated with ethylacetate (0.25 µl) and stained with HE. Arrows signifies cells in mitosis.

Following quantification of the mitotic indices (effect) per volume of exposure (10, 20 or 30 µl of extraction solvent) a dose response relationship was present for an increase in mitotic indices (Fig xx to Fig yy). With the exception of the ethylacetate group, the effects of the solvent controls were similar to that of the corresponding volumes of the test extract. For the ethylacetate the effect of the test extract produced a lower effect than the pure solvent controls. As a result, it would appear that the chemicals present within the *S. lupi* environment, as seen on TLC, are not directly responsible for carcinogenic effect of the worm *in vivo*.

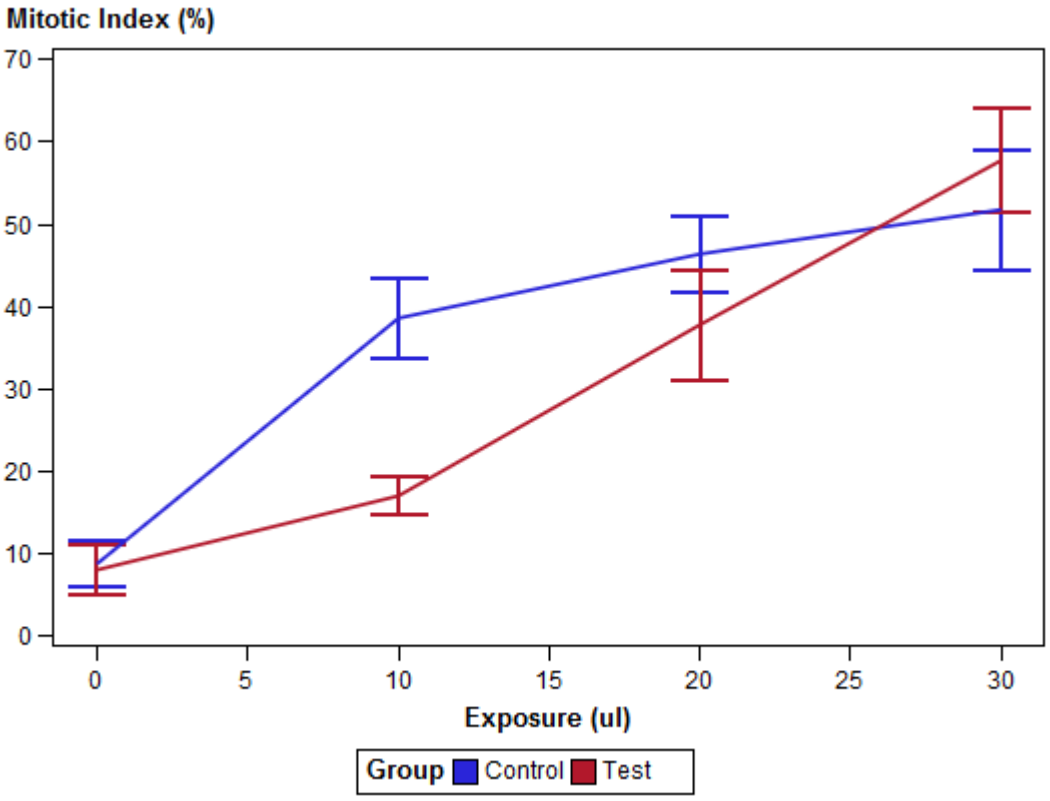


Figure 4.7-30: The effect of adult *S. lupi* extracts (dissolved in acetone) compared to the effect of the pure acetone solvent (control) on murine fibroblasts, when exposed to 40 µl of the acetone mixture for both test and control groups, to yield a final exposure volume of 10, 20 and 30 µl.

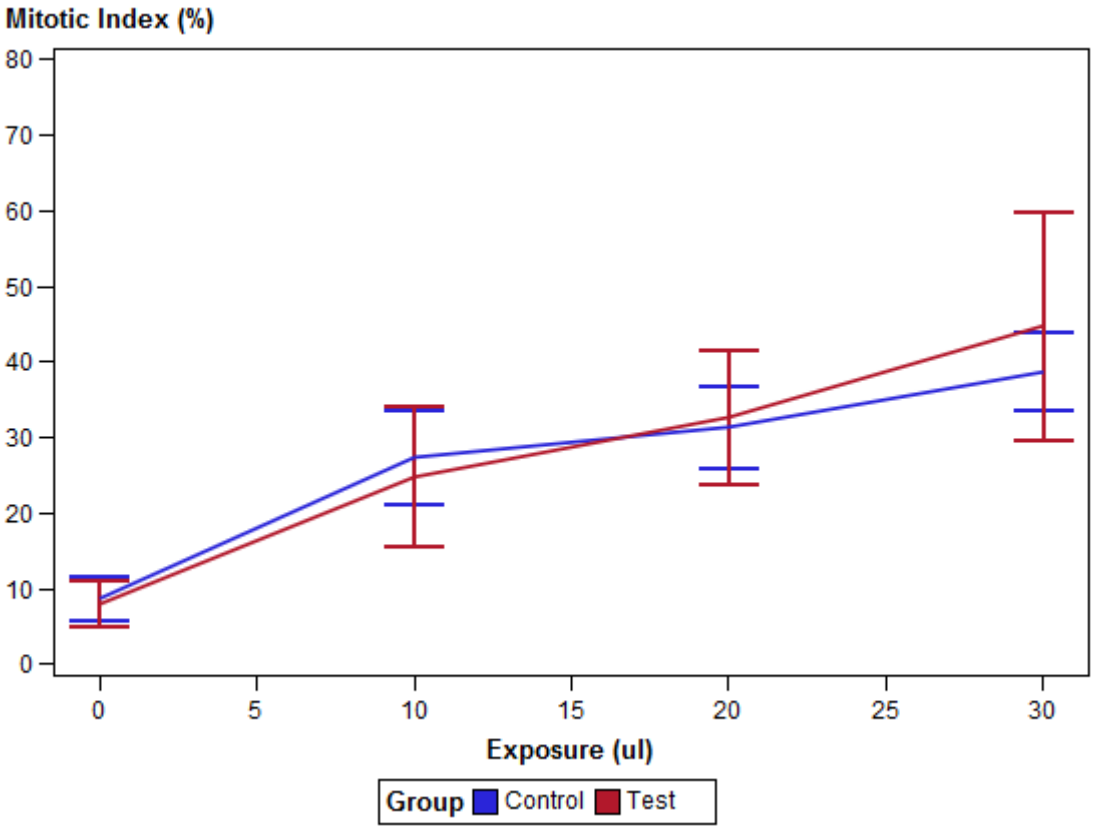


Figure 4.7-31: The effect of adult *S. lupi* extracts (dissolved in hexane) compared to the effect of the pure hexane solvent (control) on murine fibroblasts, when exposed to 40 µl of the mixture of hexane for both test and control groups, to yield a final exposure volume of 10, 20 and 30 µl.

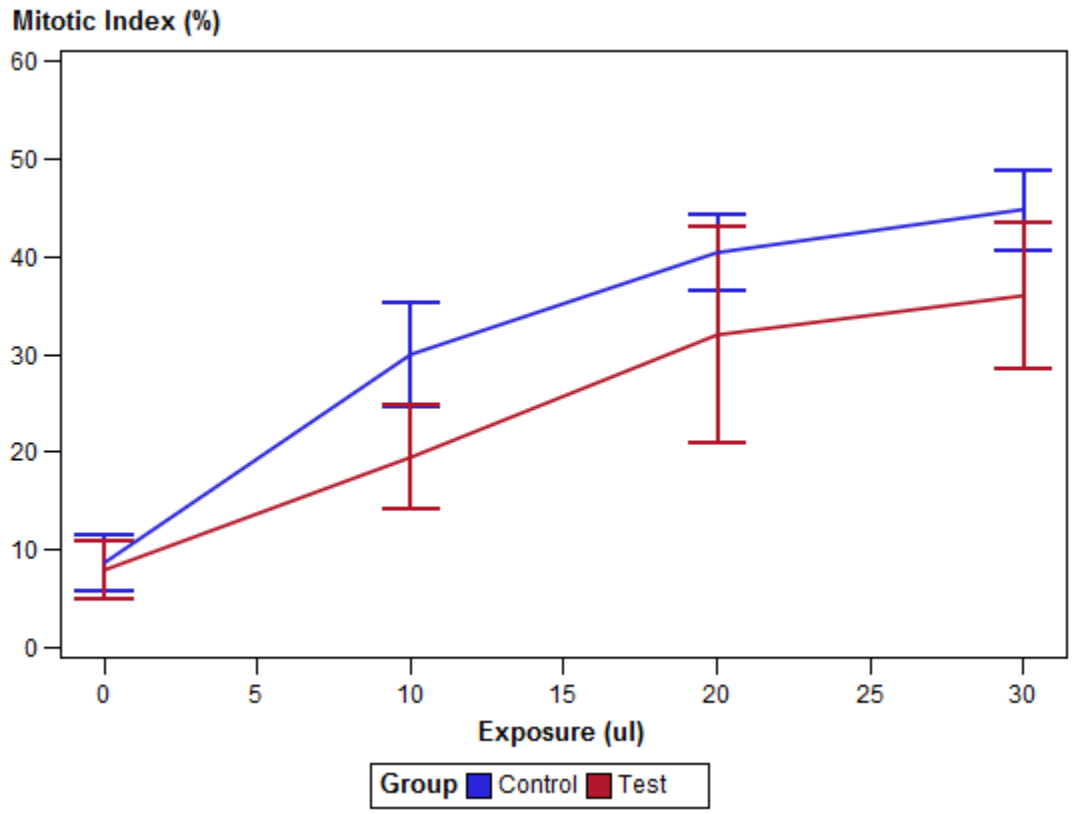


Figure 4.7-32: The effect of adult *S. lupi* extracts (dissolved in methanol) compared to the effect of the pure methanol solvent (control) on murine fibroblasts, when exposed to 40 µl of the mixture of hexane for both test and control groups, to yield a final exposure volume of 10, 20 and 30 µl.

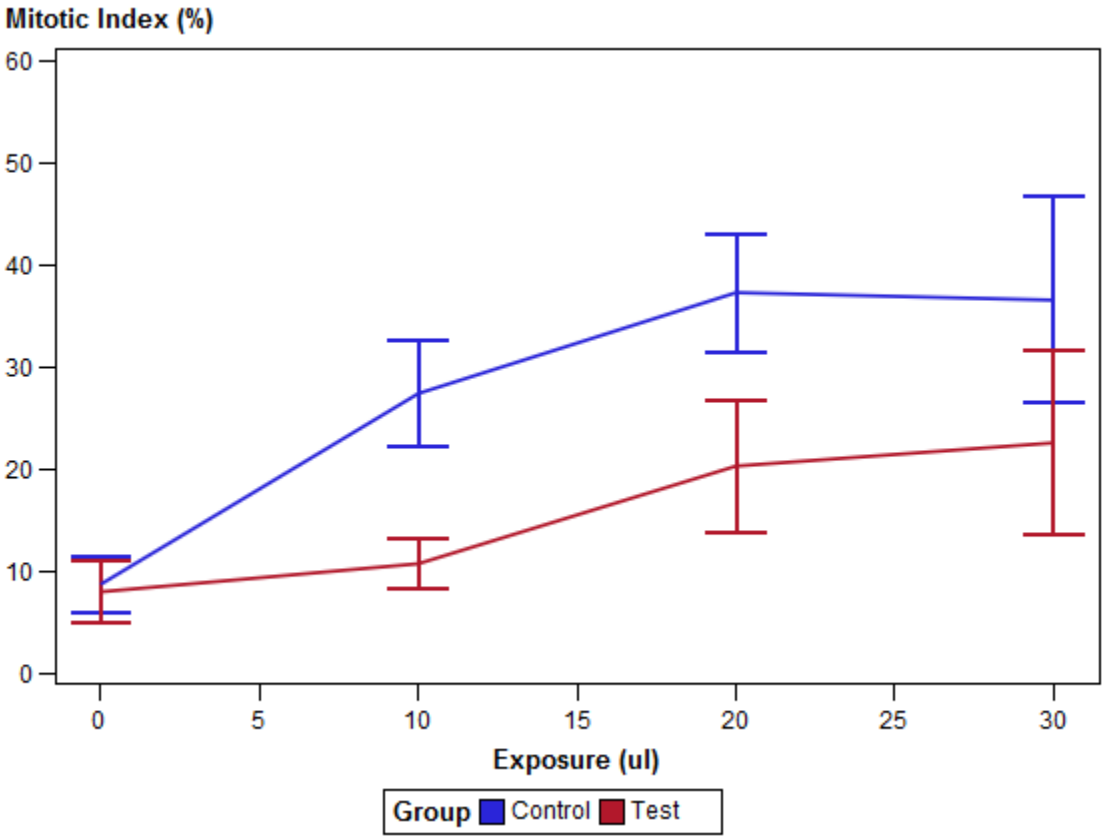


Figure 4.7-33: The effect of adult *S. lupi* extracts (dissolved in ethylacetate) compared to the effect of the pure ethylacetate solvent (control) on murine fibroblasts, when exposed to 40 µl of the mixture of hexane for both test and control groups, to yield a final exposure volume of 10, 20 and 30 µl.

5 DISCUSSION

5.1 WORM CULTURE

This study is the first report that we're aware-off, describing attempts to culture and maintain isolated intact adult *S. lupi* worms *in-vitro* for further study. The worms in question were collected opportunistically from recently euthanized dogs through dissection of their oesophageal nodules and the physical removal of worms, without the necessity of collagenase digestion. Following nodule dissection, the harvested worms were deemed viable according to our established criteria of being pink in colour, having an intact oesophagus and showing some motility. It would therefore appear that the physical harvesting of the worms had no adverse effect on parasite viability.

A qualitative difference in viability was present between the worms, as worms sourced from the dog received from the Sinoville Animal Clinic (Table 4.2-2) exhibited no motility (they were curled-up) *in-vitro* in comparison to the worms obtained from the OVAH, possibly due to the delay to time of nodule dissection in the case of the former and nodules being kept in the fridge prior to dissection. While the worms in question were not motile, they were not dead as they were pink, and had an intact oesophagus and they also resisted physical stretching. The curled-up behaviour of worms *in-vitro* was not a completely unexpected finding as this behaviour is also noticed when the worms are dissected out of oesophageal nodules (Reinecke, 1983). The reason why these worms failed to recover after a period of time is not known at this stage. Preliminary indications are that it may be best to harvest the parasite immediately post-mortem for *in-vitro* culture.

Following harvest, the worms were placed in one of following media, Ham's F12 medium, Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640 medium and / or Iscove's modification of DMEM for five to seven days, in the absence of foetal calf serum (FCS). These media were selected from prior reports of their use *in-vitro* culture of other parasites such as *Onchocerca spp. and Schistosoma spp.,* and these media

are also the most commonly used media in mammalian cell culture. The media differed from each other in their amino acid and glucose content and in their buffering systems. For example, DMEM contained only sodium pyruvate and no buffer, RPMI 1640 medium contained only sodium bicarbonate as the buffer, Iscove's modification of DMEM contained lower concentrations of bicarbonate as HEPES was included as the buffer and Ham's F12 medium contained no buffer. In terms of their nutrient contents, the media contained different amino acids to support the growth of different cell types. With current speculation tending to suggest that *S. lupi* survives off nutrients within their environment in oesophageal nodules and due to the fact that the selected cell media best represent the nutrients within tissue fluid, it was speculated that these media should meet the nutritional requirements of the parasite. Nonetheless, the parasite failed to thrive within the selected media. The worms progressively became weaker and more opaque until they died (opaque or whitish, non-motile and discolouration of the oesophagus). We suspect that the worms survived until such time as they depleted their nutrient glycogen stores. This would indicate that the worms failed to feed off the selected media. This conclusion is based on literature on *H. contortus,* whereby it was demonstrated that the colour of the parasite was due to the presence of glycogen stores (Beugnet et al., 1996). As the *Haemonchus* wormed starved, they became progressively more pale due to the depletion of their energy stores.

Further proof of the inability of the parasite to feed can be seen in their colour change from pink to transparent. The red colour of the adult *S. lupi* cuticle has been linked to the presence of haemoglobin in the perienteric fluid of the parasite (Lee and Smith, 1965). Under normal physiological conditions, haemoglobin forms bonds with oxygen and either transfers oxygen to tissues or preserves it. At a higher (alkaline) pH (7.0), de-oxygenation occurs in tissues and this result in immobility of parasites (Lee and Smith, 1965). It is suggested that the adult *S. lupi* parasite cultured *in-vitro* could not continue its normal metabolism, probably at least in part, due to the abnormal pH, leading to death of the parasite.

While we are unaware of the reason for the poor survival of the parasites in media, one possible explanation could be the intentional omission of foetal calf serum in the growth media. Foetal calf serum (FCS) is commonly added to mammalian cell cultures as a source of albumin and growth factors. Similarly, for *Schistosoma spp.,* it has been shown that the parasite needs glucose and albumin for glycogen production. The literature has also shown that supplementation of cultures with 10 % FCS is vital for the prolonged viability of *O. gutturosa* adult parasites cultured *in-vitro* using RPMI 1640 medium, Iscove's Modified Dulbecco's Medium and Minimum Essential Medium for 39 days (Townson et al., 1986). The FCS may provide parasites with necessary growth factors, e.g. platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and insulin-like growth factor (IGF), to name a few (Thuwajit et al., 2006). For this study, we omitted the FCS to see if the parasite could survive in its absence. However, based on the results achieved we strongly suggest that further attempts to culture *S. lupi* should include FCS in the media.

Despite the FCS being a plausible explanation for the poor survivability of the parasite, the absence of sufficient nutrients may not be the only reason underlying parasite death, as it does not explain why parasites survived best within the saline control. With the absence of nutrients in the saline control, other factors must be involved in parasite survival. The major difference between the saline and culture media was the pH. The saline had a pH of 6 while the culture media had pH's closer to 7.4. It is therefore plausible that pH is important for the survival of the parasite. This is not completely unsurprising as the parasite is routinely found alive in detritus and purulent material in nodules, an environment that which is known to be low in pH due to the presence of para-amino benzoic acid (PABA), a neutrophil break-down product (Dvir et al., 2010). As a result, the low pH may be an indication of parasite environmental modification.

An interesting finding was the change in colour following the placement of worms into culture media. The phenol red pH indicator changed from pink to yellow. In some cases, this colour change was also associated with a yellow precipitate at the bottom of the wells and a pungent odour. The colour change and strong odour in culture media are

similar to that observed following *in-vitro* culture of the nodular worms, *Oesophagostomum radiatum (O. radiatum)*, which was associated with the secretion of volatile fatty acids by the worms (Douvres, 1983). For this study, however, we believe that the changes were due to the culture media being contaminated by *S. lupi* excrement. This was confirmed (through culture) by the presence of *E. coli, E. faecalis, P. aeruginosa* and *S. marcescens*, all known faecal bacteria. We were, however, unable to culture the *Comamonas spp.* recently reported to be associated with *S. lupi* worms (Gottlieb et al., 2012). It is also worth noting that the ability of *S. lupi* to contaminate a sterile environment may be linked to its close association with secondary bacterial infections *in vivo*.

5.2 ANALYSIS OF THE WORM EXTRACT

Following the survival of the parasites *in-vitro*, we attempted to ascertain whether the worms were releasing substances into the media by excretion or secretion. For this part of the study, the Iscove's culture medium was selected randomly for further analysis by extraction and subjected to TLC separation using different elution solvents and different developing agents. Following elution, we were able to demonstrate that the culture media in which worms were found differed in contents to that of the pure culture media. This finding therefore confirms previous suspicions that the worms release chemical substances into their surrounding environment.

In terms of the content of the ESPs in the Iscove's culture medium, the acetone extract yielded the greatest number of bands, thereby indicating that the substances therein were of intermediate solubility. This was not a surprise finding as the worms were in a water-rich environment. Using the non-specific p-anisaldehyde or vanillin visualisation reagents, a maximum of seven bands were visible for the former and six for the latter, both eluted with the BEA solvent system. Due to the non-specific nature of the visualisation agents, these compounds are not yet identifiable. The TLC plates were also exposed to ninhydrin, a specific stain for amino acids, to ascertain whether proteins

were contained therein. One protein was found to be present in the acetone extract. As for the other solvents, said protein is not yet identifiable.

As the first step in further analysis of the protein content, un-extracted culture media was also submitted for additional analysis with the commercial analytical company Mintek. The samples were subsequently extracted and analysed by LC-MS/MS for the presence of proteins. In contrast to the TLC analysis, nine proteins were identified; three were characterized and were similar to those identified in other invertebrate organisms. The three proteins have thus far not been subjected to any further testing for the purpose of this study, nor could any published studies on the function or significance of these proteins be found. As the next step in evaluation, the effect of these proteins either in media or in isolation should be investigated especially in terms of their carcinogenic potential.

5.3 *IN-VITRO* CARCINOGENICITY ASSAY

Following the exposure of fibroblasts to adult *S. lupi* ESPs (at various dilutions, 10, 20 and 30 µl), *in-vitro* fibroblasts were investigated for an increase in mitotic rate, which would be indicative of clastogenesis. An increase in fibroblast proliferation was evident in both the adult *S. lupi* ESPs extracts and in the organic solvent groups, with a clear concentration-response relationship. Based on the similar results, it is evident that *S. lupi* ESPs had no additional clastogenic effect over and above that of the solvents alone. As a result, it would appear that the clastogenic effect of the parasite may not be due to the presence of a specific chemical secreted by the parasite. However, this does not preclude the effect being due to one of the identified proteins, which would have precipitated on extraction. These proteins still need to be evaluated.

6 Conclusion

The aim of this study was to culture adult *S. lupi* parasites *in-vitro* using serum-free media to harvest the ESPs, for carcinogenic studies. While we were unable to keep the parasite alive for longer than a few days, we were able to demonstrate that the extracted ESPs did not induce carcinogenic effects in cultured fibroblasts. We did, however, confirm that the ESPs are proteinaceous, which requires further study. As a result, we cannot as yet reject the hypothesis.

7 FUTURE STUDIES

- In an attempt to keep the parasite alive for longer periods, we propose that further nutrients be included in the culture media. These could include matrigel, a basement membrane matrix gel and/or FCS.
- Further clastogenic assays need to be undertaken with the identified protein(s), to determine their role in the carcinogenicity of the parasite *in-vivo*.

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