An evaluation of the biopesticidal characteristics of *Helichrysum marifolium* and *Helichrysum patulum* and their effects on animal metabolism

By

Abigail Speelman [B.Sc. (Hons.)]

A thesis in partial fulfillment of the requirements for the degree of Magister Scientiae in the Department of Medical Biosciences, Faculty of Natural Sciences, at the University of the Western Cape.

Supervisor: Prof. Quinton Johnson
Department of Medical Biosciences

Co-supervisor: Mr. Frans Weitz
Department Biodiversity and Conservation Biology

CONTENTS

Dedication

To my parents, for all their support, encouragement, love and interest throughout my study career.

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Keywords

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Antifungal

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UV/Visible Spectrophotometry

Animal metabolism, haematology and tissue histology

Statement

I declare that "An evaluation of the biopesticidal characteristics of *Helichrysum marifolium* and *Helichrysum patulum* and their effects on animal metabolism" is my own work, only submitted to the University of the Western Cape, with all research resources used in this project duly acknowledged by means of complete references.

Abigail Speelman
December 2002

Chapter 1: Overview and rationale for study

Pesticides and the environment

There are a number of pesticides that are suspected to be endocrine disrupters. Many of these pesticides are man-made. Endocrine disrupters are chemicals that can lead to an increase in birth defects, sexual abnormalities, and reproductive failure. Marine animals seem to be the most vulnerable to the effects of pesticides since the ocean is the final sink for many pollutants. Endocrine disrupters can exert their effects in many ways. They can either bind to the hormone's receptor and mimic the hormone, or block the action of the hormone. An experiment done of newborn female rats injected with 1mg DDT/day on days 2-4 after birth had early onset of puberty and accelerated loss of fertility. In another experiment, vinclozolin (also a pesticide) at dosage levels of 3mg/kg/day resulted in feminised male rats in the womb and an abnormal number of nipples were observed (11). Pesticides can be absorbed through the skin, by inhalation or oral ingestion. There are, however, important quantitative differences between the various derivatives. The skin poorly absorbs DDT in solution, whereas dieldrin absorption by the skin is very efficient. The major effects of insecticides in humans are qualitatively similar and cause effects on the central nervous system (9).

Endocrine disrupters encompass more than just environmental estrogens and include any agent that adversely affects any aspect of the entire endocrine system. Endocrine disrupters are usually either natural products or synthetic chemicals that mimic, enhance or inhibit the action natural hormones (5). Low levels of many endocrine disrupting chemicals can lead to high levels in body tissues of animals and humans. This is due to the fact that many endocrine disrupters are stored in fatty tissues through biomagnifications (2).

The greatest exposure to endocrine-disrupting chemicals is from food intake and since many are fat-soluble, the highest levels occur in the higher trophic levels, particularly meat, fish, and dairy products. The groups of organisms for which there is substantial evidence of endocrine disruption include snails, oysters, fish, alligators, and other reptiles, and birds such as seagulls and eagles. Many endocrine disrupting chemicals have become widespread contaminants across the globe. This is not due to their vast usage on a worldwide scale, but because some of the pesticides can be transported for thousands of kilometres on air currents (2).

Chronic administration of some of the insecticides causing effects of humans, on laboratory animals over long periods causes enhanced tumorigenesis. The chlorinated hydrocarbon insecticides, such as DDT, are considered "persistent" chemicals. Degradation is quite slow when compared to other insecticides, and bioaccumulation, particularly in aquatic ecosystems, is well documented. Because of their environmental impact, use of the chlorinated hydrocarbon insecticides has been largely curtailed in North America and Europe. Some of them are still used, however, in tropical countries. Organophosphorus insecticides, for example Diazinon, Malathion, and Trichlorfon, are useful pesticides when in direct contact with the insects or when used as "plant systemics" where the agent is translocated within the plant and exerts its effects on insects that feed on the plant. Some of these agents are used in human and veterinary medicine as local or systemic antiparasitics or in circumstances in which prolonged inhibition of cholinesterase is indicated. The compounds are absorbed by the skin as well as by the respiratory and gastrointestinal tracts. Biotransformation is rapid, particularly when compared to the rates observed with the chlorinated hydrocarbon insecticides (11).

The ban on organochlorines has led to far greater use of more toxic organophosphate insecticides (11). The polychlorinated biphenyls (PCB) have been used in a large variety of applications as dielectric and heat transfer fluids,

plasticizers, wax extenders, and flame-retardants. Their industrial use and manufacturing in the USA was terminated by 1977, but unfortunately they persist in the environment. Food is the major source of PCB residues in humans. A serious exposure to PCB's, lasting several months, occurred in Japan in 1968 as a result of cooking oil contamination with PCB-containing transfer medium (Yusho disease). Possible effects on the fetus and the development of offspring of poisoned women were reported. It is not known whether the contaminated cooking oil contained only PCB's, or also polychlorinated dibenzofurans (PCDF's) and polychlorinated quaterphenyls (PCQ's). Consequently, the effects that were initially attributed to the presence of PCB's are now thought to have been largely caused by the other contaminants. Workers occupationally exposed to PCB's have exhibited clinical signs such as dermatological problems (erythema, dryness, rash, and hyperpigmentation), some hepatic involvement, and elevated plasma triglycerides. The bulk of the evidence from human studies indicates that the PCB's pose little hazard to human health, except in situations where food is contaminated with high concentrations of these congeners (11).

Biopesticides and health

There are certain pesticides derived from natural materials such as animals, plants, bacteria and certain minerals, for example canola oil and baking soda that have pesticidal applications. Three classes of biopesticides are known: microbial biopesticides, plant pesticides and biochemical pesticides. All of these different pesticides act on a particular pest and do not have as harmful effects on other organisms as does conventional pesticides (15). Biopesticides are used mainly because of its safety in the environment (28).

Alternative therapeutic agents, in the form of herbal remedies, have been employed for generations in Asian countries in the treatment of liver diseases (31).

Recent predictions of growth in human populations and food supply suggest that there will be a need to substantially increase food production in the near future. One possible approach to meeting this demand, at least in part, is the control of pests and diseases, which currently cause a 30-40% loss in available crop production. In recent years, strategies for controlling pests and diseases have tended to focus on short-term, single-technology interventions, particularly chemical pesticides. This model frequently applies even where so-called integrated pest management strategies are used because in reality, these often are dominated by single technologies (e.g., biocontrol, host plant resistance, or biopesticides) that are used as replacements for chemicals. Very little attention is given to the interaction or compatibility of the different technologies used. Unfortunately, evidence suggests that such approaches rarely yield satisfactory results and are unlikely to provide sustainable pest control solutions for the future (13).

Microbial pesticides, which include bacteria, fungus, virus or protozoan control weeds and some kill specific insects. The most widely used microbial pesticides are subspecies and strains of *Bacillus thuringiensis*, or Bt. Each strain produces a different mix of proteins, and specifically kills one or a few related species of insect larvae. Biochemical pesticides are naturally occurring substances that control pests by non-toxic mechanisms. This may include insect sex pheromones, interfering with mating, as well as various scented plant extracts that attract insect pests to traps (13).

Certain essential plant oils, widely used as fragrances and flavors in the perfume and food industries, have long been reputed to repel insects. Recent investigations in several countries confirm that some plant essential oils not only repel insects, but also have contact and fumigant insecticidal actions against specific pests, and fungicidal actions against some important plant pathogens. Against this backdrop, natural pesticides based on plant-essential oils may

represent alternative crop protectants. Essential oils, obtained by steam distillation of plant foliage, and even the foliage itself of certain aromatic plants (notably in the families Myrtaceae and Lamiaceae, but in other plant families as well) have traditionally been used to protect stored grain and legumes, and to repel flying insects in the home. Though some of the claims made for these crude preparations have yet to be substantiated through controlled experiments, scientific investigation into the biological activities of these materials proliferated in the past decade. The emerging picture is that certain oils and their chemical constituents have demonstrable contact and fumigant toxicity to a number of economically important insect and mite pests, as well as to plant pathogenic fungi (5).

Essential oils, however, are toxic to some mammals. Some of the pure essential oil compounds are slightly toxic, with rat acute oral LD_{50} values of 2–3 g/kg plant essential oils and/or their constituents have a broad spectrum of activity against insect and mite pests, plant pathogenic and other fungi, and nematodes. Biopesticides can be considered a safer alternative to conventional pesticides, since there are 25 million cases of acute occupational synthetic pesticide poisoning in developing countries each year, as recorded by the World Health Organization (WHO) (8). As such, they have considerable potential as crop protectants and for pest management in other situations (e.g. urban pest control) (3). Chemicals used to enhance the safety of foods are of great interest to the food industry. The effectiveness of chemical preservatives in killing microorganisms, depends on the type of microorganisms and the chemical characteristics of foods. Some foods stability against attack by microorganisms is due to the fact that they contain naturally occurring substances with antimicrobial activity. Spices contain essential oils with antimicrobial activity, such as eugenol in cloves, allium in garlic, cinnamic aldehyde and eugenol in cinnamon. Some vegetables and herbs, in addition also contain substances that inhibit microbial growth. However, naturally derived preservatives are limited in use, due to the associated flavors that alter the taste of food. Chinese chive (*Allium tuberosum*)

belongs to the same family as garlic, onion, and leek, and is an important ingredient in Asian cooking. The pressed juice from Chinese chive is shown to be very effective in inhibiting a wide range of microorganisms (16). The dried bark of *Cinnamomum cassia* is used to flavor or season various foods and is a therapeutic agent for various diseases. Cinnamon is rich in essential oils and tannins, which inhibit microbial growth (14).

The world production of citrus fruit is near 80 million tons per year and the average percentage of fruits transformed into juices 34%. Because the juice yield is about half of the fruit weight, very large amounts of byproducts are formed every year. Orange peel is the primary waste fraction and is the source of orange essential oil. It is well known that phenolic products (flavonoids and phenolic acids), characteristic compounds of citrus peel, have exhibited antioxidant properties. Numerous investigations have established the remarkable antifungal properties of essential oils; phytoalexin properties have also been reported in some of the compounds of essential oils, particularly flavonoids and isoflavonoids (27).

Taxol is produced by the Pacific yew (*Taxus brevifolia*), and is a paclitaxel, which is one of a group of diterpene amides, known as taxanes. It is currently being developed as a cancer drug, due to its potent and relatively specific activity. Taxol affects a variety of cells and many human cancers, including leukemia and certain breast, ovarian and lung cancers. Taxol is found throughout the plant along with a wide number of endophytic microorganisms including potential pathogens (29).

It has been suggested that Taxol may serve as a defensive compound, retarding the growth of invading microorganisms and fungi (25).

Importance of medicinal herbs

Estimates vary, but approximately 70% of North Americans use herbal medications. Since the FDA does not regulate herbal products in the USA, dosages can vary even within the same batch, whilst labelling legislation would be significantly improved (2).

In South Africa, around 27 million people use indigenous plant medicines, and substances involving natural remedies is estimated to be valued at R 2 billion per annum. For women considering pregnancy, it is important to discuss herbal medications and potential interactions that can affect their conventional medications and to address lactation issues, but many anecdotal studies suggest that patients do not volunteer information about herbal products they are taking to their doctors. Most consumers are not aware of these potential dangers and the sales people are usually not health professionals with knowledge of drug interactions It is imperative for health professionals to expand their medication history questions to include herbal and alternative remedies in review of medical history, and to have resources available to recommend changes or discontinuation (3). Alternative remedies, including herbs and nutriceuticals, are readily available to millions of women. Many use these products during pregnancy, most of which do not have clinically proven fetal safety. The dilemma facing most regulatory authorities is that the public considers these products as either traditional medicines or natural food supplements (22).

Antimicrobials of plant origin have a great potential in therapeutics. Such constituents can treat infectious diseases, as well as mitigating side effects that often occur with synthetic antimicrobials (10). The screening of plant extracts and natural products for antimicrobial activity has shown that higher plants are representative of new anti-infective agents. It has become apparent that new antimicrobial agents will continue to select resistant strains from the pool of bacteria, which continuously undergo genetic change. Another interest in the

area of natural products is whether or not plant extracts possess activity against plant pathogens (18). Considerable research has been devoted to identifying yeast organisms that effectively control postharvest diseases of fruit, vegetables, and grains (30). Although antagonistic yeasts have been shown to protect a variety of fruit, their efficacy under semi-commercial conditions is sometimes lower than chemical control (6).

Plant products have been used as foods, powders and potions. Much success to cure diseases throughout history is due to plants. Medicinal plants have been used as early as 5000 years, back to the Sumerians (24). When aspirin (acetyl salicylic acid) was first introduced into the world in 1897 by Friedrich Bayer and Co., the historic bond between plant and human health was in existence. Aspirin is a safer synthetic analogue of salicyclic acid, which is an active ingredient of the willow bark, and was discovered independently by residents of New and Old worlds, as a remedy for aches and fevers (19). Plants are making a slow comeback in several areas of human health, namely functional foods, dietary supplements and recombinant protein manufacturing. However, they are still losing importance in areas such as the traditional drug discovery process (21). In the 1970's, 25% of all drugs dispensed in the USA contained compounds derived from flowering plants (7). Throughout human history, plants were unchallenged as sources of drug discovery, but the recent competition from combinatorial chemistry (22) and computational drug design (4) has put an end to the dominance of natural products in drug discovery (21).

Biodiversity of *Helichrysum*

Helichrysum belongs to the largest family of flowering plants, which is Asteraceae. The family consists of 1 100 genera, as well as 25 000 species. This is a widespread family and in the Western Cape alone, there are 107 genera present (2). *Helichrysum* was located in the South Western Cape, and is a

perennial herb that is usually erect. The plant is woolly or cobwebby, and often glandular.

Description of *Helichrysum*

This study encompasses analyses done on *Helichrysum*, which is a rounded, woolly plant, almost grey-white in colour. Leaves are obovate and extremely woolly. The leaves of *Helichrysum* vary in shape depending on the species. The receptacle is smooth, honeycombed or fimbrilleferous. The corollas of the female plants are often narrowly tubular.

The female plant also has a few or sometimes more central florets. Species of *Helichrysum* are very aromatic perennial herbs with densely, hairy leaves and persistent flower heads. The shape and size of the leaves and flower heads are characteristical, and differentiate the species. About 245 species occur in South Africa, of which the best-known and commonly used medicinal plants are *H. cymosum*, *H. petiolare* and *H. nudifolium* (2).

Medicinal uses of *Helichrysum*

It has been reported that pain-relieving, anti-infective, and anti-inflammatory activity exists for several *Helichrysum* species. The smoke of many *Helichrysum* species is used as ritual incense. The main plant parts used for medicinal purposes are, leaves and twigs and sometimes the roots. *Helichrysum* are used for ailments like coughs, colds, fever, and infections. Moreover, this herbal plant has been used traditionally as an anti-infective remedy. There are a number of ways of administering these traditional medicines. For coughs and colds, a tea is prepared or the leaves are boiled in milk. Smoke from burning leaves is inhaled and is a better way for administering pain relief. Leaves are widely used on wounds to prevent infection. Flavonoids, sesquiterpenoids and acylated phloroglucinols are found in the plant. These plants also have pharmacological

effects. Scientific evidence for the traditional use in wound dressing provides proven anti-microbial activity. *Helichrysum* species are distributed all over South Africa and their medicinal use often depends on local availability rather than a preference for particular species (26).

Medicinal uses in the pharmaceutical industry

It is not an exaggeration to expect that the Human Genome Project and advances in biomedical research will allow the pharmaceutical industry to develop new drugs against a wide variety of diseases (23). Yet even as conventional medicine reaches into the cell to touch every molecule, patients are reaching out to alternatives without a proven track record that promise to treat them better. This is not a complete rejection of conventional medicine, but patients are sensing they can benefit from the best of both approaches (12).

Many botanical therapeutics include plant–derived pharmaceuticals, multicomponent botanical drugs, dietary supplements, functional foods and plant-produced recombinant proteins. Conventional pharmaceuticals will soon be complimented in the treatment, prevention, and diagnosis of diseases, while at the same time adding value to agriculture (21). Alternatively therapeutic agents, in the form of herbal remedies, have been employed in Asian countries for generations, in the treatment of liver diseases (32). In the process of finding new drug candidate's medicinal chemists nowadays have a variety of options to choose from, one is to apply combinatorial chemistry techniques. Since the early 1990's synthetic and analytical methods as well as new technologies have been growing rapidly in the area of combinatorial chemistry. Applying these techniques have resulted in the production of large numbers of compounds. A trend is observed towards smaller libraries of compounds with more drug-like properties. An analysis is made to establish the contribution of combinatorial chemistry in providing new lead candidates for (pre) clinical development towards new pharmaceutical products (1).

This study focused on an evaluation of the biopesticidal characteristics of *H. marifolium* and *H.patulum* and their effect on animal metabolism.

References

1. Adang, A.E., and Hermkens, P.H. 2001. The contribution of combinatorial approaches to lead generation: An interim analysis. Curr .Med. Chem.8: 985-998.

2. Allsopp, M., Santillo, D, and Johnston, P.1997. Poisoning the Future. Impacts of Endocrine-disrupting Chemicals on wildlife and Human health.

3. Buehler, B.A. 2000. Interactions of Herbal and Alternative Remedies with conventional medicines. Teratology 61 (6): 465.

4. Clark, D.E., and Pickett, S.D. 2000. Computational methods for the prediction of drug-likeness. Drug Discov. Today 5: 49-58.

5. Crisp, T.M., Clegg, E.D., and Cooper, R.L.1997. Special report on Environmental Endocrine Disruption. An effects assessment and analysis.

6. Droby, S., Wisniewski, M., Ghaouth, A.E., and Wilson, C. 2002. Influence of food additives on the control of postharvest rots of apple and peach and efficacy of the yeast-based biocontrol product aspire. Postharvest and technology, 1-12. Israel.

7. Farnsworth, N.R., and Morris, R.W. 1976. Higher plants - the sleeping giant of drug development. Am .J. Pharm. Educ. 148: 46-52.

8. Isman, M.B. Plant Essential Oils for Pest and Disease Management. Crop Protection. 19 (8-10): 603-608. Canada.

9. Katzung, B.G (ed). 1992. Basic and Clinical Pharmacology. Department of Pharmacology: Plaa. Introduction to Toxicology: Occupational and Environmental Toxicology. Section IX, Toxicology. Appleton and Lange Publishing. USA.

10. Kokoska, L., Polesny, Z., Rada, V., Nepovim, A., and Vanek, T. 2002. Screening of some Siberian medicinal plants for antimicrobial activity. Journal of Ethnopharmacology, 82, (1): 51-53.

11. Lyons, G. 1999. Endocrine Disrupting Pesticides News No.46, December, p16-19.

12. Manachini, B. 2002. Compatibility of Chemical and Biological Pesticides. Encyclopedia of Pest Management. 134-137. Italy.

13. http://www.epa.gov/pesticides/biopesticides

14. Mathuna, D.O. 2001. The best of both approaches. The role of science in complementary and alternative medicine. European Molecular Biology Organization. 2 (12): 1054-1057. Columbus.

15. Matthews. 2001. Chemical Pesticide markets, health risks and Residues, J.Harris, Biopesticides Crop Protection. 20 (5): 452.

16. Mau, J.L., Chen, C.P., and Hsieh, P.C.2001. Antimicrobial Effect of Extract from Chinese Chive, Cinnamon, and Corni Fructus. J. Agric. Food Chem. 49: 183-188. Department of Food Science. Republic of China.

17. Navon, A. 2000. *Bacillus thuringiensis* insecticides in crop protection-reality and prospects. Crop Protection.19 (8-10): 669-676.

18. Ojala, T., Remes, S., Haansuu, P., Vuorela, H., Hiltunen, R., Haahtela, K., and Vuorela, P. Antimicrobial activity of some coumarin containing herbal plants growing in Finland. Journal of Ethnopharmacology. 73, (I-2): 299-305. Finland.

19. Pierpoint, W.S. 1994. Salicylic acid and its derivatives in plants: Medicines, metabolites and messenger molecules. Adv. Bot. Res. 20: 163-235.

20. Rao, S.R., and Ravishankar, G.A. Plant Cell cultures: Chemical Factories of Secondary Metabolites. Biotechnology Advances. 20 (2): 101-153.

21. Raskin, I., Ribnicky, D.M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D.A., Ripoll, C., Yakoby, N., O'Neal, J.M., Cornwell, T., Pastor, I., and Fridlender, B. 2002. Plants and human health in the twenty-first century. Trends in Biotechnology. 20 (12): 522-531.

22. Rousseaux, C.G. 2000. Regulatory issues concerning the safety and efficacy of herbal and alternative remedies. Teratology 61(6): 466.

23. Schreiber, S.L. 2000. Target-oriented and diversity-oriented organic synthesis in drug discovery. Science 287: 1964-1969.

24. Swerdlow, J. 2000. Nature's Medicine. Plants That Heal, National geographic Society.

25. Thomas, M.B. 1999. Ecological approaches and the development of "truly integrated" pest management. Proc. Natl. Acad. Sci. 96 (11): 5944-5951.USA.

26. Van Wyk, B.; Van Oudtshoorn, B.; And Gericke, N. 1997. Medicinal plants of South Africa. Briza Publications. South Africa.

27. Vargas, I., Sanz, P., and Prima-Yufera, E. 1999. Antimicrobial and Antioxidant Compounds in the Nonvolatile Fraction of Expressed Orange Essential Oil. Journal of Food Protection, 62 (8): 929-932.

28. http://www.expressindia.com

29. Wagner, L.J.; and Flores, H.E. 1994. Effect of Taxol and Related Compounds on Growth of Plant Pathogenic Fungi. Biochemistry and Cell Biology, 84 (10). 1173-1178. New Haven.

30. Wilson, C.L., and Wisniewski, M.E. 1994. Biological Control of Postharvest Diseases of Fruits and Vegetables. Theory and Practice. CRC Press, Boca Raton, FL.

31. http://hgic.clemson.edu

32. Zern, M.A. 2000. Herbal Remedies and the treatment of Liver Disease. Crisp Data Base National Institutes of Health. Pennsylvania.

Chapter 2: The detection and evaluation of the biopesticidal activities of *Helichrysum marifolium* plant preparations

Abstract

Plant products have been used as foods, powders and potions. Various successes to cure diseases throughout history are due to plants. The main objective of this study was to evaluate the biopesticidal characteristics of *Helichrysum marifolium* extracts and assessing its effect on animal metabolism. The plant extracts were also tested against the plant pathogens *Botrytis cinerea* (grey mould) and *Venturia inaequalis* (apple scab). Two methods were used against *B. cinerea* and *V.inaequalis*, namely a filter paper technique and a method whereby the extract was poured on an agar plate. *H. marifolium* extracts were administered to male rats, representative of a human model, to test for toxicity. Rats were given 4ml/mg/day of plant extract for a period of 45 days. Results showed significant inhibition of the germtube lengths of *V. inaequalis* ($p<0.0001$), and *B. cinerea* ($p<0.0001$). Urine pH showed significant changes ($p<0.001$) between the rats receiving *H.marifolium*, and those that received the placebo. Blood parameters were analysed for toxicological effects after animals were sacrificed. The only effect that *H. marifolium* had on blood parameters was reflected in the depression of mean cell volume. The results were an indication that *H. marifolium* had a significant inhibitory effect on plant pathogens, and has strong antifungal value. This herbal plant is worth testing for its biopesticidal value, especially due to its safety and efficacy.

1. Introduction

Helichrysum (Asteraceae) is an annual or perennial herb or shrublet, and is usually erect (4). This is a widespread family in the Western Cape alone, where there are about 107 genera present. *Helichrysum marifolium* (Van Wyk, 1997) is located in the Cape, more specifically the South Western Cape. The use of *Helichrysum* species as a herbal plant in South Africa depends on its local availability rather than a preference for its particular species. Ailments

like coughs, colds, fever, infections, are treated with this commonly used herbal plant. Moreover, this herbal plant has been used traditionally as a natural anti-infective remedy (4).

Humans have always been dependent on plants as a source of carbohydrates, proteins and fats. In addition, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives. Worldwide about 121 clinically useful prescription drugs are derived from plants (6). The screening of plant extracts and natural products for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents, as well as serving as a drug discovery platform for primary lead natural compounds (4). Sumerians have used these plants, as known in written records for at least 5000 years (16).

The objectives of this study were to investigate the effects of *H. marifolium* extracts on the growth parameters of the agriculturally important fungi, *Botrytis cinerea* and *Venturia inaequalis*. Furthermore, this investigation evaluates the effect of *H. marifolium* extracts on animal metabolism in an effort to assess its safety as a potential antifungal biopesticide.

2. Materials and Methods

2.1 Ethics

This investigation received ethical approval from the University of the Western Cape.

2.2 Plant Collection

Plant material of *H. marifolium* was collected in the South Western Cape. The site where the plant material was collected had an altitude of 60 m above sea level and was found at 34 ºC 21'54S 21º25'26E. The vegetation type is known as Southern Coast Strandveld.

2.3 Extraction procedures

A sample of the plant specimen used in this study was placed in methanol for 24 hours to extract most of the compounds found within the plant, upon collection. Filtered methanol plant extracts were placed in a round-bottom flask for rotary evaporation. Plant material was placed in an ULM 700 oven at 30-40 °C to obtain dry samples. Methanol was blended twice into the remaining plant material, which was filtered and evaporated again. All the methanol extracts were then dried and frozen for 24 hours and stored in a cold room at 5 °C for 72 hours.

2.4 Plant and soil sample digestion

Soil and plant samples were digested in the laboratory using an acid-digestion mixture. Acid digestion of organic material is an oxidising system and has the advantage that phosphorus, and even nitrogen sometimes can be determined on the final solution along with other nutrients. In this procedure sulphuric acid was used in the digestion mixture to reduce the possibility of the sample drying out. Hydrogen peroxide in sulphuric acid mixture was used, which is a less harmful oxidant, requires a catalyst, and also ensures that nitrogen was retained during the reaction.

2.4.1 Digestion procedure

The plant and soil samples were weighed out individually. Grounded plant material (0.8 g) was used for the digestion, and 4 g of soil samples for each of the plants were weighed out. A digestion mixture was made up beforehand, consisting of 0.42 g Se and 14 g of LiSO4. H_2O, which was added to 350 ml H_2O_2. This was all added together and mixed well with 420 ml of concentrated H_2SO_4. About 8.8 g of the digestion mixture were added to the plant and soil samples, respectively. The samples were then digested at 200 °C with incremental temperature elevation being applied up to 350 °C. This was digested for a few hours until a colourless solution was obtained. The samples were cooled afterwards, and then diluted with distilled water. This was then

filtered into 100ml volumetric flasks. The glass tubes were rinsed with distilled water to obtain all of the sample and this was also filtered into the volumetric flask. The solution was then filled up to 100 ml and placed into plastic containers for analyses.

2.5 Elemental profiles

Solaar Atomic Absorption Spectrometry (AA) was used for soil and plant analysis. A number of standards were prepared for analyses. Ca, Mg, K, Cu, Zn and Na respectively, were prepared by dilution. To make up this solution, we needed to dilute 10 ml of H_2SO_4, with distilled water to 980 ml to obtain a 1% H_2SO_4. 20 ml of Ca was measured and diluted with 1% H_2SO_4. The same was done for Mg, K, and Na. All of the solutions were made up to 100 ml in a volumetric flask using 1% H_2SO_4. About 15 ml of each soil and plant sample were diluted to 75 ml with distilled water and the dilute samples used for Atomic Absorption Spectrometry.

2.6 Herbarium studies

The remaining plant samples were stored in Formaldehyde (FAA), for herbarium studies. The FAA ensures that the plant was preserved for further identification.

2.7 Plant anatomy

Sections of leaves were cut into a thickness of 10-20 microns, using CO_2 and Hamilton's solution to freeze the sample. Temporary slides were then made from these sections and photos were captured digitally, using an Olympus photomicroscope.

2.8 Antifungal studies

2.8.1 Preparation of fungi

Exactly 50 ml of water was used with Tween to allow the fungal spores to disperse more easily. The spores were then collected with a 1ml pipette and added to the Tween and water suspension, after which the suspension was shaken. A direct cell count of each spore concentration was made by filling the Neubauer chamber The microscope objective (x10) was focused on the squares and all the spores contained within the 5 groups of 16 small squares (= 80 small squares) were counted. About 80 squares containing spores were counted under 100x magnification using a Nikon electronic absorption microscope.

2.8.2 Methods of assessing extracts against fungi

2.8.2.a Filter paper technique

This method involved cutting the filter paper into strips of 2mm wide to which the *H. marifolium* extract was applied. The extract was then left to dry, while the plates were streaked. This method was used for the fungi *Botrytis cinerea* and *Venturia inaequalis*. The filter paper was then put onto the plates that were streaked with the fungi. After 24 hours, the length of fungal germ tubes was measured. The length of the germtubes (µm) was indicative of the efficacy of the extract.

2.8.2 b Extracts on agar plate

This method involved the use of 1ml of the extract poured onto the agar plate. The agar was tilted to ensure that there is equal distribution of the extract throughout the plate. The fungi were then poured onto the plate that was covered by extract, after the latter had dried. After 24 hours, the germ tube lengths of the fungi were measured to assess the effect of the *H. marifolium* extract.

2.9 Animal Studies

The Medical Research Council (MRC) supplied 20 Wistar male rats with an average mass of 300-400 g for this investigation. The rats were divided into two groups of ten each and housed in pairs of two in plastic cages with mesh wire flooring and tops in a temperature-controlled room. The cages were 35 cm x 35 cm with a height of 23 cm. The animals had a period of two weeks to acclimatize to the room. A control group receiving only distilled water as a substitute for the herbal medicine extract was used, and a second group was medicated with a water extract of *H. marifolium*. Four milliliters of herbal extract was then administered to each individual rat by means of a Terumo spinal needle, 1.20 x 90 mm in diameter, with a round bulb-shaped edge, in order to avoid any damage to the animals. The extract was given at days 15, 30 and 45.

2.9.1 Metabolic Analyses

Ten rats were weighed individually and each put into metabolic cages on days 15, 30 and 45. Each rat was given 40 g of food and 60ml of distilled water for 24 hours and was due for the collection of metabolic data. After 24 hours had elapsed, the rats were taken out of the metabolic cages and were weighed again. The pH of the urine was also measured. Food consumption and water intake were also determined. The rats were sacrificed after 45 days using Chloroform and Ether. Terminal blood samples were taken from the left ventricle. About 3ml was placed in an EDTA vacutainer for the analyses of Red Cell Count (RCC), haemaglobin, haematocrit, Mean Cell Volume (MCV), Mean Corpuscular haemaglobin (MCH), Mean corpuscular haemaglobin concentration (MCHC), RDW, White blood Cell Count (WBC), neutrophil levels, basophil levels, lymphocyte levels, monocyte levels, eosinophil levels, Neutrophil Absolute count (NAC), Monocyte Absolute count (MAC), Eosinophil Absolute count (EAC), Basophil Absolute count (BAC), Lymphocyte Absolute count (LAC) and platelet levels.

2.9.2 Animal Histology

At termination, the liver and testis of the animals were removed for analyses. These organs were stored in Bouins' fluid and prepared for histological sectioning. The tissue was then processed with a histokinette for 22 hours and embedded in wax. Tissue were cut with disposable blades at 5 microns and stained with Heamotoxylin and Eosin stain and mounted with DPX.

2.10 Statistics

Data was analyzed using Microsoft Excel Stat (2000). The significant differences between rat weights of the control and experimental group, necessitated baseline corrections to be made to data for the respective collections made over the experimental period. Control and experimental animal groups were then compared with one another and a minimum significance of $P< 0.05$ was determined for all metabolic parameters using the Mann-Whitney test. Baseline corrections were not applied to blood data, which was directly compared. Significant differences were also determined at a minimum level of $P< 0.05$ with the Mann-Whitney test.

The antifungal data were sorted using the stem and leaf method and p-values were determined using the Mann-Whitney test.

3 Results

3.1 Plant anatomy

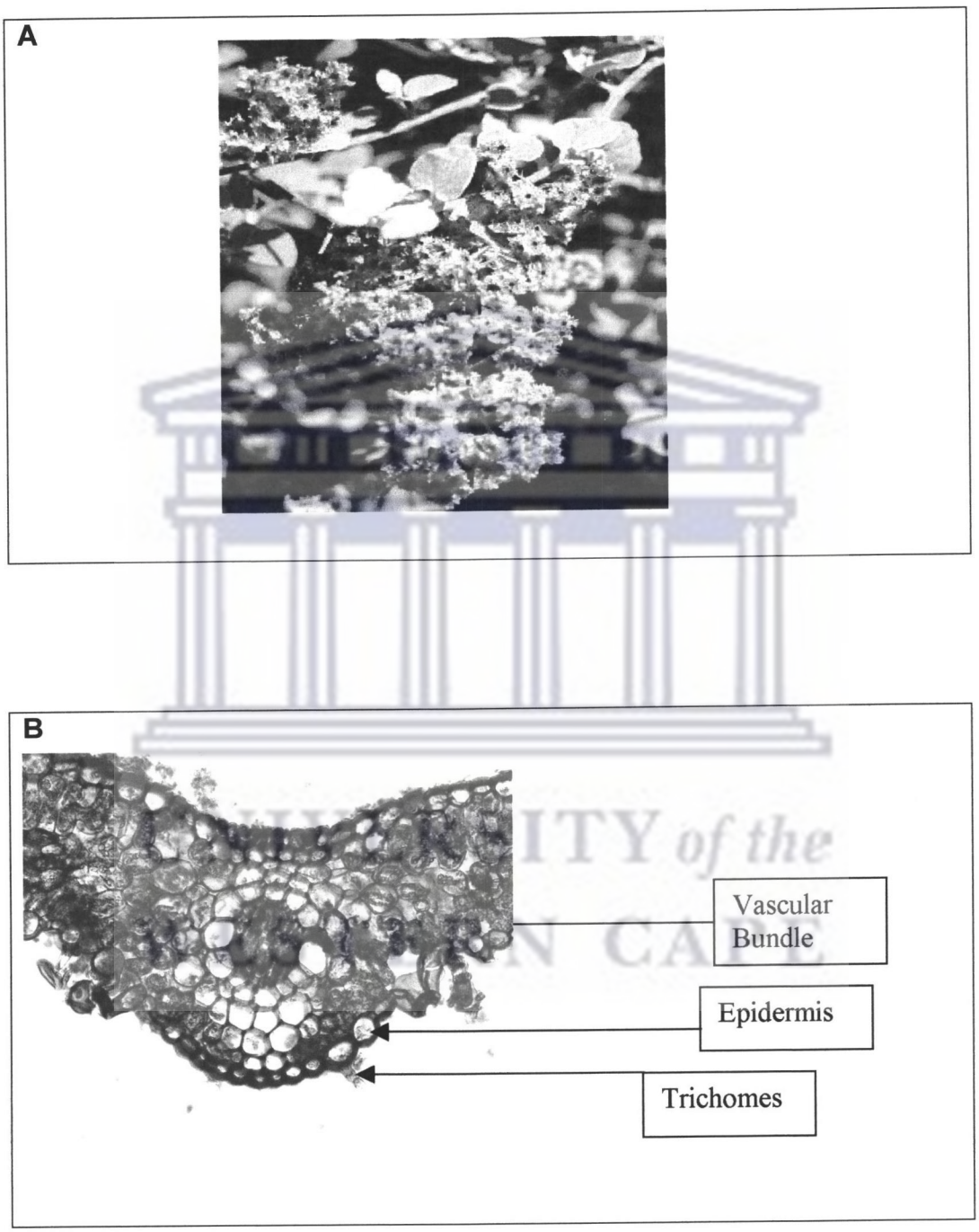
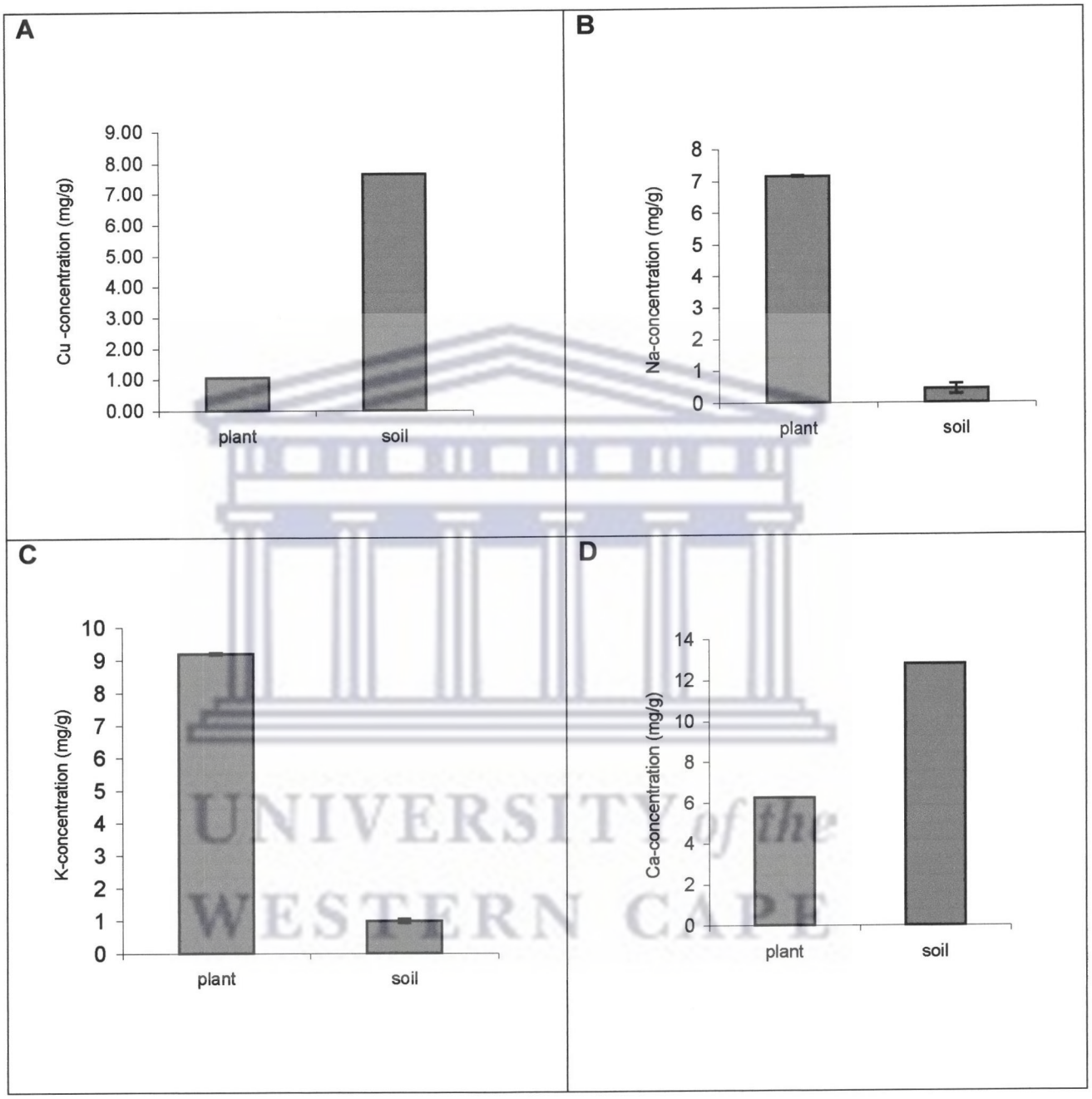


Figure 1a:Intact plant of Helichrysum sp., b: Cross section of the leaf of *Helichrysum marifolium* using freeze microtomy.

3.2 Elemental analysis



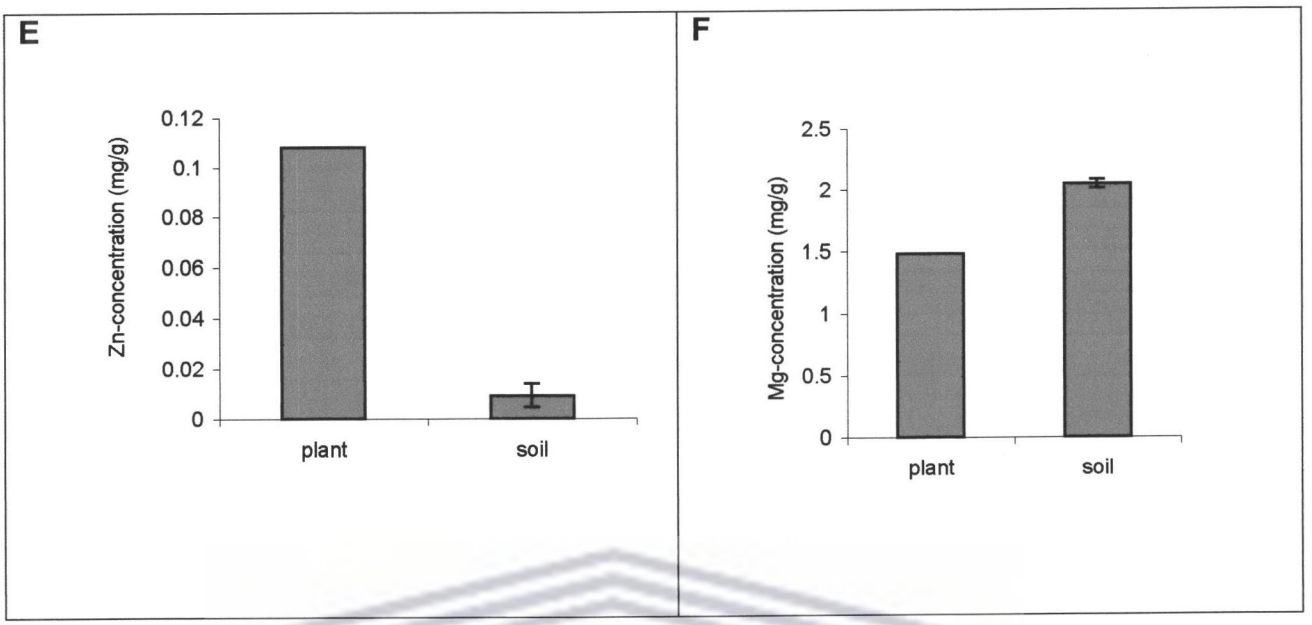


Figure 2 a-f. The elemental levels of *H. marifolium* leaves and the soil in which it grows.

3.3 Antifungal results

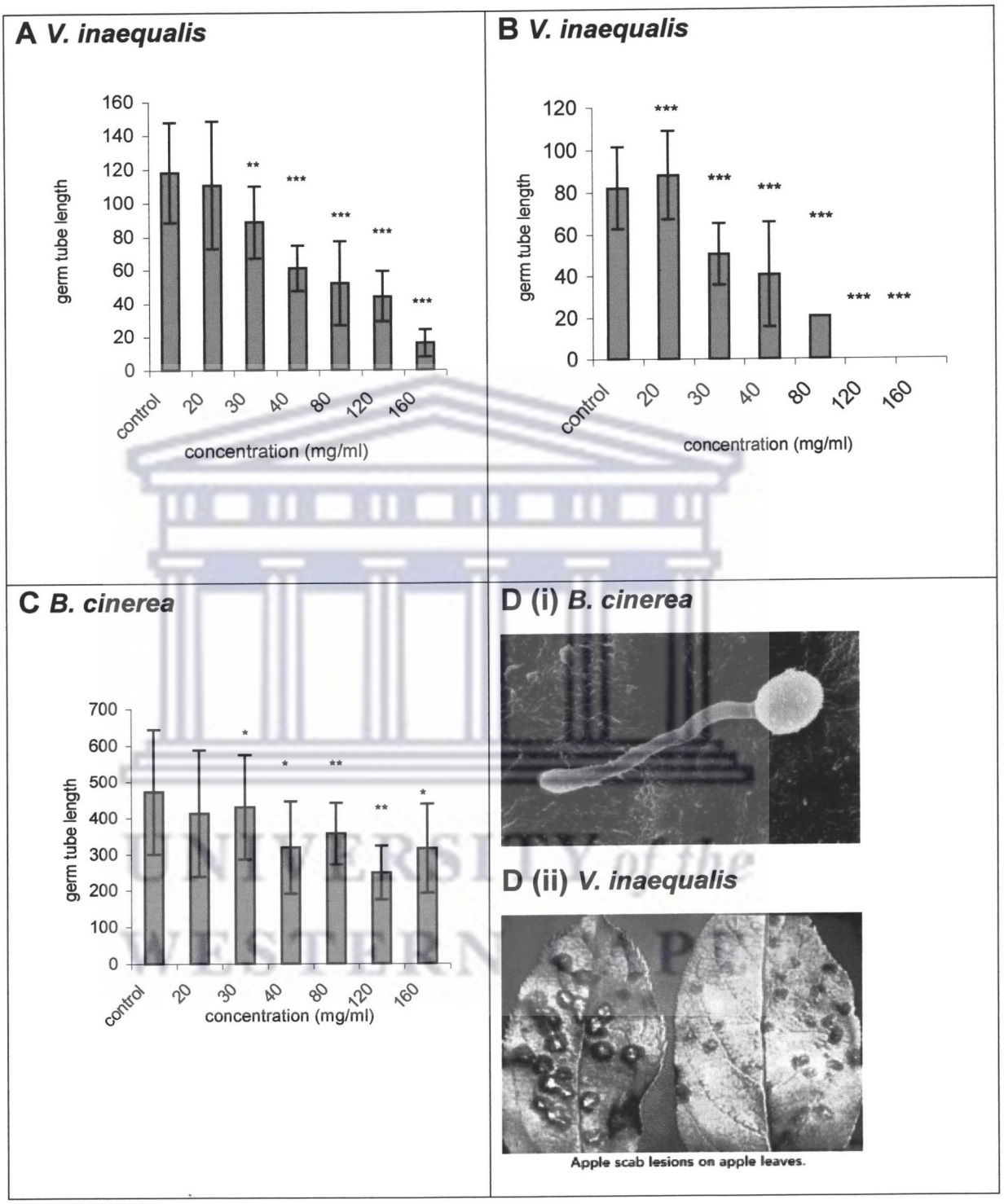


Figure 3 a-c: Antifungal results in µm in control and experimental groups. Figure 3d (i)/(ii) displays the organisms and its effect on apple leaves. Significance of the experimental group in relation to the control: *p<0.05, **p<0.005, ***p<0.0001.

3.4 Metabolic outcomes

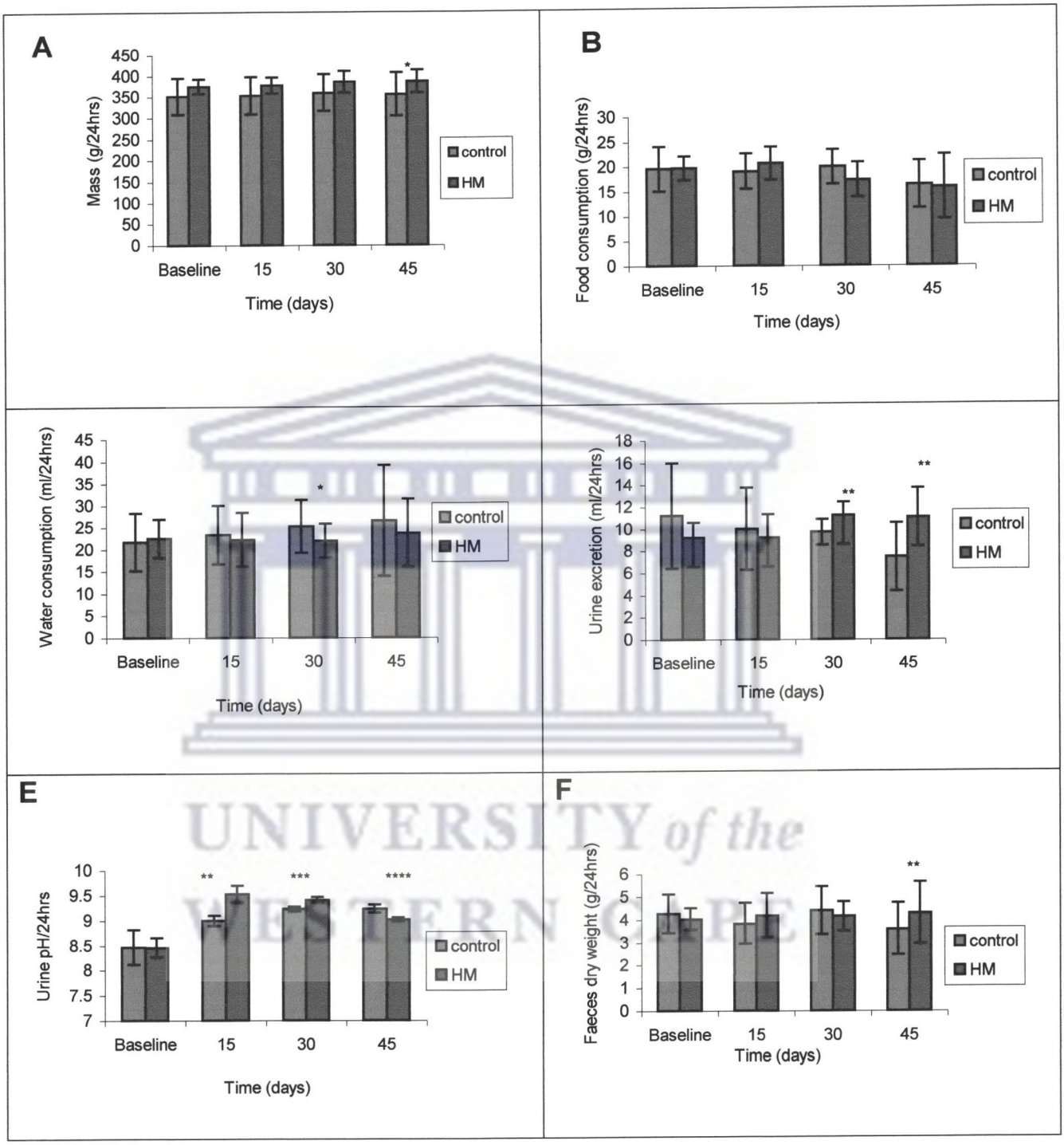
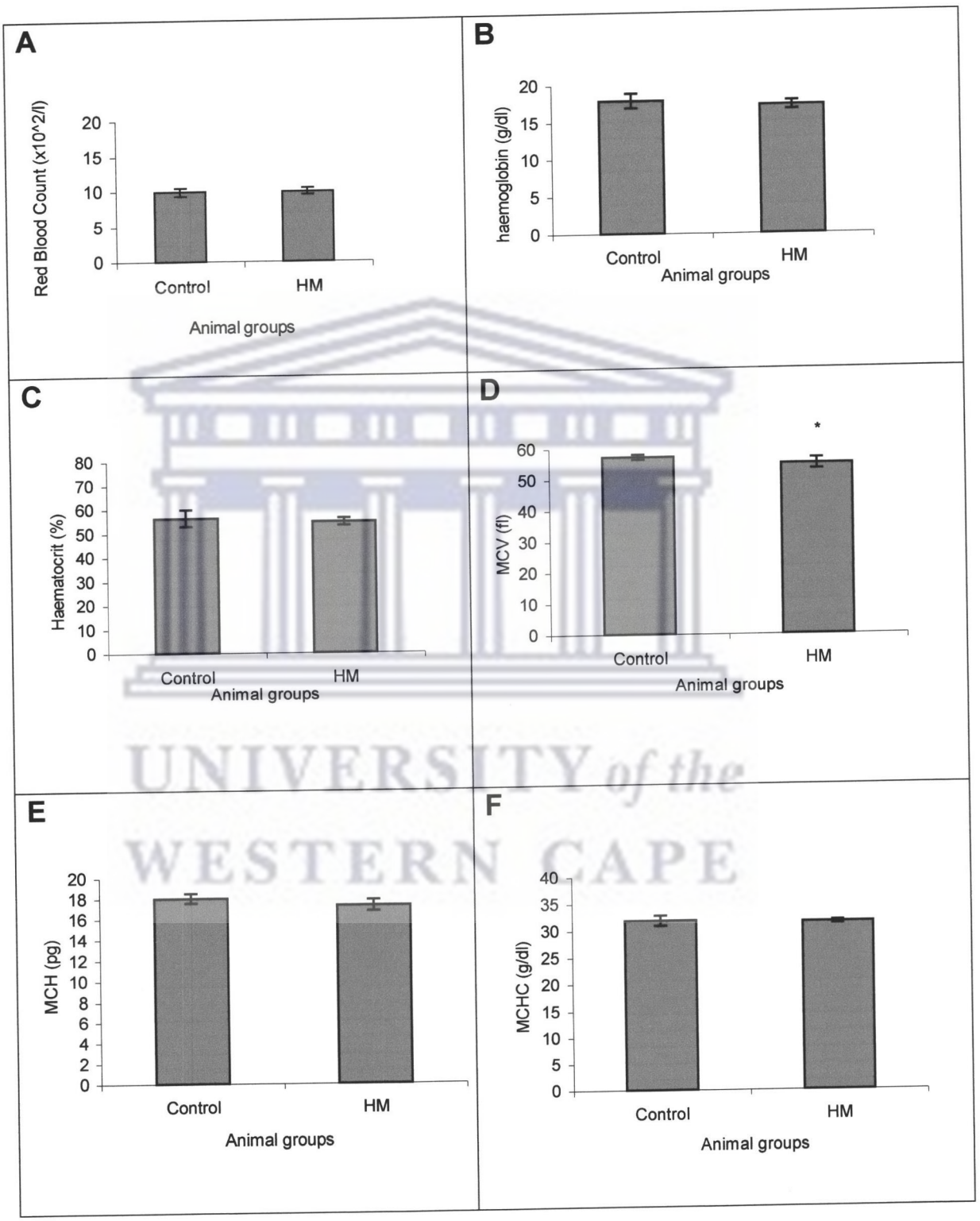


Figure 4 a-f: Metabolic parameters of animals that received H. marifolium (HM) extract compared to those who received a placebo. Significance of experimental groups in relation to control group: *p<0.05, p<0.005 p<0.001.

3.5 Haematological profiles

Red blood cell parameters



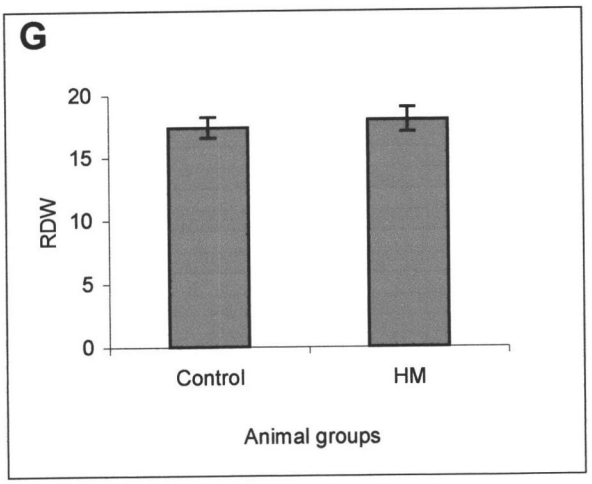
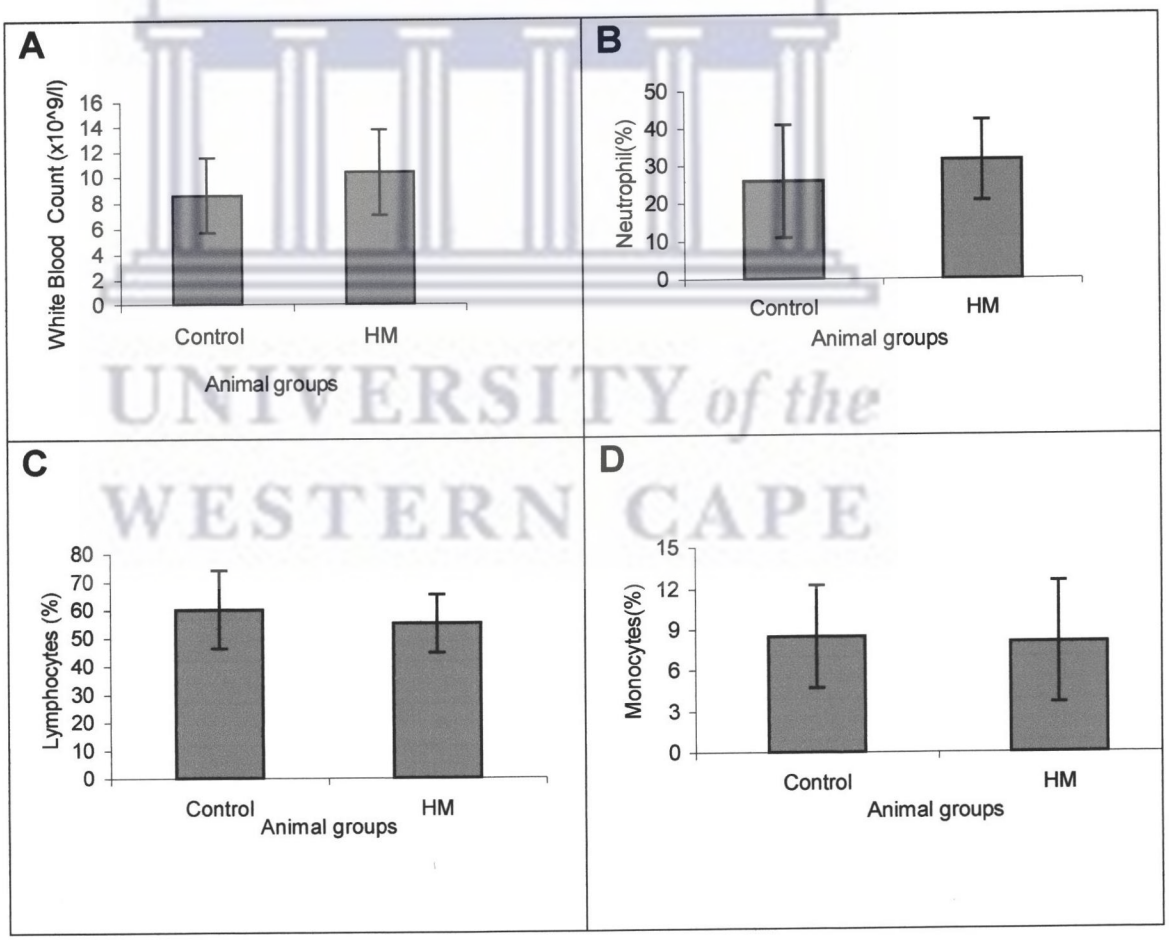
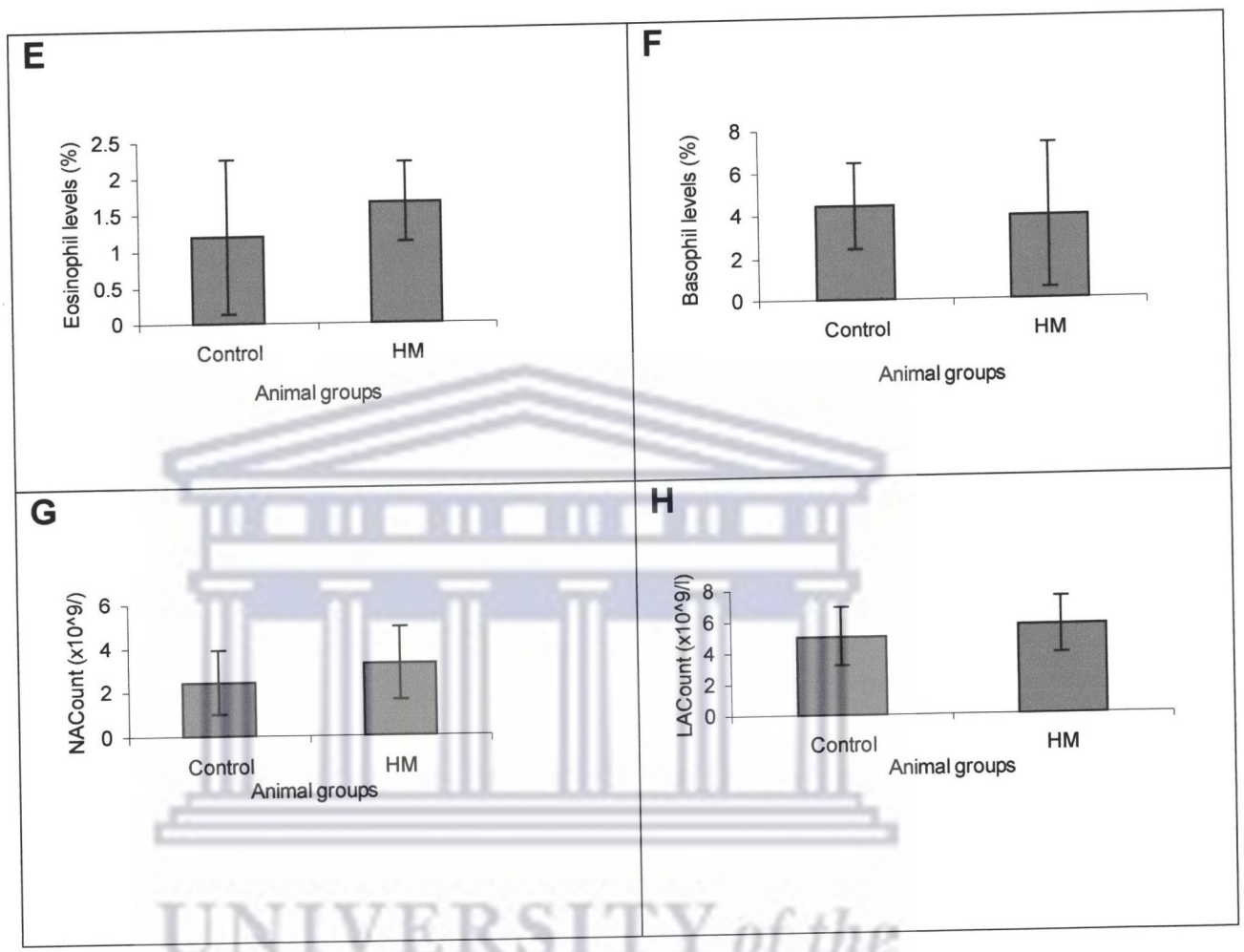


Fig 5. a-g: The various red blood parameters of animals that were fed *H. marifolium* (HM) compared to those that received the placebo. Significance in relation to control and experimental group*p<0.01.

White blood cell parameters





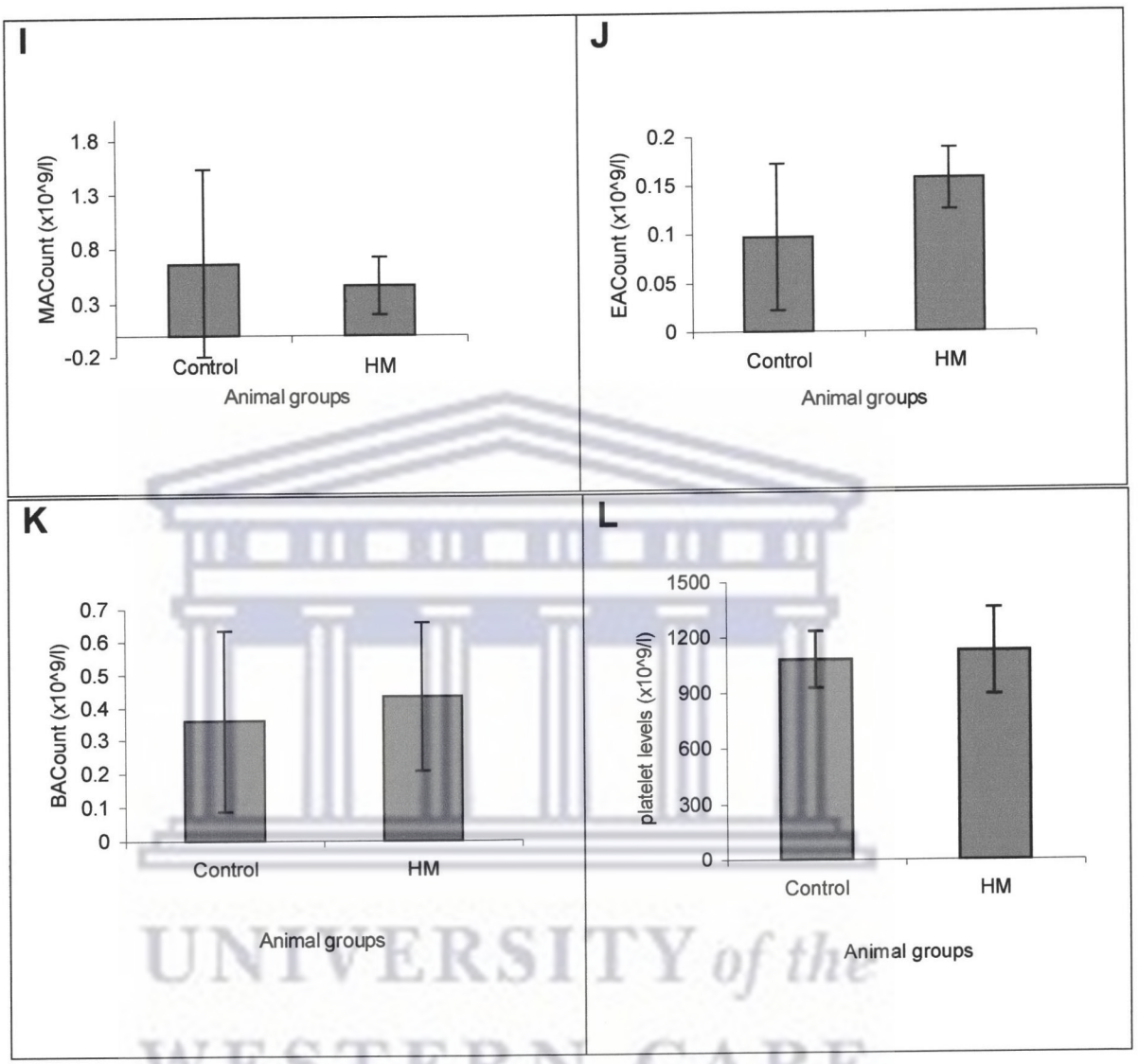


Figure 6 a-l: The various white blood parameters of animals that were fed *H.marifolium* (HM) compared to those that received the placebo. Significance in relation to control and experimental group*p<0.01.

3.7 Animal tissue histology

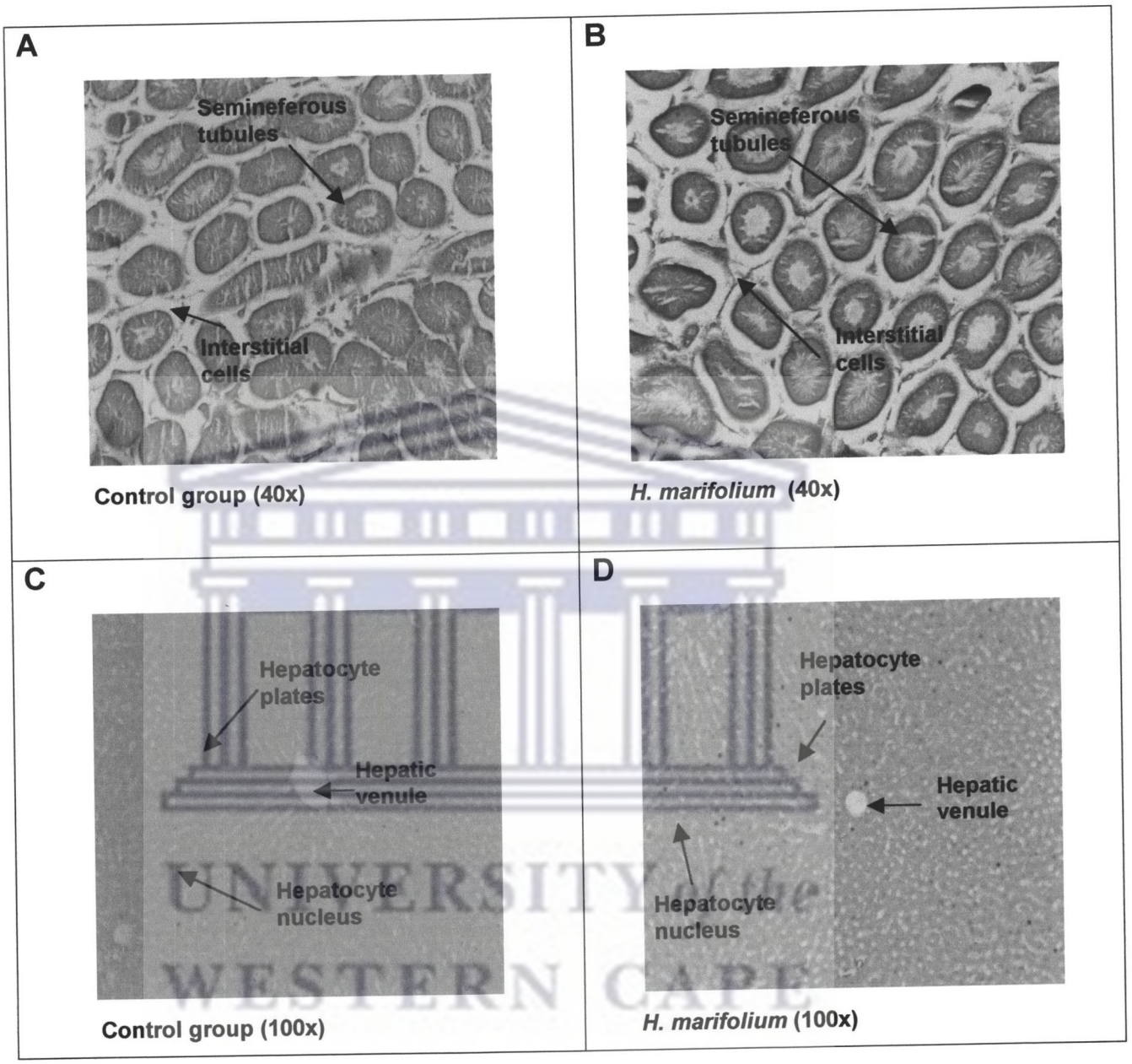


Figure 7 a-d: Cross sections of the testis (a,b) and liver (c,d) of animals that were given *H. marifolium* extract and those that received the placebo.

4. Discussion

The study provides evidence that *H. marifolium* crude methanol extract consist of compounds with antifungal activity against important plant pathogens such as *Botrytis cinerea* and *Venturia inaequalis*, where germ tube lengths were an indication of the effect of the plant extract against the plant pathogen.

There are certain pesticides derived from natural materials such as animals, plants, bacteria and certain minerals, for example canola oil and baking soda have pesticidal applications. Three classes of biopesticides are known: microbial biopesticides, plant pesticides and biochemical pesticides. All of these different pesticides act on a particular pest and does not have as harmful effects on other organisms as does conventional pesticides (6).

The study included the anatomy of *H. marifolium*, as indicated in fig.1b. *H. marifolium* contains certain organs known as trichomes, which develop very early in the life of a leaf. Since trichomes develop so early, the number of trichomes depends on the growth of the leaf (4).

Elemental analyses were done on plant and soil samples. There were no significant changes ($p>0.05$) between the soil and plant samples of *H. marifolium* for each of the elements analysed.

B. cinerea is a fungus that has the ability to go unnoticed in vineyards and cause grey mould. *B. cinerea* usually grows on dead plant material and attacks a variety of crops, although this does not occur in vineyards. The fungus moves in vineyards mainly as conidia, which is a structure derived from sclerotia. Sclerotia are tough survival structures, which may develop on any infected plant material, where it can survive throughout winter. It germinates in late winter and form

conidia. Conidia are little spores (Fig. 3d(i)) with a diameter of about 0.01mm. It gets released, by wind or insects, land on plant parts, and germinates (3).

Apple scab, caused by the fungus *Venturia inaequalis*, is one of the most serious diseases of apple. Disease development is favored by wet, cool weather that generally occurs in spring and early summer. Both leaves and fruit can be affected. Infected leaves may drop resulting in unsightly trees, with poor fruit production. This early defoliation may weaken trees and make them more susceptible to winter injury or other pests. Infected fruits are blemished and often severely deformed. Infected fruits may also drop early (2). Apple scab survives the winter in the previous year's diseased leaves on the orchard floor. In the spring, the fungus in old diseased leaves produces millions of spores. The spores get dispersed through rain and any wet conditions, favorable for it to germinate (2).

Antifungal results are indicated in Fig. 3 a-c. Germ tube lengths are indicated in Fig 3a showing *H. marifolium's* inhibiting effect on *V. inaequalis*. Significant inhibition of germ tube lengths occurs at concentrations of 30 mg/ml ($p<0.005$), 40 mg/ml ($p<0.0001$), 80 mg/ml ($p<0.0001$), 120 mg/ml ($p<0.0001$), and 160 mg/ml ($p<0.0001$). Fig. 3b shows very significant inhibition of *V. inaequalis* at concentrations of 20 mg/ml ($p<0.0001$), 30 mg/ml ($p<0.0001$), 40 mg/ml ($p<0.0001$) and 80 mg/ml ($p<0.0001$). Concentrations of 120 mg/ml and 160 mg/ml ($p<0.0001$), however, it completely inhibits growth of the fungus. It is clear from Fig. 3 a-b, that despite different techniques, the *H. marifolium* extract significantly inhibited growth of *V.inaequalis*.

H. marifolium's effect on *B. cinerea* is indicated in Fig. 3. c, and shows significant inhibition at 30 mg/ml ($p<0.05$), 80 mg/ml ($p<0.005$) and 160 mg/ml ($p<0.05$).

Scientific evidence for the traditional use of *Helichrysum sp.* in wound dressing provides proven anti-microbial activity. *Helichrysum* species are distributed all over South Africa and their medicinal use often depends on local availability rather than a preference for particular species (11). The smoke of many *Helichrysum* species is used as ritual incense. The main plant parts used for medicinal purposes are, leaves and twigs and sometimes the roots. Ailments like coughs, colds, fever, infections, headache and menstrual pains are treated with this commonly used herb. There are a number of ways of administering these traditional medicines. For coughs and colds, a tea is prepared or the leaves are boiled in milk. Smoke from burning leaves is inhaled and is a better way for administering pain relief. Leaves are widely used on wounds to prevent infection. Flavonoids, sesquiterpenoids, and acylated phloroglucinols are found in the plant. These plants also have pharmacological effects. It has been reported that pain-relieving, anti-infective, and anti-inflammatory activity exists for several *Helichrysum* species (8).

More than 85% of higher plants have not been sufficiently surveyed for potentially useful biological activity and the plant kingdom has not received sufficient attention as a resource of possible medicinal agent. Over half of the world's 25 best selling pharmaceuticals for 1999 owed their origin to a natural source material (10).

Medicinal plant is one in which one or more of the plant's organs, contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. This needs to be scientifically proven, although many medicinal plants are used by traditional healers and are not tested scientifically (8).

Natural products have historically been used in numerous applications in the fungal, weed and insect control sectors of agriculture. Plants have an excellent track record in providing novel leads for crop protection, particularly in the field of

insecticides. This can be attributed to the evolution of secondary metabolites, which address the specific needs of the host plant in protecting it form insect attack (9). Antimicrobials of plant origin have a great effect, therapeutically, and are effective in the treatment of infectious dieases, while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (7).

Whole animal models are used in most cases for *in vivo* experiments, thus to collect biological material. This is needed to study enzymes, cells, tissues and organs, which are obtained from when the animal is dead. Therefore, animals must be sacrificed. Most large pharmaceutical companies dispose of a large battery of *in vivo* tests for the secondary evaluation of selected leads. In vivo tests are done mostly for the determination of activities of the cardiovascular, intestinal, liver and respiratory system, as well as for the determination of anti-inflammatory, antiviral, and anti-diabetic effects. Many *in vivo* tests also remain the stepping-stone for many corresponding tests in human clinical traits (9).

Metabolic data were collected at days 15, 30 and 45 of this study. The mass (Fig. 4a) of the animals was taken. The *H. marifolium* extract, compared to the control group, had no effect on animal mass, except significantly elevating ($p<0.05$), at day 45. The plant extract, however, had no effect on food consumption (Fig. 4b). On the other hand, water consumption was significantly ($p<0.05$) lower on day 30 (Fig. 4c) in the *H. marifolium* group compared to the control group. Even though this trend was observed, the animals on the plant extract excreted significantly more ($p<0.001$) urine on days 30 and 45 (Fig. 4d). This could mean that the herbal plant is a possible diuretic. Urine pH is indicated in Fig. 4e, and shows a significant difference at day 15 ($p<0.005$), day 30 ($p<0.001$), and day 45 ($p<0.001$) between the control group and the animal group fed on *H. marifolium*. This group excreted significantly more alkaline urine than the group on placebo. Faecal excretion (Fig. 4f) changed significantly ($p<0.05$) at day 45.

Blood parameters that were analysed involved, white blood cell count, red blood cell count, haemotocrit, eosinophil, neutrophil, basophil, monocyte, lymphocytes and platelet levels. It is a measure of the total volume of the erythrocytes relative to the total volume of whole blood in a sample. The result is expressed as a proportion, either unitless (e.g. 0.42) or with volume units (e.g. 0.42 L/L, or 42 cL/L [centiliters/liter]). The most aesthetically pleasing of all the leukocytes, the basophils are also the least numerous, the normal range of their count in peripheral blood being 0 - 200/μL. They are easily recognized by their very large, deep purple cytoplasmic granules, which overlie, as well as flank, the nucleus (eosinophil granules, by contrast, only flank the nucleus, but do not overlie it). It is tempting to assume that the basophil and the mast cell are the blood and tissue versions, respectively, of the same cell type. Actually it is controversial as to whether this concept is true or whether these are two different cell types. Eosinophils are granulocytes that are less numerous than neutrophils and make up 2-4% of leukocytes in normal blood. Eosinophils have a diameter of 12-15 microns and contain a characteristic bilobed nucleus. The main identifying feature of eosinophils is the presence of many large and elongated granules that are stained red by eosin. An increase in the absolute number of eosinophils in circulation is associated with allergic reactions and parasitic infections. These cells also produce substances that modulate inflammation .Neutrophils are short-lived cells (6-7 hours in blood and 1-4 days in tissue) that are 12-15 microns in diameter with a multilobed nucleus. Immature neutrophils have a non-segmented horseshoe shaped nucleus. Basophils are often difficult to find in blood smears because they are so few in circulating blood (less than 1% of blood leukocytes) (15).

Lymphocytes are spherical cells with a diameter of 6-18 microns with a spherical nucleus and very little cytoplasm. Some large lymphocytes are thought to be memory cells that differentiate into effector T cells or B-lymphocytes having specific antigen receptors on the cell surface. T cells participate in cellular immunity, while B cells are involved in humoral immunity as they differentiate into

plasma cells that make specific antibodies. Lymphocytes vary in life span with some living only a few days, while others circulate for many years. These cells are the only white blood cells that return to the blood stream after migrating to the tissues. These agranulocytes vary in diameter from 12-20 microns and have an oval, horseshoe or kidney shaped nucleus with more cytoplasm than lymphocytes. The nucleus does not stain as darkly as that of lymphocytes in blood smears (15). Monocytes represent 3-8% of circulating white blood cells. After leaving the circulatory system, monocytes differentiate into macrophages in connective tissue. These cell fragments originate from large cells in the bone marrow called megakaryocytes, have a lifespan of about 10 days and are non-nucleated. Platelets promote blood clotting and repair gaps in the walls of blood vessels. Upon injury to the vessel endothelium, the platelets aggregate and form a platelet plug. They then release substances that induce further platelet aggregation and blood clotting. After the vessel is repaired by the formation of new tissue, the clot is removed by plasma and platelet derived enzymes. Normal platelet count ranges from 200,000 to 400,000 per microliter of blood (15).

It seems the only effect that *H. marifolium* had on blood parameters was reflected in its depression of MCV (Fig. 5 d), $p<0.01$. For the rest, *H. marifolium* extract had no significant effects on RBC and WBC. Organs that were histologically sectioned are mentioned in Fig. 7 a-d. This includes organs of both the control group and the animals that received an extract of *H. marifolium*. These organs were chosen because it is believed that the testis and liver are the organs most susceptible to potential toxicological insult.

Compounds from natural sources such as *Helichrysum marifolium*, have the potential to enrich the collection for candidates for biological assessment. They not only bring with them their own diversity, but also a set of challenges which, if successfully resolved, will provide the crop-protection industry with opportunities to safeguard the supply of food well into the next century (9).

5. References

1. Buehler, B.A. 2000. Interactions of Herbal and Alternate Remedies with conventional medicines. Teratology; 61(6): 465. Nebraska.

2. Ellis, M.A; Chatfield, J; and Draper, E. Scab of Apple and Crabapple. Ohio State University Extension Fact Sheet. Plant Pathology.

3. Holz, G. 2001.The Occurrence And Control Of *Botrytis cinerea* (Grey Mould) In Grapes: A South African Perspective. Winetech. Department of Plant Pathology. Stellenbosch. South Africa.

4. Karousou, R., A.M. Bosabalidis and S. Kokkini. 1992. Sideritis syriaca ssp. syriaca: glandular trichome structure and development in relation to systematics. Nordic Journal of Botany 12(1): 31-37.

5. Kokoska, L; Polesny, Z; Rada, V; Nepovim, A; and Vanek, T. 2002. Screening of some Siberian medicinal plants for antimicrobial activity. Journal of Ethnopharmacology. Volume 82, Issue 1. Pages 51-53. Czech Republic.

6. Matthews. 2001. Chemical Pesticide markets, health risks and Residues, J.Harris, Biopesticides Crop Protection. Volume 20,Issue 5, Page 452.

7. Ojala, T.; Remes, S.; Haansuu, P.; Vuorela, H.; Hiltunen, R.; Haahtela, and Vuorela, P. 2000. Antimicrobial Activity of some Coumarin containing plants growing in Finland. Journal of Ethnopharmacology. Volume 73,Issues 1-2, Pages 299-305.

8. Rao, S.R.; and Ravishankar, G.A. Plant Cell cultures: Chemical Factories of Secondary Metabolites. Biotechnology Advances. Volume 20,Issue2, Pages 101-153. Japan, India.

9. Rice, M.J.; Legg, M.; and Powell, K.A. 1998.Natural Products in Agriculture- A View from the Industry. Pestic.Sci.52, 184-188. Great Britain.

10. Rousseaux, C.G. 2000. Regulatory Issues concerning the safety and efficacy of herbal and alternative remedies. Teratology, 61(6): 466. Canada.

11. Sofowora, A. 1982. Medicinal Plants and Traditional Medicine in Africa. John Wiley And Sons Ltd. Nigeria.

12. Van Wyk, B.; Van Oudtshoorn, B.; and Gericke, N. 1997. Medicinal plants of South Africa. Briza Publications. South Africa.

13. http://handlin.cvm.okstate.edu/histology/MR/HiMRp3.htm

14. Vlietinck, A.J. 1999.Screening Methods for Detection and evaluation of Biological Activities of Plant Preparations. Department of Pharmaceutical Sciences, University of Antwerp (UIA), Universitetitsplan, 1,B-2610 Antwerp, Belgium.Ed. Bohlin, L, and Bruhn, J.G. Bioassay Methods in Natural Product Research and Drug Development.

15. www.e-histology.net

16. Swerdlow, J. 2000. Nature's Medicine. Plants That Heal, National geographic Society.

Chapter 3: The detection and evaluation of the biopesticidal activities of *Helichrysum patulum* plant preparations

Abstract

It has been reported that pain-relieving, anti-infective, and anti-inflammatory activity exists for several *Helichrysum* species. The objective of this study is to evaluate the biopesticidal characteristics of *Helichrysum patulum* extracts and assessing its effect on animal metabolism. *H. patulum* was tested against the plant pathogens *Botrytis cinerea* (grey mould) and *Venturia inaequalis* (apple scab). Two methods were used to test the herbal plant against these plant pathogens. *H. patulum* was administered to male rats, representative of a human model, to test for toxicity. Rats were given 4 ml/ mg/ day of plant extract for a period of 45 days. Various parameters were tested, including rat weights, faecal and urinary excretion. The extract of *H. patulum* caused significant inhibition of *V. inaequalis* ($p<0.0001$), whilst *B. cinerea* was also significantly inhibited ($p<0.0001$). Blood parameters were analysed for any toxicological effects after animals were sacrificed. The only effect *H. patulum* had on blood parameters was the depression of haemaglobin levels ($p<0.05$). The safety and efficacy of this herbal plant therefore suggest that it could be a potential biopesticide with further research.

1. Introduction

Helichrysum (Asteraceae) is an annual or perennial herb or shrublet, and is usually erect (2). This is a widespread family in the Western Cape alone, where there are about 107 genera present. *Helichrysum marifolium* (Van Wyk, 1997) is located in the Cape, more specifically the South Western Cape. The use of *Helichrysum* species in South Africa depends on its local availability rather than a preference for its particular species. Ailments like coughs, colds, fever, infections, headache, and menstrual pains are treated with this commonly used herbal plant. Moreover, this herb has been used traditionally in wound dressing as a natural anti-infective remedy (2).

Man has always been totally dependent on plants as source of carbohydrates, proteins and fats. In addition, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives. Worldwide about 121 clinically useful prescription drugs are derived from plants (4). The screening of plant extracts and natural products for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents, as well as serving as a drug discovery platform for primary lead natural compounds (3).

The objectives of this study were to investigate the effects of *H. patulum* extracts on the growth parameters of the agriculturally important fungi *B. cinerea* and *V. inaequalis*. Furthermore, this investigation evaluates the effect of *H. patulum* extracts on animal metabolism in an effort to assess its safety as a potential antifungal biopesticide.

2. Materials and Methods

2.1 Ethics

This investigation received ethical approval from the University of the Western Cape.

2.2 Plant collection

H. patulum plant material was collected in the South Western Cape. The site where the plant material was collected had an altitude of 60 m above sea level and was found at 34 ºC 21'54S 21º25'26E.

2.3 Extraction procedure

A sample of the plant specimen used in this study was placed in methanol for 24 hours to extract most of the compounds found within the plant, upon collection. Filtered methanol plant extracts were placed in round-bottom flask for rotary evaporation. Plant material was placed in an oven at 30-40 °C to obtain dry samples.

Methanol was blended twice into the remanining plant material, which was filtered and evaporated again. All the methanol extracts were then dried and frozen for 24 hours after which it was freeze-dried for another 24 hours, and stored in a cold room at 5 °C for further analysis.

2.4 Plant and soil digestion

Soil and plant samples were digested in the laboratory using an acid-digestion mixture. Acid digestion of organic material is an oxidising system and has the advantage that phosphorus, and even nitrogen, sometimes can be determined on the final solution along with other nutrients. The procedure is preferable to dry-ashing. In this procedure, sulphuric acid is used in the digestion mixture to reduce the possibility of the sample drying out. Hydrogen peroxide in sulphuric acid was used, which is a less harmful oxidant, and is slower, requiring a catalyst, and also ensures that nitrogen is retained during the reaction.

2.4.1 Digestion procedure

The plant and soil samples were weighed out individually. Grounded plant material (0.8 g) was used for the digestion, and 4 g of soil samples for each of the plants were weighed out. A digestion mixture was made up beforehand, consisting of 0.42 g Se and 14 g of $LiSO_4$. H_2O was added to 350 ml of H_2O_2. This was all added together and mixed well with 420 ml of concentrated H_2SO_4. About 8.8 g of the digestion mixture was added to the plant and soil samples, respectively. The samples were then digested at 200 °C with incremental temperature elevation being applied up to 350 °C. This was digested for a few hours until a colourless solution was obtained. The samples were cooled afterwards, and then diluted with distilled water. This was filtered into 100 ml volumetric flasks. The glass tubes were rinsed with distilled water to obtain all of the sample and this was also filtered into the volumetric flask. The solution was then filled up to 100 ml and placed into plastic containers, ready for analyses.

2.5 Elemental profiles

Solaar Atomic Absorption Spectrometry (AA) was used for soil elemental analysis. A number of standards were prepared for analyses of Ca, Mg, K, and Na. 10 ml of H_2SO_4, was mixed with 980 ml distilled water to obtain a 1% H_2SO_4. Twenty milliliters of Ca was measured and diluted with 1% H_2SO_4. The same was done for Mg, K, and Na. A combination of Ca and K was used and diluted at various concentrations, as well as a combination of Mg and Na. All of the solutions were made up to 100 ml in volumetric flasks using 1% H_2SO_4. Fifteen milliliters of each soil and plant sample were diluted to 75 ml with distilled water and the dilute samples used for AA analyses.

2.6 Herbarium studies

The remaining plant samples were stored in Formaldehyde (FAA) for herbarium studies. FAA ensures that the plant is preserved for further identification.

2.7 Plant anatomy

Sections of leaves were cut into a thickness of 10-20 microns, using CO_2 and Hamilton's solution to freeze the sample. Slides were then made from these sections and photos were captured digitally, using an Olympus photomicroscope.

2.8 Antifungal studies

2.8.1 Preparation of fungi

Exactly 50 ml of water was used with Tween to allow the fungal spores to disperse more easily. The spores were then collected with a 1ml pipette and added to the suspension, of Tween and water, after which the suspension was shaken. Filling the Neubauer chamber made a direct cell count of each spore concentration.

The microscope objective (x10) was focused on the squares and all the spores contained within the 5 groups of 16 small squares (= 80 small squares) were counted. About 80 squares containing spores were counted under 100x magnification using a Nikon electronic absorption microscope.

2.8.2 Methods of applying extracts to fungi

2.8.2 a Filter paper technique

This method involved cutting the filter paper into strips of 2mm wide to which the *H. patulum* extract was applied. The extract was then left to dry, while the plates were streaked. This method was used for the fungi, *Botrytis cinerea* and *Venturia inaequalis*. The filter paper was then put onto the plates that were streaked with the fungi. After 24 hours, the length (µm) of fungal germ tubes was measured. The length of the germ tubes was indicative of the efficacy of the extract.

2.8.2.b Extracts on agar plate

This method involved the use of 1ml of the extract, which was poured onto the agar plate. The agar was tilted to ensure that there is equal distribution of the extract throughout the plate. The fungi were then poured onto the plate that was covered by the extract, after the latter had dried. After 24 hours, the germ tube lengths (µm) of the fungi were measured to assess the effect of *H. patulum* extract.

2.9 Animal studies

The Medical Research Council (MRC) supplied 20 Wistar male rats with an average mass of 350-400g for this investigation. The rats were divided into two groups of ten each and housed in pairs of two in plastic cages with mesh wire flooring and tops, in a temperature-controlled room. The cages were 35 cm x 35 cm with a height of 23 cm. The animals had a period of two weeks to acclimatize to the room. A control group receiving only distilled water as a

substitute for the plant extract was used, and a second group was medicated with a water extract of *H. patulum.* Four milliliters of the herbal plant was then administered to each individual rat by means of a Terumo spinal needle, 1.20 x 90mm in diameter, with a round bulb-shaped edge, in order to avoid any damage to the animals. The medicine was given at days 15, 30 and 45.

2.9.1 Metabolic Analysis

Ten rats were weighed individually and each put into metabolic cages at days 15, 30 and 45. Each rat was given 40 g of food, and 60 ml of distilled water for the 24 hour collection of metabolic data. After 24 hours had elapsed, the rats were taken out of the metabolic cages and were weighed again. The pH of the urine was also measured. Food consumption and water intake were also determined. The rats were sacrificed after 45 days using chloroform and ether. Terminal blood samples were taken from the left ventricle. About 3ml was placed in an EDTA vacutainer for the analyses of Red Cell Count (RCC), haemaglobin, haematocrit, Mean Cell Volume (MCV), Mean Corpuscular haemaglobin (MCH), Mean corpuscular haemaglobin concentration (MCHC), RDW, White blood Cell Count (WBC), neutrophil levels, basophil levels, lymphocyte levels, monocyte levels, eosinophil levels, Neutrophil Absolute count (NAC), Monocyte Absolute count (MAC), Eosinophil Absolute count (EAC), Basophil Absolute count (BAC), Lymphocyte Absolute count (LAC), and platelet levels.

2.9.2 Animal histology

At termination, the liver and testis of the animals were removed for analyses. These organs were stored in Bouins' fluid and prepared for histological sectioning. The tissue was then processed with a histokinette for 22 hours and embedded in wax. Tissue were cut with disposable blades at 5 microns and stained with Heamotoxylin and Eosin stain and mounted with DPX.

2.10 Statistics

Data was analyzed using Microsoft Excel Stat (2000). The significant differences between rat weights of the control and experimental group,

necessitated baseline corrections to be made to data, for the respective collections made over the experimental period. Control and experimental animal groups were then compared with one another and a minimum significance of $P< 0.05$ was determined for all metabolic parameters using the Mann-Whitney test. Baseline corrections were not applied to blood data, which was directly compared. Significant differences were also determined at a minimum level of $P< 0.05$ with the Mann-Whitney test.

The antifungal data were sorted using the stem and leaf method and p-values were determined using the Mann-Whitney test.

3. Results

3.1 Plant histology

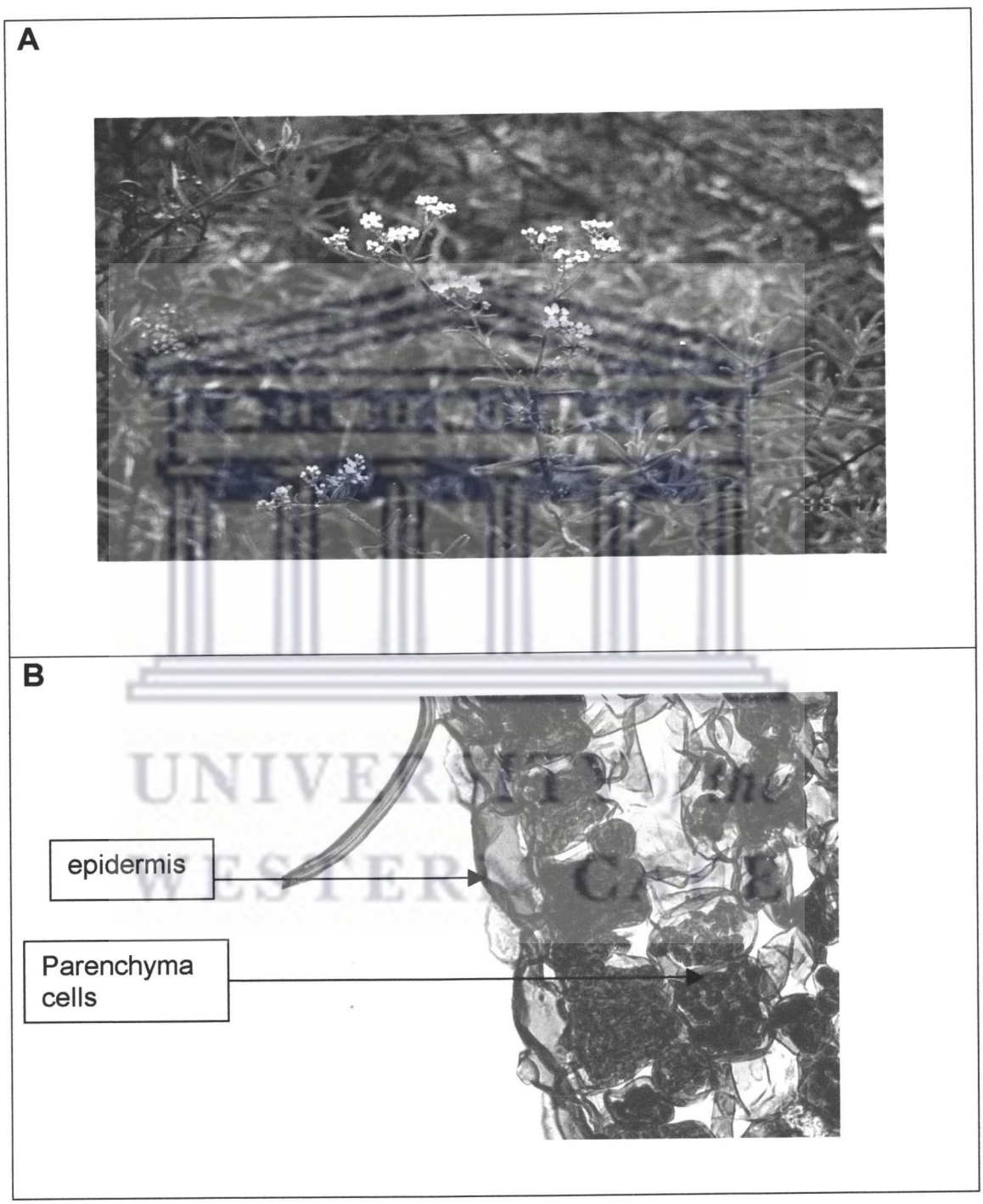


Fig. 1 a, b: Intact plant of *Helichrysum sp.* and a cross section of *H. patulum* using freeze microtomy.

3.2 Elemental Analysis results

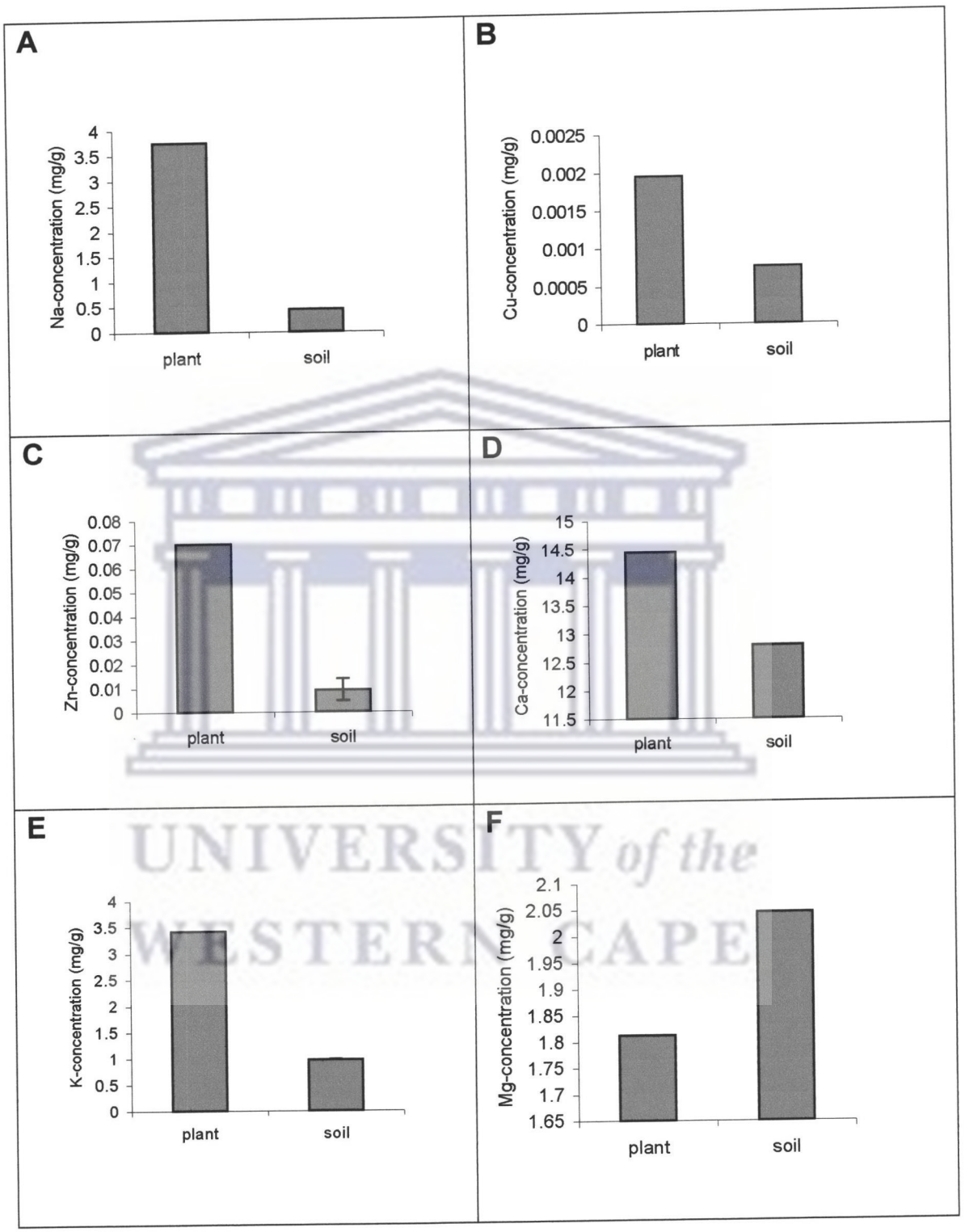


Fig. 2 a-f. The elemental levels of *H. patulum* leaves and the soil in which it grows.

3.3 Antifungal results

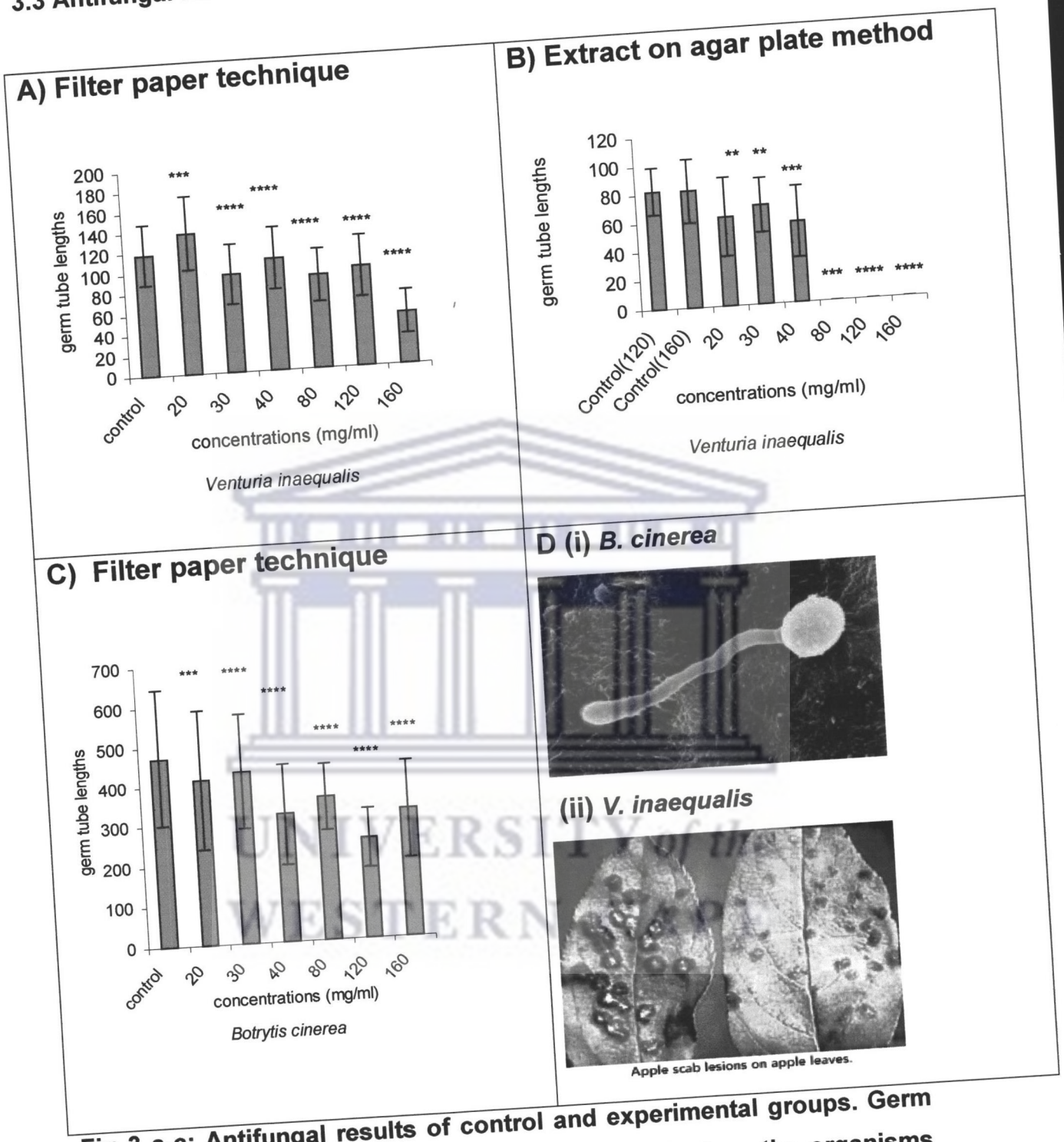


Fig 3 a-c: Antifungal results of control and experimental groups. Germ tube lengths were measured in µm. Fig 3d i/ii displays the organisms and its effect on apple leaves.

Significance in relation to control and experimental groups:*p<0.05, **P<0.01, ***p<0.005, ****p<0.0001

3.4 Metabolic outcomes

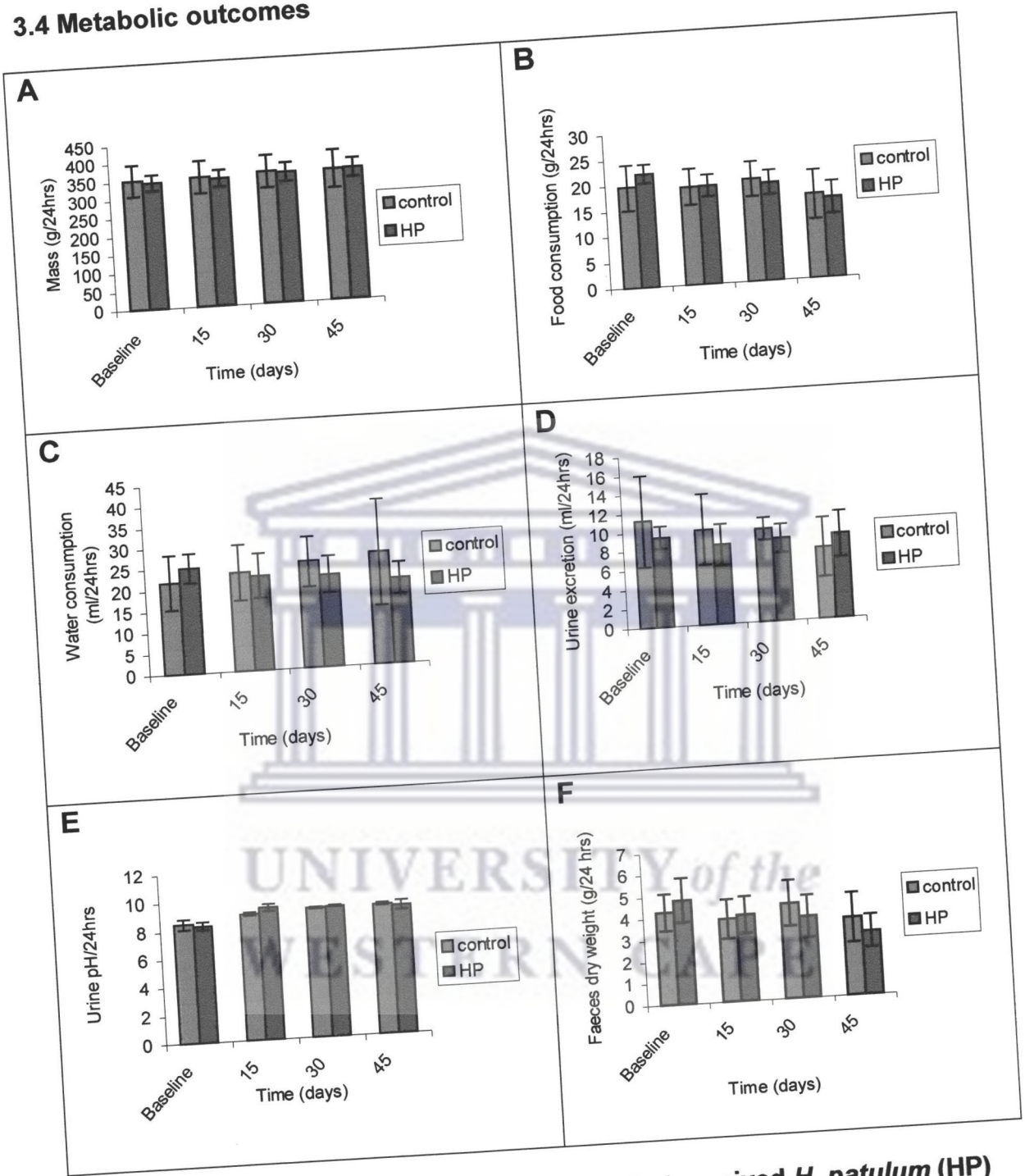


Fig. 4 a-f: Metabolic parameters of animals that received *H. patulum* (HP) extract compared to those who received a placebo. Significance of experimental groups in relation to control group: *p<0.05.

3.5 Haematological profiles

Red blood cell parameters

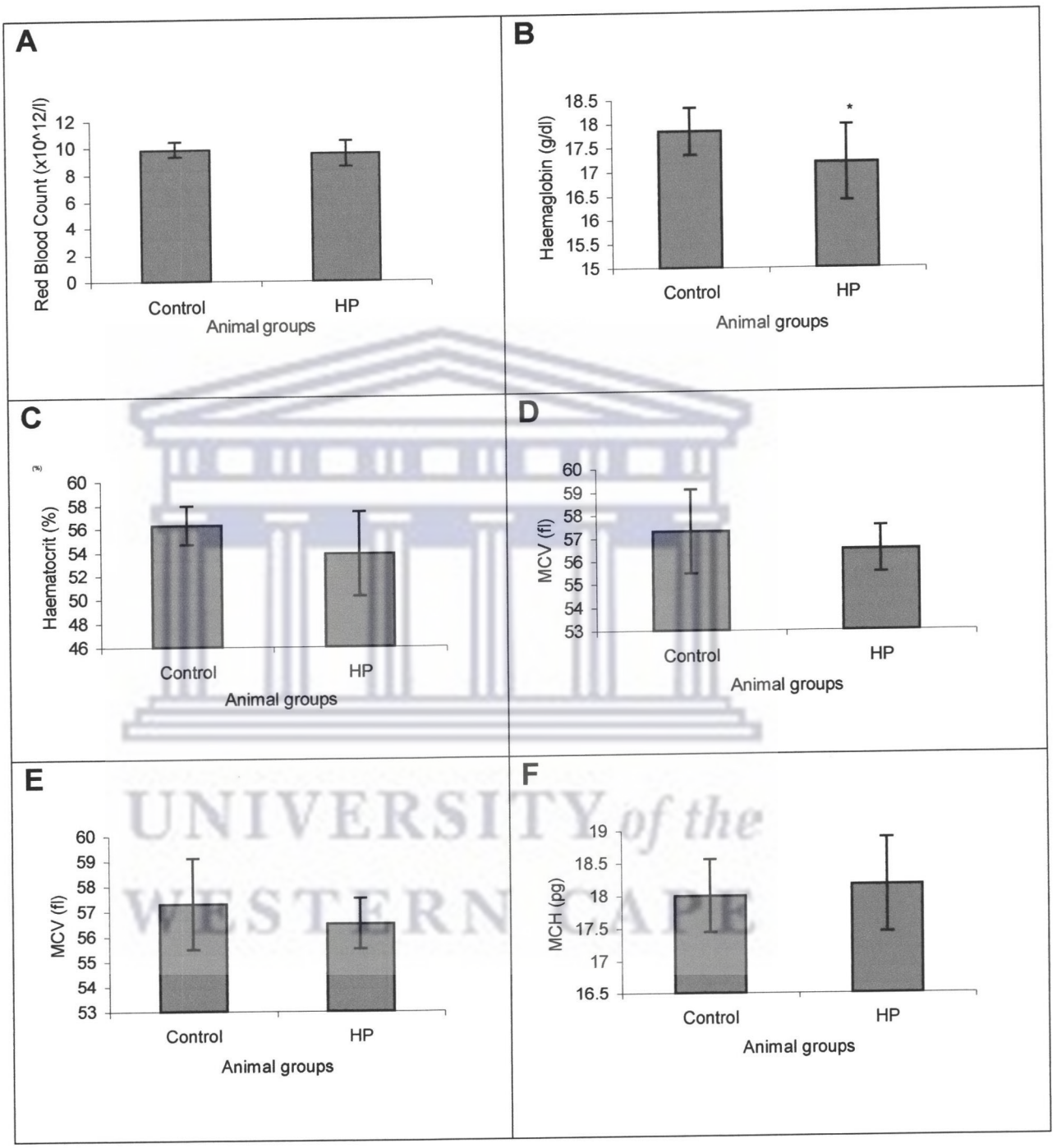


Fig 5. a-f: The various red blood parameters of animals that were fed *H. patulum* (HP) compared to those that received the placebo. Significance in relation to control and experimental groups: *p<0.05.

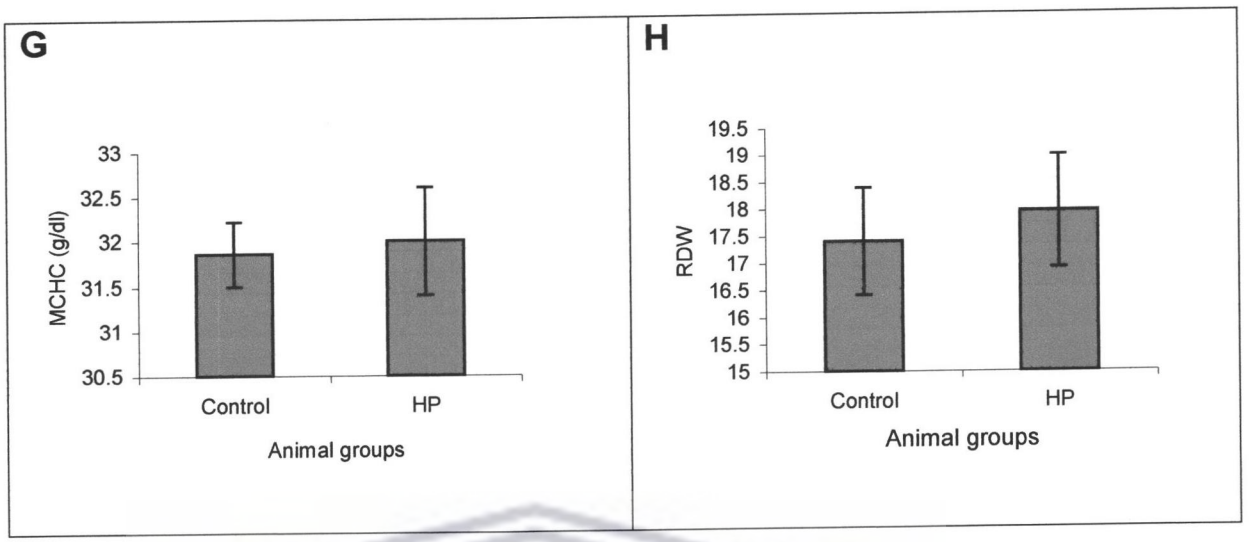
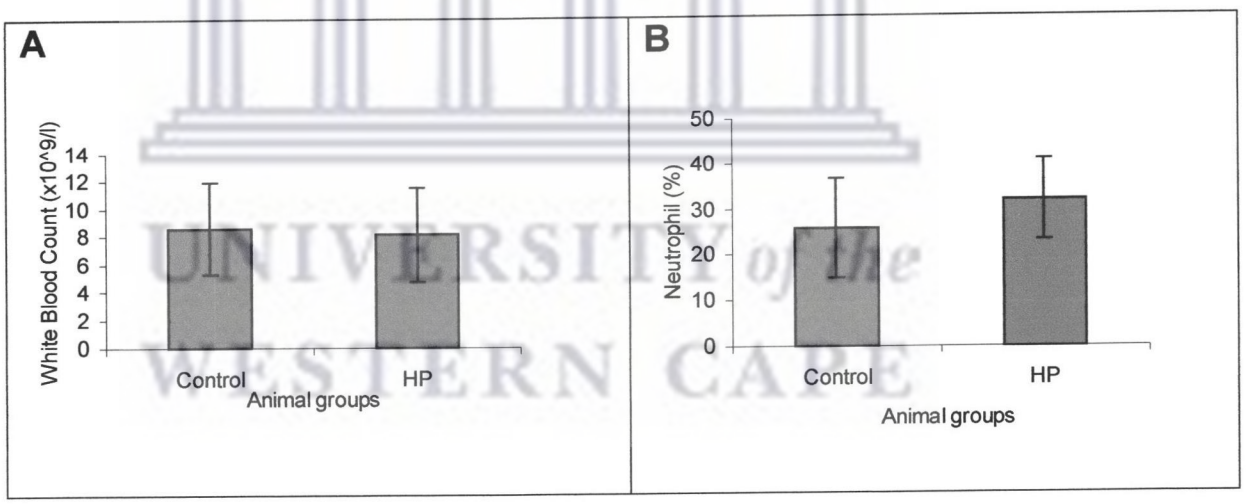
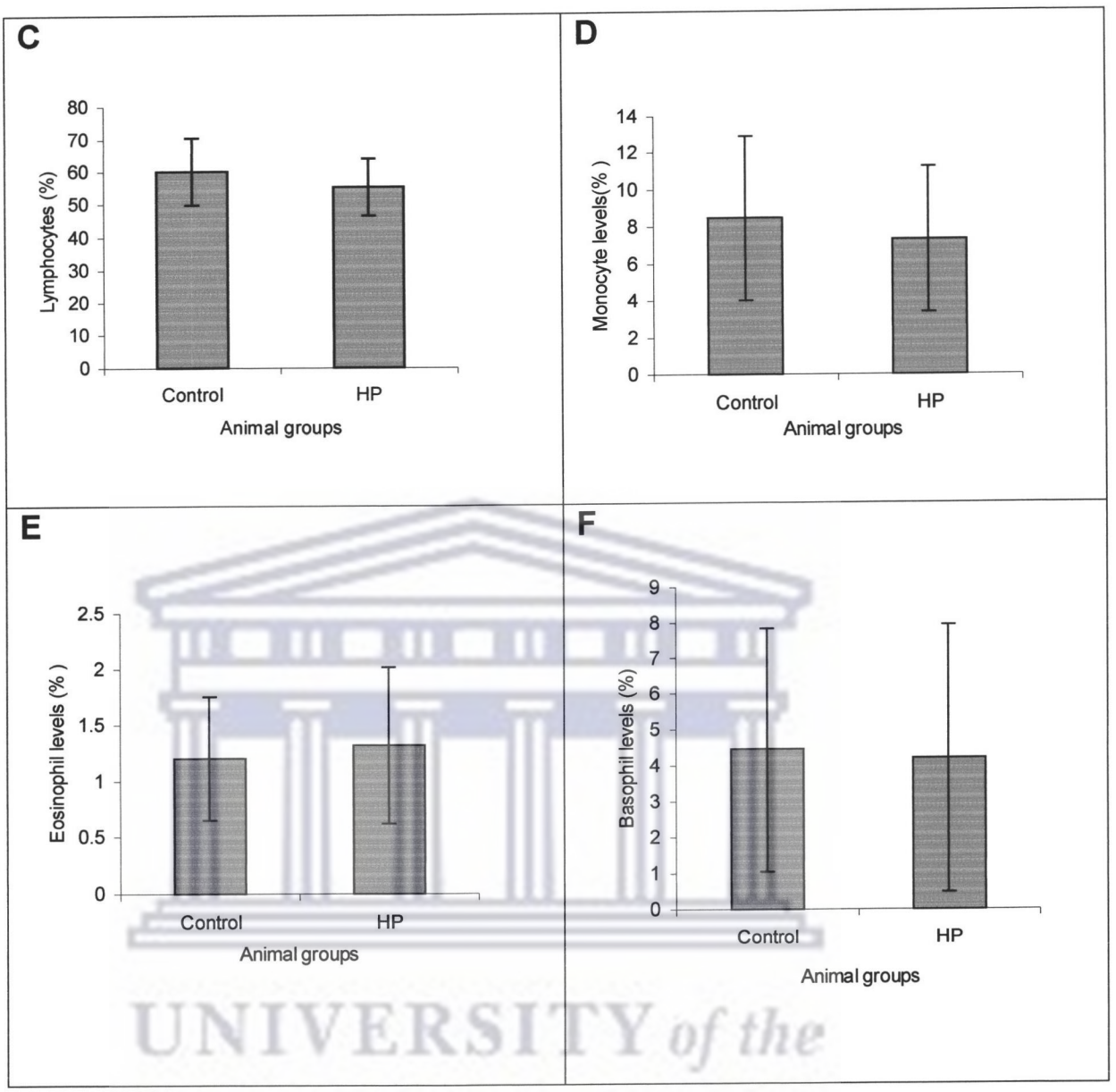


Fig 5. g-h: The various red blood parameters of animals that were fed *H. patulum* (HP) compared to those that received the placebo. Significance in relation to control and experimental groups: *p<0.05.

White blood cell parameters





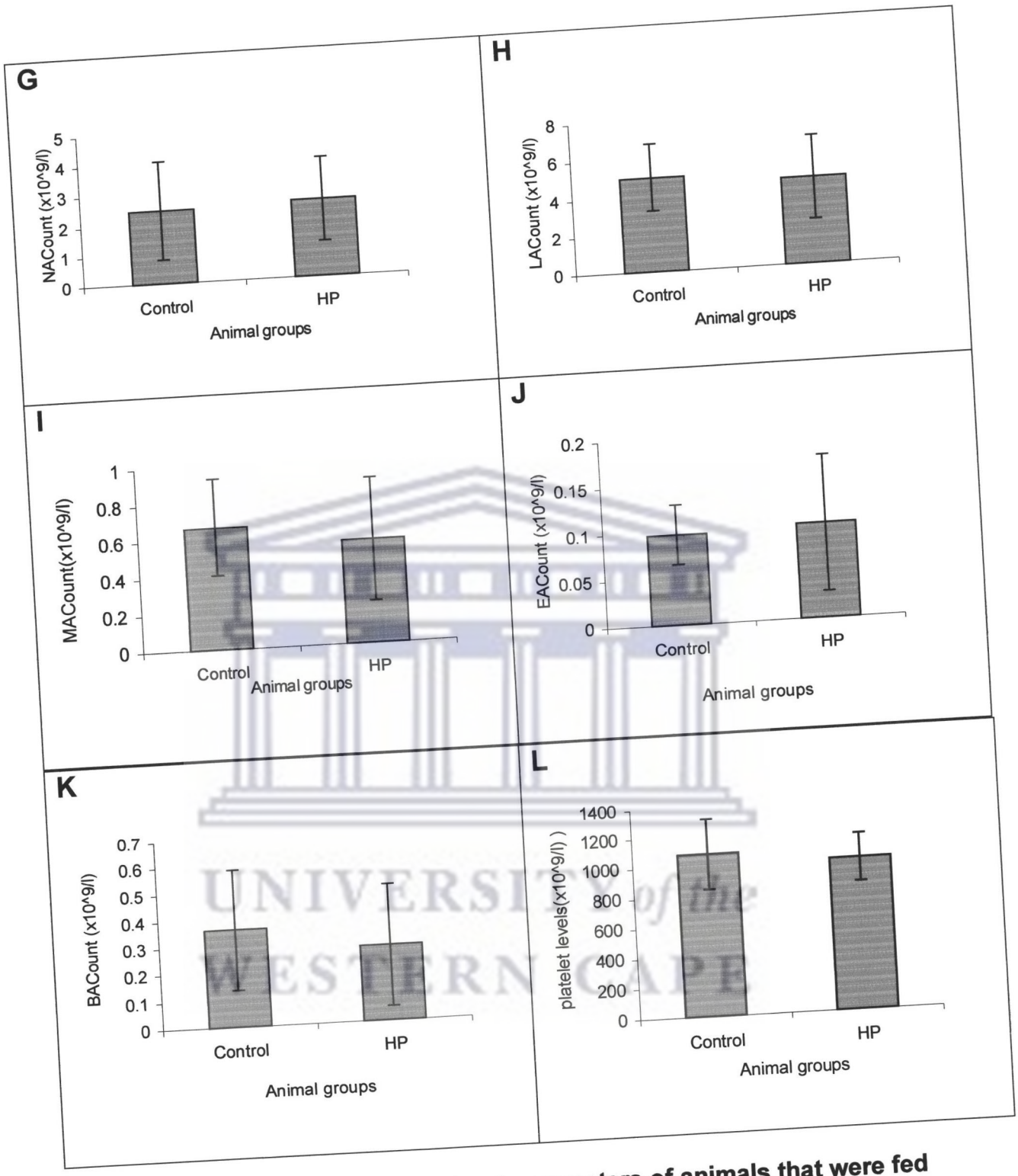


Figure 6 a-l: The various white blood parameters of animals that were fed *H. patulum* (HP) compared to those that received the placebo. Significance in relation to control and experimental group: *p<0.01

3.6 Animal tissue histology

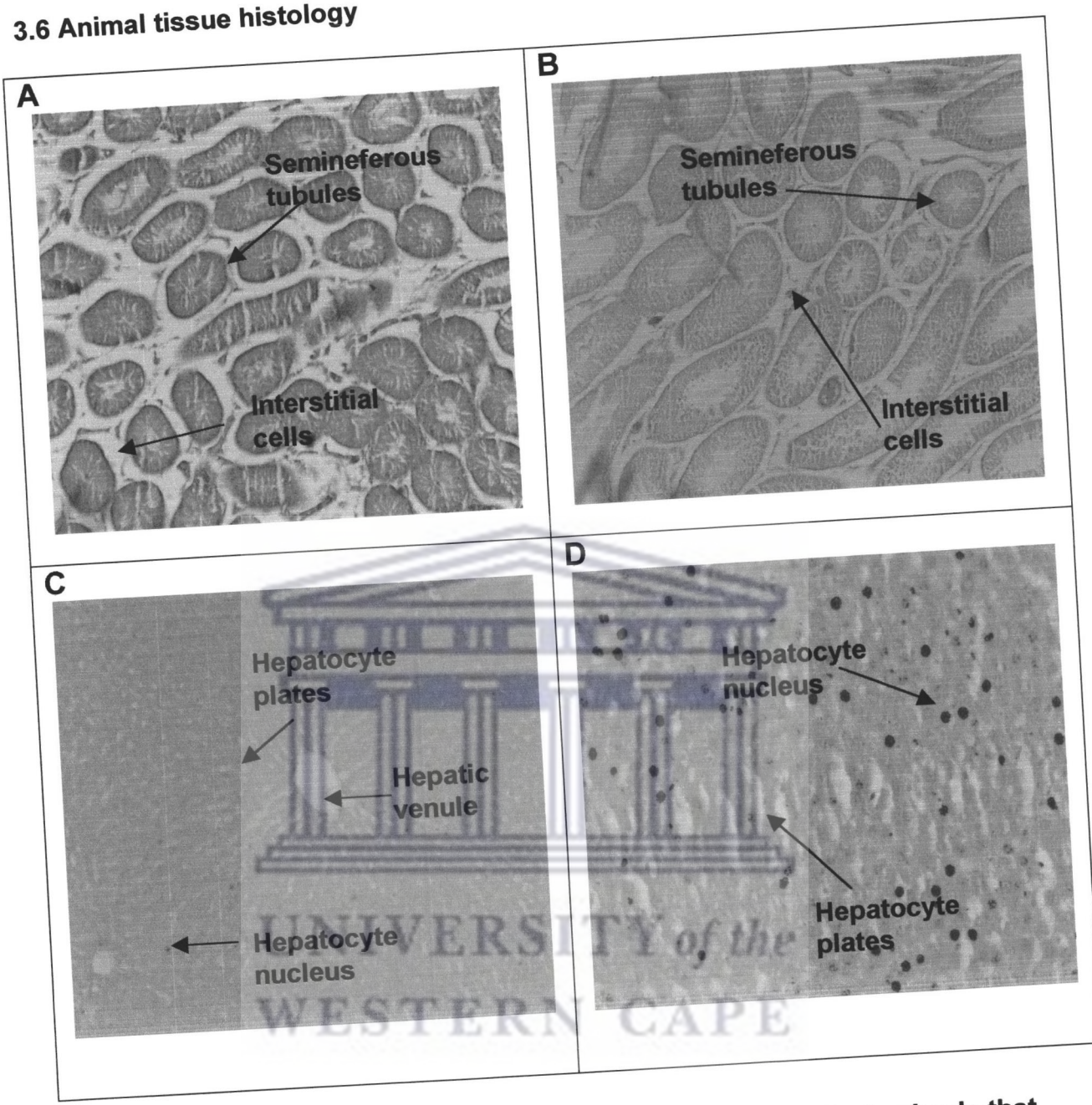


Figure 7 a-d: Cross sections of the testis (a,b) and liver (c,d) of animals that were given *H. patulum* extract and those that received the placebo.

4. Discussion

This study provides evidence that *H. patulum* crude methanol extract consist of compounds with antifungal activity against important plant pathogens such as *B. cinerea* and *V. inaequalis*. The germ tube lengths were an indication of the plant's effect on the plant pathogen.

The anatomy of the plant is indicated in Fig. 1 b. Parenchyma cells is indicated in Fig. 1b, and is a unspecialized cell, that lacks a secondary cell wall, but a primary cell wall is present, and all types of cells develop from parenchyma cells. Elemental analysis showed no significant changes between plant and soil ($p>0.05$).

B. cinerea, in a fungus that has the ability to go unnoticed in vineyards, and cause grey mould. *B. cinerea* usually grows on dead plant material, but this does not occur in vineyards. It attacks a variety of crops. The fungus moves in vineyards mainly as conidia, which is a structure, derived from sclerotia. Sclerotia are tough survival structures that may develop on any infected plant material, where it can survive throughout winter. It germinates in late winter and form conidia. Conidia are little spores (Fig. 3d(i)) with a diameter of about 0.01mm. It gets released, by wind or insects, land on plant parts, and germinates (3).

Apple scab, caused by the fungus *Venturia inaequalis,* is one of the most serious diseases of apple. Disease development is favored by wet, cool weather that generally occurs in spring and early summer. Both leaves and fruit can be affected. Infected leaves may drop resulting in unsightly trees with poor fruit production. This early defoliation may weaken trees and make them more susceptible to winter injury or other pests. Infected fruits are blemished and often severely deformed. Infected fruits may also drop early (2). It survives the winter in the previous year's diseased leaves on the orchard floor. In the spring, the

fungus in old diseased leaves produces millions of spores. The spores get dispersed through rain and any wet conditions, favorable for it to germinate (2).

Antifungal results show significant inhibition of the germ tube lengths against the plant pathogens *V. inaequalis* and *B. cinerea*, as indicated in Fig. 3 a - c. In Fig. 3a the plant extract was tested against *V. inaequalis*, using a technique known as the filter paper technique, and a significant decline at 20 mg/ml ($p<0.005$), 30 mg/ml ($p<0.001$), 40 mg/ml ($p<0.001$), 80 mg/ml ($p<0.001$), 120 mg/ml ($p<0.001$) and 160 mg/ml ($p<0.001$) was observed. Fig 3 b showed that the herbal plant inhibited growth significantly at concentrations 30 mg/ml ($p<0.01$), 40 mg/ml ($p<0.01$), 80 mg/ml ($p<0.005$), 120 mg/ml ($p<0.0001$) and 160 mg/ml ($p<0.0001$).

Fig. 3c shows the effects of the plant extract against the plant pathogen *B. cinerea*. The plant extract of *H. patulum* also showed to be significant against *B. cinerea,* with a decline in germ tube lengths ($p<0.0001$). Significant inhibition against this plant pathogen by *H. patulum* was observed. The most diluted concentration 20mg/ml ($p<0.005$) showed a significant inhibition of the germ tube length. Significant inhibition of the germ tube lengths occurred from concentrations of 20 mg/ml to the strongest concentration of 160 mg/ml ($p<0.0001$).

After it was established that *H. patulum* effectively inhibits the growth of *B. cinerea* and *V. inaequalis*, metabolic studies were done which involved parameters such as faecal weight, mass of the animals, urine excretion and pH, and water consumed (Fig. 4 a-f). Haematological analyses were also executed (Fig. 5 a-g, and Fig. 6 a-l). No significant changes were observed within metabolic studies and haematological parameters (13).

Animal histology is indicated in Fig. 7 a-d. Seminiferous tubules are the exocrine part of the testis, which also consists of an endocrine part, called the Leydig cells or interstitial cells. The interstitial cells are responsible for the secretion and synthesis of the male steroid hormone testosterone (13).

The smoke of many *Helichrysum* species is used as ritual incense. The main plant parts used for medicinal purposes are, leaves and twigs and sometimes the roots. Ailments like coughs, colds, fever, and infections. There are a number of ways of administering these traditional medicines. For coughs and colds, a tea is prepared or the leaves are boiled in milk. Smoke from burning leaves is inhaled and is a better way for administering pain relief. Leaves are widely used on wounds to prevent infection. Flavonoids, sesquiterpenoids, and acylated phloroglucinols are found in the plant. These plants also have pharmacological effects. It has been reported that pain-relieving, anti-infective, and anti-inflammatory activity exists for several *Helichrysum* species. Scientific evidence for the traditional use in wound dressing provides proven anti-microbial activity. *Helichrysum* species are distributed all over South Africa and their medicinal use often depends on local availability rather than a preference for particular species (10). It is thought that the leaves of *Helichrysum* contain the active substance used for medicinal purposes.

More than 85% of higher plants have not been sufficiently surveyed for potentially useful biological activity and the plant kingdom has not received sufficient attention as a resource of possible medicinal agent. Over half of the world's 25 best selling pharmaceuticals for 1999 owed their origin to a natural source material (7)

Medicinal plant is one in which one or more of the plant's organs, contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. This needs to be scientifically proven, although

many medicinal plants are used by traditional healers and are not tested scientifically (5).

Natural products have historically been used in numerous applications in the fungal, weed and insect control sectors of agriculture. Plants have an excellent track record in providing novel leads for crop protection, particularly in the field of insecticides. This can be attributed to the evolution of secondary metabolites, which address the specific needs of the host plant in protecting it from insect attack (4).

Whole animal models are used in most cases for *in vivo* experiments, thus to collect biological material. This needed to study enzymes, cells, tissues and organs which are obtained from when animals are dead. Therefore animals must be sacrificed. Most large pharmaceutical companies dispose of a large battery of in vivo tests for the secondary evaluation of selected leads. *In vivo* tests are done mostly for the determination of activities of the cardiovascular, intestinal, liver and respiratory system, as well as for the determination of anti-inflammatory, antiviral, and antidiabetic effects. Many *in vivo* tests also remain the stepping-stone for many corresponding tests in human clinical traits (7).

Metabolic results showed no significant changes in either the control group or those receiving the water extract of *H. patulum*, as indicated in Fig. 4b. In the blood analysis, haemaglobin levels were significantly lower in the experimental group than in the control group ($p<0.05$), as shown in Fig.5b. Animal histology is indicated in Fig 6 and liver and testis in both groups were not very different. These two organs were chosen because they are most susceptible to potential toxicological insult.

Compounds from natural sources have the potential to enrich the collection for candidates for biological assessment. They not only bring with them their own diversity, but also a set of challenges which, if successfully resolved, will provide

the crop-protection industry with opportunities to safeguard the supply of food well into the next century (4). The outcome suggests that *H. patulum* would be a challenging candidate for further biopesticidal testing.

5. References

1. Buehler, B.A. 2000. Interactions of Herbal and Alternate Remedies with conventional medicines. Teratology 61(6): 465. Nebraska.

2. Ellis, M.A., Chatfield, J., and Draper, E. 2000.Scab of Apple and Crabapple. Ohio State University Extension Fact Sheet. Plant Pathology.

3. Holz, G. 2001.The Occurrence and Control of *Botrytis cinerea* (Grey Mould) in Grapes: A South African Perspective. Winetech. Department of Plant Pathology. Stellenbosch. South Africa.

4. Karousou, R., Bosabalidis A.M. and Kokkini. S. 1992. *Sideritis syriaca* ssp. syriaca: glandular trichome structure and development in relation to systematics. Nordic Journal of Botany 12(1): 31-37.

5. Kokoska, L; Polesny, Z; Rada, V; Nepovim, A; and Vanek, T. 2002. Screening of some Siberian medicinal plants for antimicrobial activity. Journal of Ethnopharmacology. 82(1): 51-53. Czech Republic.

6. Matthews. 2001. Chemical pesticide markets, health risks and residues, J.Harris, Biopesticides Crop Protection 20 (5): 452.

7. Ojala, T., Remes, S., Haansuu, P., Vuorela, H., Hiltunen, R., Haahtela, and Vuorela, P. 2000. Antimicrobial Activity of some Coumarin containing plants growing in Finland. Journal of Ethnopharmacology. 73 (1-2): 299-305.

8. Rao, S.R., and Ravishankar, G.A. Plant Cell cultures: Chemical Factories of Secondary Metabolites. Biotechnology Advances. 20 (2): 101-153. Japan.

9. Rice, M.J., Legg, M., and Powell, K.A. 1998. Natural Products in Agriculture- A View from the Industry. Pestic. Sci.52, 184-188. Great Britain.

10. Rousseaux, C.G. 2000. Regulatory Issues concerning the safety and efficacy of herbal and alternative remedies. Teratology 61(6): 466. Canada.

11 Sofowora, A. 1982. Medicinal Plants and Traditional Medicine in Africa. John Wiley And Sons Ltd. Nigeria.

12. Van Wyk, B.; Van Oudtshoorn, B.; and Gericke, N. 1997. Medicinal plants of South Africa. Briza Publications. South Africa.

13 http://handlin.cvm.okstate.edu/histology/MR/HiMRp3.htm

14. Vlietinck, A.J. 1999. Screening Methods for Detection and evaluation of Biological Activities of Plant Preparations. Department of Pharmaceutical Sciences, University of Antwerp (UIA), Universiteitsplan, 1,B-2610 Antwerp, Belgium. Ed. Bohlin, L, and Bruhn, J.G. Bioassay Methods in Natural Product Research and Drug Development.

13. www.e-histology.net

14. Swerdlow, J. 2000. Nature's Medicine. Plants That Heal, National geographic Society.

Summary

Plant pathogens have been a problem on crop fields for centuries and have been the cause of a significant loss of revenue. Many of these pathogens have become resistant to the conventional synthetic pesticides or there is a need to find effective, safe and affordable alternatives.

Plants were selected mainly on the basis of it being known to repel insects. It is also used for various ailments. There are certain pesticides derived from natural materials such as animals, plants, bacteria and certain minerals, for example canola oil and baking soda have pesticidal applications. Certain essential plant oils, widely used as fragrances and flavors in the perfume and food industries, have long been reputed to repel insects. Recent investigations in several countries confirm that some plant essential oils not only repel insects, but also have contact and fumigant insecticidal actions against specific pests, and fungicidal actions against some important plant pathogens.

The screening of plant extracts and natural products for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents, as well as serving as a drug discovery platform for primary lead natural compounds. More than 85% of higher plants have not been sufficiently surveyed for potentially useful biological activity and the plant kingdom has not received sufficient attention as a resource of possible medicinal agents.

The smoke of many *Helichrysum* species is used as ritual incense. The main plant parts used for medicinal purposes are leaves and twigs and sometimes the roots. Ailments like coughs, colds, fever, infections, headache and menstrual pains are treated with this commonly used herb. There are a number of ways of administering these traditional medicines. For coughs and colds, a tea is prepared or the leaves are boiled in milk. Smoke from burning leaves is inhaled and is a better way for administering pain relief. Leaves are widely used on wounds to prevent infection.

Two members of the family Asteraceae, *Helichrysum marifolium* and *Helichrysum patulum,* were extracted with methanol and tested as a potential biopesticide. Two methods were used to tests the plant extracts on two pathogens, namely *B. cinerea* and *V. inaequalis*. The results showed the plants significantly inhibited the growth of both *B. cinerea* and *V. inaequalis*.

Animal studies were done to determine whether the plant have any adverse effects on living organisms, rats being a representative of a human model. In order for these herbs to be used outside of this study, its toxicology has to be taken into consideration, and thus the tests on animals were performed. Animals were sacrificed and blood samples were taken and organs were removed for analysis. Various blood parameters were also tested. Organs were histologically analysed. Histological sections determined any different effects on a control group versus the groups receiving medicine, respectively. Testis and liver were removed for analysis, because these are tissues that are the most sensitive to potential toxicological insult.

H. marifolium and *H. patulum* showed little or no effect on animals and can therefore be used as potentially safe and effective biopesticides. This assertion though should be underpinned by longer-term investigation.