UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

An investigation into the topical and systemic safety and efficacy of a new carbohydrate derived fulvic acid (CHD-FA) product.

by

Riaz Ahmed Sabi

Dissertation presented in the partial fulfilment of the requirements for the Degree of Masters in Science in Pharmacology at the University of Pretoria.

July 2008

Promoter: Prof C.E. Medlen

ABSTRACT

Humic substances are a group of ubiquitous compounds formed during the decay of plant and animal residues in the environment. These substances can be divided into humic acid, fulvic acid and humin on the basis of its solubility in water as a function of pH. Fulvic acid is the fraction that is soluble in water under all pH conditions and is usually extracted from brown coal and is therefore associated with high levels of heavy metals. These heavy metal levels have become a point of concern and a unique method for synthesizing fulvic acid from a carbohydrate source has been subsequently developed and patented in an attempt to address this problem. The purpose of this study was therefore, to test the topical and systemic safety and efficacy of this new carbohydrate derived fulvic acid (CHD-FA) product.

Initial *in vitro* screening using the Kirby-Bauer disk diffusion method indicated that the product was active against the *Staphylococcus aureus* test organisms used (ATCC 12600 and P3938). Results showed that not only was it as effective as the oxacillin control, but in the case of a more virulent strain of *S. aureus*, the CHD-FA (3.9% w/v) proved more active.
Subsequently, the ability of the product to cross full-thickness skin was ascertained using Franz-type diffusion cells fitted with 100µm skin disks obtained from rats. At the end of a 12-hr period, results indicated that up to 20% of the CHD-FA at a pH of 2.9 was able to cross the skin barrier.

Divided into 6 phases, extensive animal safety studies were conducted on the product. In phase I, toxicity and sensitivity to topical application was examined by applying the product to the ears of mice over a period of 30 days. Phase II and III was undertaken to study the long term effects (6-weeks and 6-months) of CHD-FA ingestion (150 mg/kg body mass) whilst phase IV tested the effects of

CHD-FA ingestion on pregnant rats and their off-spring. Phase V was an acute toxicity study in which rats received a single oral dose of CHD-FA (150mg/kg bw) and then observed for a period of 7 days. Phase VI was a 6-week chronic study in which the animals received 100mg/kg bw daily for the duration of the experiment. Results obtained in all studies showed that CHD-FA is safe for topical as well as, oral use at the doses tested. In addition, it is safe for use during pregnancy.

Finally, the *in vivo* anti-microbial efficacy of the CHD-FA was examined using an infected wound healing model. A pilot study indicated that the animals used should be immunocompromised and the wounds inoculated with a *Staphylococcus aureus* (ATCC 12600) concentration of $1x10^{10}$ CFU/ml.
Results from the main study showed that topical applications of the CHD-FA (pH 1.98) product at a concentration of 1.75% w/v over the 5-day period produced a wound healing pattern similar to that of the positive fuscidic acid control. In contrast, animals receiving an oral treatment of the product did not produce a pattern different from that of the water control group.

In conclusion, CHD-FA has been shown to be safe and effective in treating *S. aureus* infections *in vitro* and in animal models. This study indicates that the next phase of human studies is certainly warranted.

ACKNOWLEDGEMENTS

I would like to thank my promoter and supervisor, Prof C.E. Medlen, for her guidance and support throughout the course of this research.

In addition, I`d like to extend a note of gratitude to Mr. P. Selahle, Mrs L. Sentle and Dr. R. Auer for their invaluable help and guidance in all the animal studies.

Thanks are due as well to Mr. A.C. Fernandes for all his help and guidance pertaining to all the microbiology studies.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at the University of Pretoria a great experience.

Finally, thanks to my mother and father for their patience and encouragement.

NOMENCLATURE

ALT	Alanine-amio tranferase
ANOVA	Analyses of variance
AST	Aspartate-amino tranferase
ATCC	American Type Culture Collection
CHD-FA	Carbohydrate Derived Fulvic Acid
CLSI	Clinical and Laboratory Standards Institute
GGT	Gamma-glutamyl transferase
Hb	Hemoglobin
HBSS	Hanks Buffered Saline Solution
Ht	Hematocrit
MCHC	Mean Cell Hemoglobin Concentration
MCV	Mean Cell Volume
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant Staphylococcus Aureus
RCC	Red Cell Count
RDW	Red blood cell Distribution Width
THR C	Thrombocytes
UEA	Aqueous cream
U.S. FDA	United States Food and Drug Administration
UPBRC	University of Pretoria Biological Research Council
WCC	White Cell Count
WHO	World Health Organization

Table of Contents

CHAPTER I: LITERATURE REVIEW

History of infectious diseases and antimicrobial treatment

There is little doubt as to the profound influence infectious diseases have had on the course of human history. Throughout history, humanity has fallen victim to pandemics of cholera, plague, influenza, typhoid, tuberculosis and other infectious maladies so widespread, most people rarely made it to middle age. Other less dangerous ailments such as ear, skin and throat infections often resulted in deafness, disfigurement and even death due to scepticema and other complications [1]. Many diseases, including smallpox, leprosy, tuberculosis, meningococcal infections and diphtheria were epidemic in ancient Greece and Egypt. The black plague (caused by *Yersinia pestis*) changed the social structure of medieval Europe. This epidemic spread to Italy and Egypt in 1347 on merchant ships carrying rats and fleas infected with the plague bacillus. During the next 5 years, plague, also known as the *Black Death*, extracted its devastating toll on the population of Europe. The final tally amounted to roughly 24 million people dead in Europe - that is 1 in every 10 people. The Middle East wasn't spared either- the epidemics of plague killed about a quarter of the population there [2].

We are, thankfully, the first generation ever to have the means of protecting itself against some of the most deadly and common infectious diseases. This is largely due the discovery of antibiotics. Although antibiotic chemotherapy has completely transformed the approach to infectious diseases, the use of substances with anti-infective properties is, however, not a totally new concept. Substances with anti-infective potential have been applied medically for

thousands of years. For example, the Chinese were aware of the therapeutic properties of mouldy soybean curd applied to carbuncles and boils, over 2500 years ago [3]. Ancient Greek physicians, including Hippocrates, routinely used substances with antimicrobial activity. These included wine, myrrh and inorganic salts in their treatment of wounds. Heavy metals, such as arsenic and bismuth were found to be useful against a number of infections, including syphilis, in the early 1900's [3].

The modern era of chemotherapy did not really begin though, until the discovery and initial clinical use of the sulphonamides [3]. The introduction of sulphonamides in the mid-1930's and of penicillin and streptomycin a decade later ushered in a glorious period in modern medicine [4]. The knowledge to prevent or cure diseases such as malaria, tuberculosis, HIV, pneumonia and measles are now widely known and implemented. Not only has modern antibacterial therapy markedly reduced the morbidity and mortality of infections but its widespread use has contributed significantly to the development of modern surgery, trauma therapy and organ transplantation [4].

The widespread use of antimicrobials has however, not come without its own set of problems. In addition to interacting with concurrent medication, some of these drugs can on occasion, cause serious adverse reactions. Widespread use has also provided a selective pressure for the increasingly widespread antimicrobial resistance among bacteria [4]. As early as half a century ago, a few years after penicillin was put on the market, scientists began noticing the emergence of a penicillin-resistant strain of *Staphylococcus aureus*, a bacterium common to the human body. Resistant strains of gonorrhoea, dysentery-causing Shigella and Salmonella followed rapidly in the wake of *Staphylococcus* 20 to 25 years later [1].

Bacterial resistance can result from modification of the antibacterial targets or from bypassing of that target. It can also be contingent on impermeability, efflux or enzymatic deactivation. Some bacteria are inherently resistant while in others,

resistance may arise via mutation or horizontal gene transfer by plasmids, transposons and lysogenic bacteriophage. Bacteria are able to exchange genes in nature via three processes. The first is called conjugation and involves cell to cell contact as DNA crosses a sex pilus from donor to recipient. The second is known as transduction. During this process, a virus transfers the genes between mating bacteria. Finally, in transformation, DNA is acquired directly from the environment, having been released from another cell. The formation of biofilms by bacteria is another means by which bacteria can acquire resistance.

Several factors have been identified as contributors of resistance. Overuse of broad-spectrum antibiotics, such as second- and third-generation cephalosporins, greatly hastens the development of methicillin resistance; even in organisms that have never been exposed to the selective pressure of methicillin *per se* (thus the resistance was already present). Other factors contributing towards resistance include incorrect diagnosis, unnecessary prescriptions, improper use of antibiotics by patients, and the use of antibiotics as livestock food additives for growth promotion [5].

Since bacterial resistance is closely linked to antibiotic use, it can be considered an un-evitable antibiotic side effect. The bacterial potential to spread and share the genetic resistance determinants in hospital wards, community and even globally, makes the problem of resistance interesting not only for biomedical research, but also for societal studies, health administrators and even governments. It is estimated that about 20 to 50% of human antibiotic use is unnecessary [5].

After a flurry of discoveries between 1930 and 1970, the past 30 or so years have yielded fewer and fewer breakthroughs in the fight against infectious diseases. The 1970's brought acyclovir which is very effective against herpes zoster (shingles, cold sores and genital herpes). Today, antimicrobial compounds have increased to more than 150. The cost of development has been staggering though. Pharmaceutical companies routinely spend US$ 500 million on research

and development for every new compound that makes it to market. For every success, there are many failures and drug resistance is already nibbling away at medications which took decades to develop [6].

In less than a decade, newly used inexpensive drugs such as trimethoprim have gone from being highly effective in the treatment of dysentery in developing countries to becoming unusable in several of these areas. In the past, new drug discoveries have allowed us to be one step ahead of the bacterial pathogens. Nonetheless, the rapid evolution of resistance has limited the duration of the effectiveness of specific agents against certain pathogens. It is a deepening and complex problem accelerated by the overuse of antibiotics in developed nations and the paradoxical underuse of quality antimicrobials in developing nations owing to poverty. In its World Health Report on Infectious Diseases (2000), the World Health Organization (WHO) has acknowledged the need to increase research for new drugs and vaccines.

In order to maintain our dominance and negate the threat of microbial resistance, the development of new, cheaper and more readily available antimicrobials are essential. Failure in this task would certainly result in a post antibiotic era.

Humic substances and fulvic acid

Structure and origin

Humic substances are a group of compounds found in almost all soils and water surfaces [7]. Described as dark brown in colour, humic substances are the most widely-spread natural complexing ligands occurring in nature and are formed from the decomposition of organic matter. Generally divided into three components, based on their solubility in water as a function of pH, these are referred to as humic acid, fulvic acid or humin. Humic acids and fulvic acids represent the alkali soluble fragments and humic acids are commonly extracted

using diluted alkali and precipitated with an acid to separate it from the soluble fulvic acids [8].

It is widely believed that all dark coloured humic substances are part of a system of closely related, but not completely identical, high-molecular-weight polymers. According to this concept, differences between humic acids and fulvic acids can be explained by variations in molecular weight, numbers of functional groups (carboxyl, phenolic OH) and extent of polymerization. Carbon and oxygen contents, acidity and degree of polymerization all change systematically with an increase in the molecular weight.

Fulvic acids are therefore the component which is soluble in water under all pH conditions and is, in general, lower in molecular size and weight. This means that fulvic acids have higher oxygen but lower carbon contents than the high-molecular-weight humic acids. In addition, fulvic acids contain more functional groups of an acidic nature (COOH) and its total acidities are considerably higher than for humic acids. Another important difference is that the oxygen molecules found in fulvic acids is contained in mainly known functional groups (COOH, OH, C=O) where as with humic acids, the oxygen occurs as a structural component of the nucleus [7]. It is further distinguished in that it is light yellow in colour as compared to the dark brown/black colour of the humic acids [8].

It is apparent that humic substances consist of a heterogeneous mixture of compounds and as such, no single structural formula is possible. As far as fulvic acid is concerned, the hypothetical structure postulated (known as Buffle`s model) contains both aromatic and aliphatic structures, both extensively substituted with oxygen containing functional groups [7].

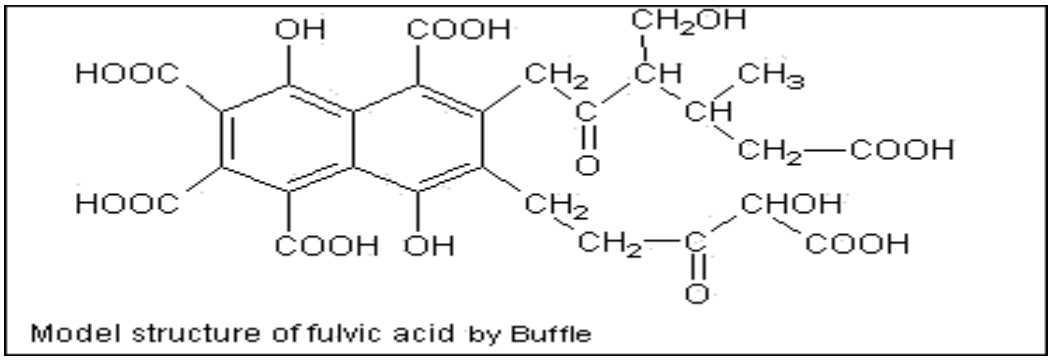

Figure 1: Hypothetical structure of fulvic acid as described by Buffle [9].

Medicinal properties

The therapeutic properties of humic substances have long been known. It has been reported that the ancient Babylonians used it for medicinal applications and that the Romans used mud baths rich in humic and fulvic acids to treat a number of ailments [10]. "Shilajit", "asphaltum", "vegetable asphalt" or "mumie" are names used for humic substance found in the Himalayan and Caucasus mountains and has been used for centuries to treat numerous ailments and improve the immune system. Among the many indications for which these humic acid type compounds are used include: burns, injuries, bone fractures, dislocations, disease of the skin, neuralgia, arthritis, poisoning and as an anti-inflammatory, antibacterial, anticancer, a diuretic and an immune stimulating agent. Other diverse conditions claimed to respond to these compounds are diabetes, cholesterolemia, eczema, amnesia, epilepsy, asthma, dysmenorrhoea and digestive disorders - including ulcers

Most studies conducted to date on fulvic acid, as far as medicinal applications are concerned, have been conducted on a fulvic acid product derived from bituminous coal via a controlled wet oxidation process [11]. These studies have shown that fulvic acids have the ability to protect against cancer and cancer causing viruses and have often demonstrated a reversal of cancers and tumours when using humic substance therapies [12]. In another study, the antibacterial

activity of bituminous coal derived fulvic acid was tested on eight microbial pathogens using the macro-broth tube dilution method. The organisms used included *Enteorcoccus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus pyogenes*, *Klebsiella pneumone*, *Proteus mirabilis* and *Candida albicans.* The results showed that all eight organisms were sensitive to the bituminous coal derivative fulvic acid at concentrations of 1,5% [13]. The anti-inflammatory properties of bituminous coal derived fulvic acid were demonstrated in a study conducted by Van Rensburg [12]. In this study the anti-inflammatory activity of topically applied bituminous coal derived fulvic acid at 4.5% and 9% was compared with that of diclofenac sodium at 1% and betamethasone at 0.1% in a murine model of contact hypersensitivity. The fulvic acid creams compared favourably with both the control creams in suppressing the cutaneous inflammatory response.

The anti-inflammatory property of fulvic acid was also confirmed in a second study, which was done on pyotraumatic dermatitis in cats and dogs [14]. In addition to these studies, a pilot study was undertaken to establish the safety and efficacy of topically applied bituminous coal derived fulvic acid cream (4.5%) compared to hydrocortisone cream (0.1%) in healthy volunteers. Fulvic acid had no significant effect on any of the safety parameters and did not induce sensitization when applied on the skin. The 4.5% fulvic acid cream caused inhibition of the elicited inflammatory reaction at 15min and differed significantly from the 9% cream at 24h. These changes compared favourably with that caused by hydrocortisone [15].

Upon examination of the research conducted on bituminous coal derived fulvic acid, the potential benefits seem astounding. The problem with extracting fulvic acid from brown coal in this manner is the associated high heavy metal levels that may result in the extracted solution. In an attempt to avoid the high levels of heavy metals found with the traditional method, a new and novel fulvic acid was developed by Fulvimed Pvt Ltd, using a patented process entailing the wet

oxidation of a metal free carbohydrate source (CHD-FA). The purpose of this study is to examine the antimicrobial efficacy and toxicity profile of this new product.

CHAPTER II: *IN VITRO* EFFICACY STUDIES

Radial diffusion inhibition study (Kirby Bauer disk diffusion)

INTRODUCTION

The radial diffusion inhibition method is a common method used for determining microbial sensitivity towards antibiotics in clinical laboratories[16]. The principle of the disk diffusion technique is that the diameter of a zone of inhibition that develops around a well filled with a sample of the antibiotic (or an antimicrobial-impregnated paper disk) relates approximately linearly to the antimicrobial's $\log_2$ MIC. Zone diameters are usually interpreted as signifying susceptibility, intermediate susceptibility or resistance to each antimicrobial agent tested according to published criteria. These criteria can only be used however, if standard procedures are followed and the method cannot be used in the case of anaerobic bacteria [17, 18]. Conditions that must be constant from test to test include the agar used, the amount of organism used, the concentration of chemical used and incubation conditions (time, temperature and atmosphere). The amount of organism used is standardized using a turbidity standard and can be determined using a spectrophotometer to obtain an optical density.

AIMS

The purpose of this study was, therefore, to test if certain strains of *Staphylococcus aureus* (ATCC 12600 and patient strain P3938) are susceptible to CHD-FA.

MATERIALS AND METHODS

Plates were prepared using 1.2g of MacConkey agar with salt (0.85%) (pH 7.2) (Mast Group Ltd, Merseyide, UK), to which, 23ml distilled water was added. The resulting solution was then autoclaved (for sterilization) and allowed to cool before being poured into balanced sterile petri dishes and allowed to solidify. The result was standardized agar plates with an agar depth of 4mm [19].

The test microorganism (sourced from the NHLS of South Africa) cultures were maintained on prepared MacConkey agar with salt (0.85%), which was left for a period of 24-hrs before being used. Colonies were removed from the 24-hr culture with an inoculum needle and placed in 2ml of saline. The test microorganism was added to saline until the suspension had an optical density (o.d) of 0.07, which was determined using a calorimeter with a 520nm filter. The ATCC 12600 is a strain maintained in a laboratory for purposes of experimentation whilst the P3938 example is a specimen obtained from a patient. 70µl of this suspension was then added to the prepared agar plates, and spread evenly over its surface, resulting in 35 x 10^6 colony forming units [20].

A well was then punched into the agar using a 0.45mm punch and 50ul of a 3.9% CHD-FA (pH 2.9) was placed into the well.

A 1µg oxacillin antimicrobial disc (Mast Group Ltd, Merseyide, UK) served as a positive control whilst a second well containing 50 µl distilled water served as a negative control. The samples in the wells were then allowed to diffuse into the media. The plates were incubated for a period of 18-24hrs at 37^0C.

Finally, the zones of inhibition (z.i.) were read using an electronic calliper, taking two diameter readings at 90^0 angles to each other. The average of the two readings was taken as a result and each screening was carried out in triplicate with the mean z.i. recorded to the nearest millimetre.

RESULTS

Figure 1 shows that both oxacillin and CHD-FA are effective in inhibiting the growth of the laboratory strain of *S. aureus* (ATCC 12600). The oxacillin resulted in a z.i of 25mm whilst CHD-FA resulted in a z.i. of 15mm. Although oxacillin was significantly more effective in this laboratory strain, it was ineffective against the patient strain. Figure 2 shows the CHD-FA as significantly more effective against the patient strain of *S. aureus* (P3938), resulting in a z.i. of 15mm whilst a z.i. of 2mm resulted in the case of oxacillin.

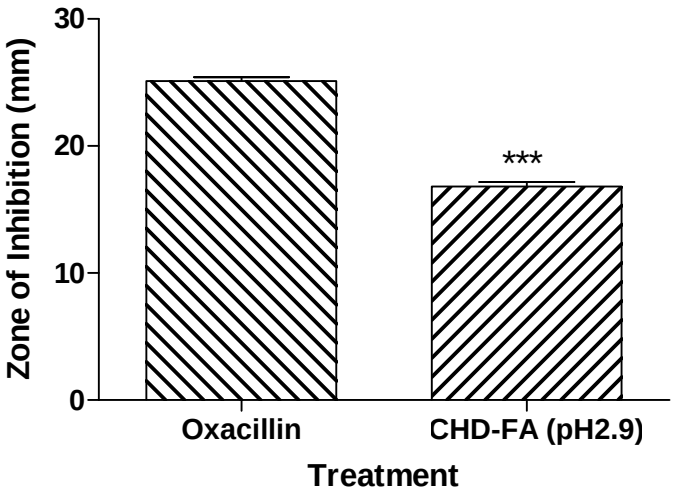

Figure 2: Mean Inhibition zone sizes (±SD) indicating sensitivity of *S. aureus* (ATCC 12600) when treated with either CHD-FA or Oxacillin. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Oxacillin: positive control

CHD-FA: carbohydrate derived fulvic acid.

Indicating a significant difference between CHD-FA (pH 2.9) and that of the oxacillin control (*** $p<0.001$).

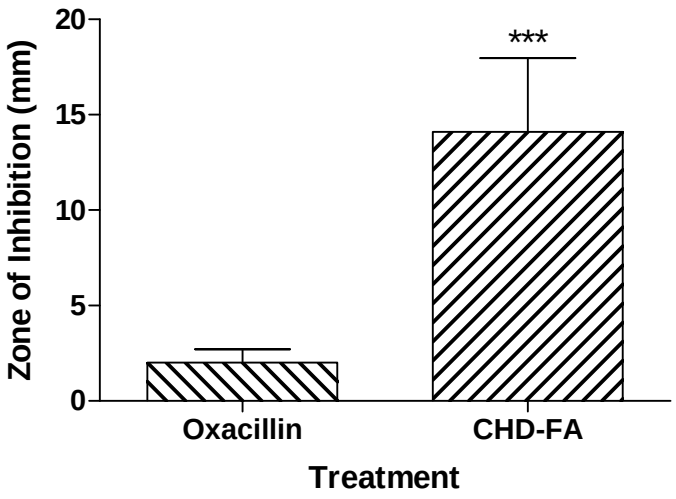

Figure 3: Mean Inhibition zone sizes (±SD) indicating sensitivity of *S. aureus* (P3938) when treated with either CHD-FA or oxacillin. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Oxacillin: positive control
CHD-FA: carbohydrate derived fulvic acid.

Indicating a significant difference between CHD-FA (pH 2.9) and that of the oxacillin control (*** $p<0.001$)

DISCUSSION

Published guidelines by the Clinical and Laboratory Standards Institute (CLSI) dictates that when using *S. aureus* in susceptibility tests, a z.i. of 10mm or less is considered resistant; a z.i. of between 11-12 is intermediate and a z.i of 13 or more is considered as susceptible in the case of oxacillin [21]. Using these criteria, the results indicate that although both oxacillin and CHD-FA are effective against a laboratory strain of *S. aureus*, CHD-FA is significantly more effective against the patient strain, which is highly pathogenic. In addition, CHD-FA compares favourably to *Aloe vera* which in a similar study, obtained a z.i. of 18 mm. The volume (100µl) of *aloe vera* used in the study was double the volume used for the CHD-FA (50µl) though and this could be an indication that the CHD-FA is more active against *S. aureus* than *aloe vera* [22].

It is clear therefore, that CHD-FA is active against the test organisms *in vivo* and further study is warranted.

Full skin diffusion study (Franz-Type diffusion cells)

INTRODUCTION

Human skin has an area of 1.8 to 2 m^2 and a weight of almost 9 kg, thus making it the largest body organ. Its primary role is to provide protection from the external environment and to prevent water loss from the underlying tissues and thus forms a unique interface layer between the human body and outside world [23, 24].

The skin offers a number of opportunities as a route of drug administration, both for topical application in local treatment of skin disorders and for transdermal application of drugs for systemic effect with the only limitation being insufficient permeability [25].

As illustrated in figure 4, the skin can be divided into four layers; subcutaneous tissue, dermis, epidermis and a discontinuous layer of sebum [25]. The epidermis consists of an outer layer called the stratum corneum consisting of 10 to 25 layers of dead, elongated, fully keratinized corneocytes, which are embedded in a matrix of lipid bilayers [26].

It has been shown that it is the latter layer which is the main barrier to penetration through the skin. When a topical formulation is placed on the skin, the active drug is required to penetrate through the stratum corneum into the underlying viable tissue. The limiting factor for these processes is the slow diffusion through the dead horny layer of skin. The stratum corneum behaves as a hydrophobic membrane and the rates of permeation of skin by low and high molecular weight organic non-electrolytes are primarily determined within this layer [25, 27].

The most commonly used device used to determine penetration through skin in *in vitro* studies is the Franz type diffusion cell. The system consists of a donor compartment containing the permeate, separated from a receptor compartment by a membrane placed between the two compartments. The increase in

concentration of the permeate in the receptor phase is measured as it diffuses from the donor cell to the receptor cell [28, 29].

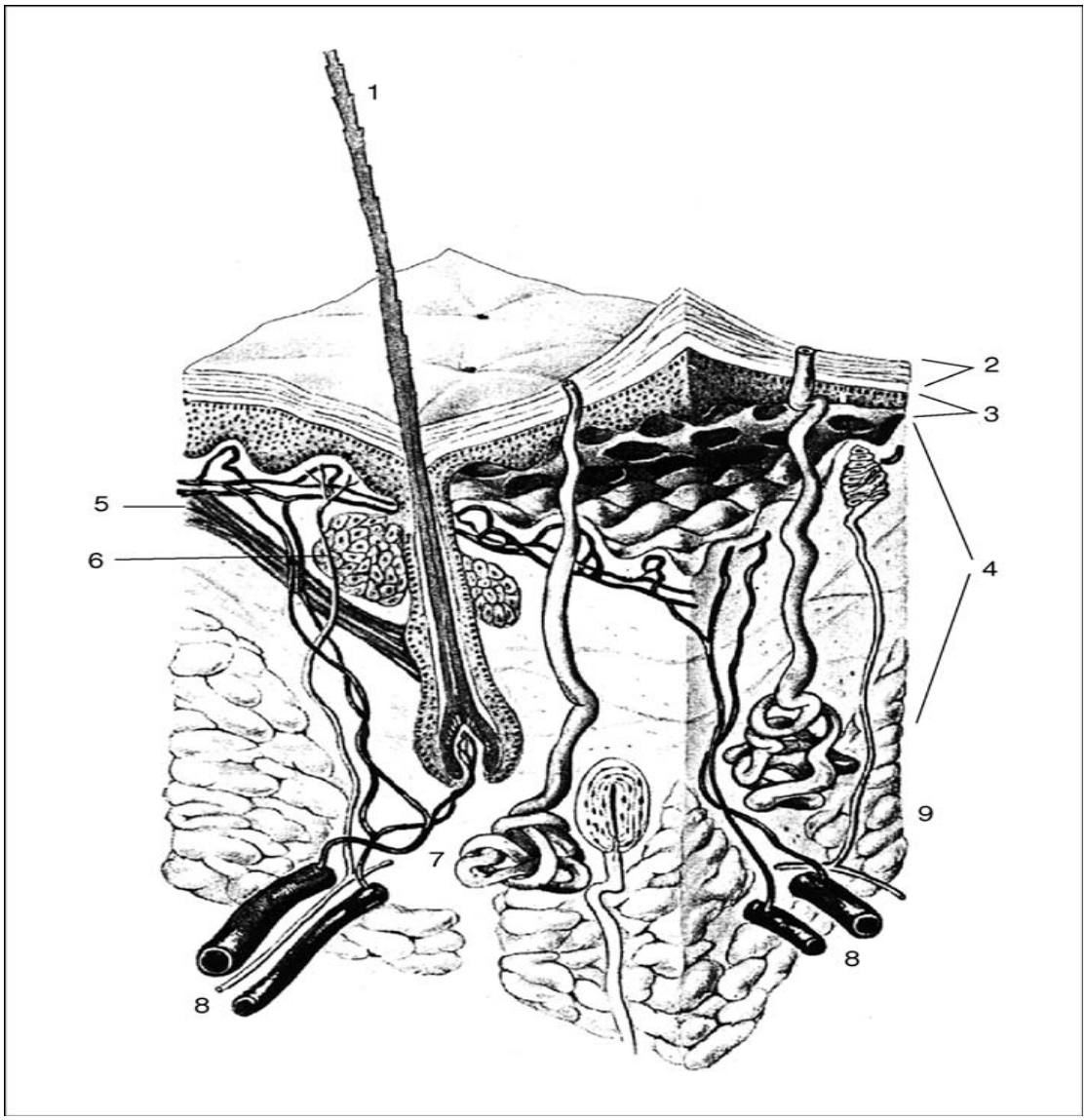

Figure 4 : Schematic cross sectional view of skin structure. In this scheme the following structures are labelled: hair shaft (1); epidermis having an outermost layer, stratum corneum (2), and sequential inner layers, stratum granulosum, stratum spinosum and stratum basale (3); dermis (4); arrector pilimuscle (5); sebaceous gland (6); sweat gland (7); blood vessels (8); and adipose tissue (9).
Source: [30]

AIM

The purposes of this study are to test the permeability of the skin to CHD-FA and ascertain the ability of CHD-FA to cross full thickness skin.

MATERIALS AND METHODS

Formulation of calibration graphs

Samples of CHD-FA (pH 2.9) and CHD-FA buffered to pH 5.5 with sodium acetate were scanned using a Perkin Elmer Lambda 25 uv/vis spectrophotometer (between 200 and 700nm) to determine the points of maximum absorption. Once the points of maximum absorption were obtained (318nm), calibration graphs were constructed for each of the samples using stock solutions of the samples diluted with water.

Preparation of rat full thickness skin samples

Rats were asphyxiated via CO_2 overdose and the skin was taken from the dorsal region, dissected from the underlying layers of muscle of the Sprague-Dawley rats used in this study. The attached, subcutaneous fat tissue was first removed by means of careful rubbing using a flat sharp blade and the skin cut into disks, approximately 100µm thick and $1cm^2$. The discs of skin were then stored frozen at -20^oC for the remainder of the study, to be used as needed.

Preparation of diffusion cells

Diffusion cells were prepared in the manner outlaid by Tonojo *et al* (1997)[29]. Figure 5 represents a setup similar to the one used in this study, with the exception being the continuous flow water bath. Water temperature in this study was therefore regulated manually. Cells were fitted with the 100µm skin disks; epidermis facing the donor cell, whilst the lower skin surface facing the receptor cell.

Donor chambers were filled with either 0.7ml of a 3.9% (w/v) fulvic acid solution, or 0.7ml of a 3.9% (w/v) fulvic acid solution buffered with sodium acetate to a pH of 5.5. Blank samples were also prepared, with the donor chambers receiving 0.7ml of saline only. The receptor chambers received 1ml of degassed Hanks Balanced Salt Solution (HBSS, Highveld Biologicals (Pty) Ltd Johannesburg, SA). The diffusion cells were then placed in a water bath at 37^0C with the contents of the cells being stirred by means of magnetic stirrers.

Samples from the receptor cell were removed at the end of 12 hours and analyzed using the spectrophotometer, using a wavelength of 318nm. Results were recorded as accumulated amount after 12 hours.

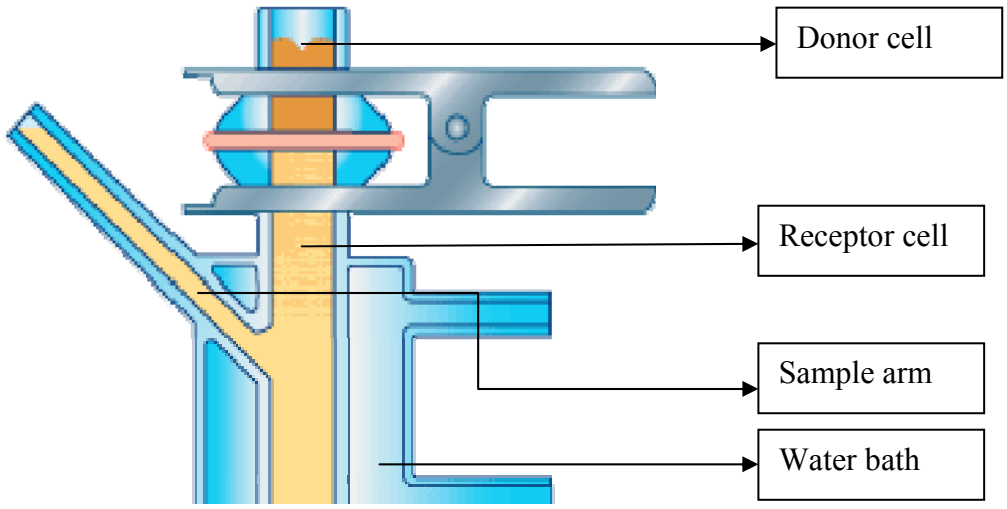

Figure 5: Franz-type diffusion cell (http://www.labecx.com/t-Diffussion_Cells.aspx?gclid=CNL4m476ipQCFRQXsgodmAP1XA)

RESULTS

Calibration graphs yielded strong correlation coefficients of 0.999 and 0.995 for the CHD-FA un-buffered and CHD-FA buffered, respectively (figs 3 and 4).

Figure 8 shows that the absolute amount of fulvic acid in the receptor chamber at the end of the 12-hr test period was greater in the case of the un-adjusted CHD-FA (0.0075 ± 0.0004167 g.ml vs. 0.0025 ± 0.002446 g.ml). This is further emphasized in the percentage of initial donor concentration found in the receptor chamber at the end of the test period-less than 10% for the buffered CHD-FA vs. 20% for the un-buffered CHD-FA (Figure 5).

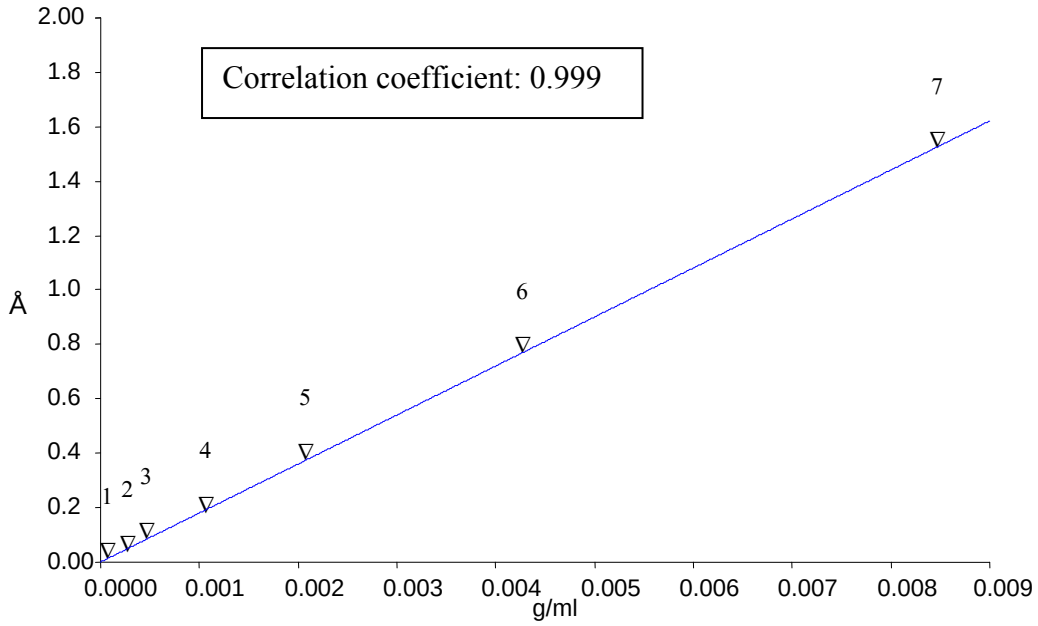

Figure 6: Calibration graph of CHD-FA buffered with sodium acetate to a pH of 5.5

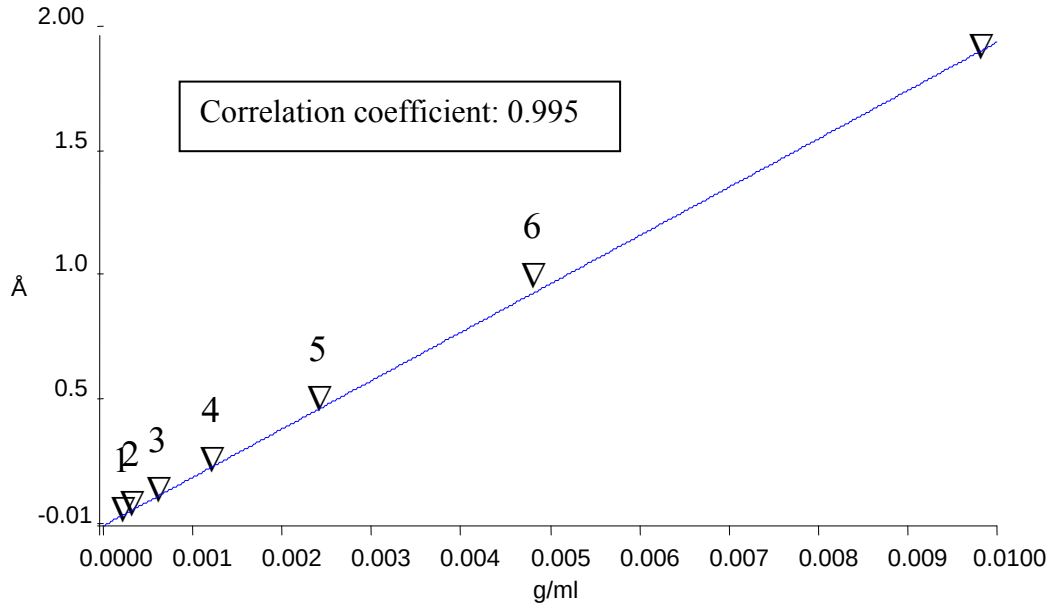

Figure 7: Calibration graph of CHD-FA un-buffered (pH of 2.9**)**

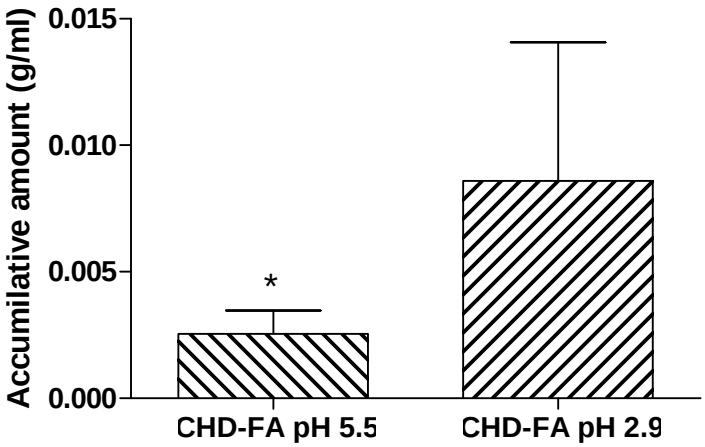

Figure 8: Accumulated amount (g/ml) of CHD-FA at either a pH of 5.5 or 2.9, found in the receptor cell at the end of a period of 12hrs. Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

CHD-FA pH 5.5; carbohydrate derived fulvic acid adjusted with sodium acetate to a pH of 5.5.
CHD-FA pH 2.9; carbohydrate derived fulvic acid, un-adjusted and measuring pH 2.9.

Indicating a significant difference in the accumulative amount compared to the CHD-FA 2.9 group (* $p<0.05$).

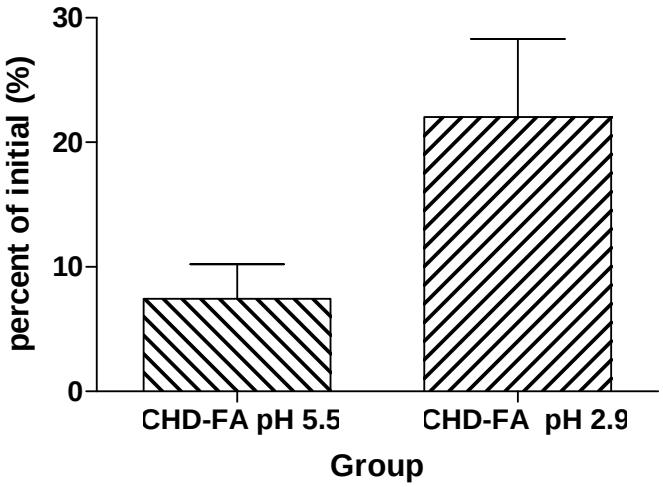

Figure 9: Percent of initial (%) CHD-FA at either a pH 5.5 or 2.9, found in receptor cell at the end of a period of 12-hrs. Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

CHD-FA pH 5.5: carbohydrate derived fulvic acid buffered with sodium acetate to a pH of 5.5.
CHD-FA pH 2.9: carbohydrate derived fulvic acid, un-buffered and measuring pH 2.9.

DISCUSSION

The skin forms a barrier to the external environment, maintaining body fluids within our system and excluding harmful substances. On the other hand, the skin is a site of administration of drugs for topical and systemic chemotherapy. In the pharmaceutical, cosmetic and agrochemical fields, therefore, it is an important issue to predict the rate at which substances penetrate the skin.

The rate and amount of percutaneous absorption of a compound strongly depends on both the physiologic characteristics of the skin (e.g., skin thickness, hydration and temperature) and the physico-chemical nature of the compound (e.g., hydrophobicity, polarity, physical state, water solubility and molecular mass). In general, substances with greater hydrophobicity are absorbed more readily by the skin than less hydrophobic substances. Dermal absorption generally increases as log P does from −1 to 3.5 [31]. Highly lipophilic substances (log $P > 5$) can pass easily through the stratum corneum but are generally too water insoluble to pass through the remaining sub-layers and enter the bloodstream. In addition, the physico-chemical properties of the vehicle have a direct effect on the drug release from topical formulations. One of the factors that are very often ignored in dermal formulation design and in the prediction of skin permeability is that many potential permeates are weak acids or bases and may therefore be ionized. The skin surface pH is around 5.5 and often a pH between 4 and 7 is chosen for the aqueous phase of a dermal formulation. According to the pH-partition theory, only the unionised forms of drugs are able to permeate through phospholipid membranes [31].

The un-adjusted CHD-FA has a pH of 2.9 and is therefore mainly unionized and very hydrophobic. Results show that the un-buffered CHD-FA was able to penetrate easier through the skin barrier when compared to the buffered CHD-FA, as predicted by the pH partition theory. The results indicate therefore that the un-buffered CHD-FA could be used in the treatment of sub-cutaneous infections.

CHAPTER III: *IN VIVO* TOXICITY PROFILE OF CHD-FA

INTRODUCTION

Toxicity profiling is an essential component in all drug development. A drug that is highly effective yet toxic is worthless as a therapeutic agent. Agencies such as the U.S. Food and Drug Administration (FDA), Medical Research Council of South Africa (MRSA) and the World Health Organisation (WHO) require extensive pre-clinical studies to be conducted before a drug is allowed to be tested in humans.

Preclinical animal safety studies are conducted to prove that the drug in question is, in the words of the regulations, "reasonably safe for the proposed clinical trials," and should be designed to test the margin of safety, the type of toxicity (to asses the benefit/risk ratio) and to test the reversibility of any toxic effects [32, 33].

In general, animal toxicity studies are intended to inform the clinical investigators about the potential toxicities associated with the investigational drugs so that those effects may be monitored during the clinical investigations. Baseline evaluation of safety includes monitoring for signs and symptoms of adverse events and laboratory screening (e.g., chemistry, hematology, and urinalysis).

Studies required by the U.S. FDA include acute toxicity studies in two rodent species, one month toxicity studies in a rodent and a non-rodent species, reproductive toxicity studies and long term toxicity studies [18, 32, 33].

The duration of treatment in animal studies should always be at least as long as the duration of treatment in the intended clinical study and at the later stages of development, longer than the therapeutic indication. This provides an extra element of safety [32].

AIM

The aim of these studies is to evaluate the safety of CHD-FA, in accordance with FDA standards in preparation for any future clinical trials.

MATERIALS AND METHODS

The animals (rats and mice) used for these studies were housed at the UPBRC for at least one week before each study was initiated. Food and water was provided *ad libitum* with a 12 hour day/night light cycle. The animals were weighed daily and monitored for pain and distress (behavioural changes). Morbidity was determined on a regular basis by the trained personnel of UPBRC.

Toxicity was determined by evaluation of reduced food and water intake, weight loss [more than 20% original weight], observation of abnormal movement (particularly as it pertains to the ability of the animal to obtain food and water) and ease of breathing. Animals were terminated if 25% or more so showed signs of distress or adverse reactions, as described above. The final decision to terminate the animals was made by Dr Auer and was done if any signs of discomfort were observed. Animals were euthanized by anaesthetic overdose (isoflurane) if deemed necessary.

All blood samples were taken immediately for analysis and analysed at the Clinical Pathology Laboratories, Faculty of Veterinary Sciences, University of Pretoria.

Analysis of variance (ANOVA) was used to analyse data.

Toxicity studies were divided into 6 phases:

PHASE I: TOPICAL APPLICATION; TOXICITY AND SENSITIZATION STUDY IN MICE.

For this study 60 female 6-8 weeks old Balb C mice were used. The animals were divided into 3 groups of 20 mice each. Two containers of carbohydrate derived fulvic acid (CHD-FA) creams were prepared using an aqueous cream (UEA), one of which was neutralized with sodium acetate to a pH of 5.5. A predetermined amount was applied to the left ears twice on day one and once on day 7 to determine whether they have been sensitized against the product. From day 8 they received the products topically on their left ears twice a day for 30 days (up to day 38). A 28-day study is considered a sub chronic study, which is well accepted for eliciting any toxicity on long-term treatment.

Group 1 received UEA only.
Group 2 received fulvic acid.
Group 3 received fulvic acid neutralized to a pH of 5.5.

The amount used per application was approximately 200mg cream (i.e. 8mg fulvic acid/mouse, or 400mg/kg body weight) twice a day. Blood samples (500 µl/mouse) were drawn before termination of the mice, at the end of the study to determine kidney and liver enzyme levels (creatinine and gamma-glutamyl transferase).

Phase II: Oral application (sub chronic, 6 weeks) and Phase III: Oral application (chronic, 6 months) in rats.

This study began with a 6-week toxicity (sub chronic) study using 20 female 6-8 weeks old Sprague Dawley rats. At the end of this period, the study was extended to 6 months. Animals in the experimental group (20) received a single oral dose (by gavage) of 150 mg/kg body weight/day of CHD-FA (potassium

salt) neutralized to a pH of 5.5 in distilled water as a 1.0 ml bolus per day for 6 weeks. Control animals (20) received an oral dose (by gavage) of 1.0 ml distilled water per day to control for the procedure used in the experimental group. Animals were evaluated at the end of the initial 6 week period. Both groups showed little signs of toxicity and therefore the decision was made to extend the study to 6 months in order to determine the long term effects of the CHD-FA (Phase III).

A third group of 20 animals was added later and received half the dose of the experimental group for a period of 6-weeks.

The animals were weighed daily and monitored for pain and distress (as stated before). Animals were euthanized by CO_2 asphyxiation 6 months after treatment was initiated.

Toxicity was determined by evaluation of reduced food and water intake resulting in weight loss (more than 20% original weight), observation of abnormal movement (particularly as it pertains to the ability of the animal to obtain food and water) and ease of breathing.

Blood samples (500 µl/rat) were drawn from the animals at the beginning of the study (i.e. two (2) days before the first administration of the test drug) and at the end of the study for haematological analysis (haematocrit, red blood cells, white blood cells, and platelet counts) and kidney and liver enzyme levels (creatinine, urea, aspartate aminotransferase and gamma-glutamyl transferase).

Phase IV: Teratogenicity study (oral application)

A group of 20 pregnant rats was treated orally by gavage with 150 mg fulvic acid /kg bw/day, dissolved in water and neutralized to a pH of 5.5 as a 1.0 ml bolus per day, 3 days before fertilization up to 12 days of pregnancy (i.e. a total of 15 days on fulvic acid treatment). The control group comprised of 20 female rats, and received a daily oral dose of 1 ml water 3 days before fertilization to 12 days of pregnancy (i.e. a total of 15 days on water only). Animals, who failed to

mate, were terminated. Puppies were monitored daily for clinical and behavioural abnormalities for a period of two weeks after birth. All the animals were terminated by CO_2 overdose at the end of the experiment, according to standard procedures used by the UPBRC. Puppies were weighed upon termination and examined for gross abnormalities by pathologists at Golden Vetpath.

Phase V: 7-day acute toxicity study

A 7-day toxicity study was completed using 40 female Sprague Dawley rats (6-8 weeks old) divided into two groups of 20. The first group of 20 animals received a single oral dose (by gavage) of 150 mg/ kg bw/day of fulvic acid (potassium salt) neutralized to a pH of 5.5 in distilled water as a 1 ml or less bolus (depending on body mass) on day one. Control animals (20) received an equivalent oral dose (by gavage) of distilled water.

At the end of the study, the animals were euthanized by CO_2 asphyxiation, and dissected to determine whether any abnormally enlarged organs were present (mainly the liver, kidney, spleen). Animals were euthanized by CO_2 asphyxiation on day 7 after a final blood sample was taken from each of the animals. Blood samples (500 µl/rat), taken from the heart, were drawn by trained personnel from the UPBRC from the animals at the end of the study (day 7) for haematological analysis (haematocrit, red blood cells, white blood cells, and platelet counts) and kidney and liver enzyme levels (creatinine, urea, AST, ALT and GGT).

Phase VI: 6-week chronic toxicity study

A 6-week toxicity study was completed using 40 female old Sprague Dawley rats (6-8 weeks). Animals in this experimental group (20) received a single oral dose (by gavage) of 100 mg/ kg bw/day of fulvic acid (potassium salt)

neutralized to a pH of 5.5 in distilled water as a 1.0 ml bolus, per day for 6 weeks. Control animals (20) received an oral dose (by gavage) of 1.0 ml distilled water per day.

At the end of the study, the animals were euthanized and dissected to determine whether abnormally enlarged organs were present (mainly the liver, kidney, spleen). Animals were euthanized by CO_2 asphyxiation on the final day of the 6 weeks, after a final blood sample was taken from each of the animals. Blood samples (500 µl/rat), taken from the heart, were drawn by trained personnel from the UPBRC from the animals at the end of the study for haematological analysis (haematocrit, red blood cells, white blood cells, and platelet counts) and kidney and liver enzyme levels (creatinine, urea, AST, ALT and GGT).

RESULTS

Phase I: Topical application; toxicity and sensitization study

There were no deaths or any signs of toxicity observed after application or at any stage during the study.

Weight gain patterns of all of the groups were similar (Figure 10).

Haematological analyses indicate a significant decrease in the red cell count (RCC) of the buffered CHD-FA treated group compared to that of both the other groups. A significant decrease is also observed, in the white cell count (WCC) and the clotting factor (Thr C) (Table 1).

Examination of the liver enzymes reveals significant decreases in AST and GGT between the untreated group and the two treatment groups (Figures 11 and 12).

The results obtained for the kidney functions shows a significant increase in both creatinine and urea levels when compared to the control (Figures 13 and 14).

Phase II and III: Oral application; a chronic toxicity study

There were no deaths or any signs of toxicity observed after application or at any point during the study.

Over the six-month period, the weight growth patterns between the control and CHD-FA treated group showed no significant differences (Figure15).

Analyses of haematological parameters show similar patterns for both control and experimental groups with most parameters staying within published normal values (Table 2). The exceptions to this trend was the red cell count of the experimental group, which at 6 weeks was slightly elevated at 9.6 $x10^{12}$/l (normal value range from 7.21-8.45 x 10^{12}/l) and the white cell counts which were lower than the normal expected values of between 7.30-12.7 $x10^{9}$/l for both groups. The elevated red cell count returned to normal levels at the end of

6-months and was not significantly different from the control group at the same time period.

Examination of the liver function showed no significant differences in GGT levels (Figure 17) but an increase in the AST levels was observed at the 6-week mark (Figure 16). As it was the only parameter that was elevated, a decision was made to continue. At the end of the study, the levels of AST in the treated group returned to normal levels (Figure 16).

Kidney function showed no significant differences in both urea or creatinine levels between the control and experimental group (Figures 18 and 19).

The third group of animals receiving half the dose (75mg/kg body mass) of CHD-FA for 6-weeks, showed no significant differences in any of the parameters when compared to the control group.

Phase IV: Teratogenicity study (oral application)

The data collected in this study indicates no significant differences between the groups treated with CHD-FA and that of the control (water).

The weight growth patterns of the females during the pregnancy period, between the groups were almost identical (Figure 20).

Although having slightly smaller litter numbers on average, the CHD-FA treated group showed significantly greater pup weights on average (Figure 21). No other significant differences were observed between the groups.

In addition to the above analyses, morphological evaluation, including macroscopical necropsy, was conducted on the organs of the pups. Organs evaluated included the adrenal glands, brain, heart, gonads, intestines (large and small), kidney, liver, lung, spleen, stomach and thymus. Histological examination of all the organs mentioned was also conducted.

The conclusions of the pathologists were that there was no indication of any developmental defects or pathological anatomical abnormalities associated with the CHD-FA treatment conducted on the female rats.

These results indicate therefore, that CHD-FA is safe to use in rats during pregnancy.

Phase V: 7-day acute toxicity study

There were no deaths or any signs of toxicity observed after application or at any point during the study.

Weight gain patterns of both groups were similar (Figure 24).

Comparison of red blood cell count, hematocrit, haemoglobin and white cell count shows no significant differences between the control and treatment groups (Table 4).

Examination of the liver enzymes reveals no significant differences in AST, ALT and GGT between the untreated group and the treated group (Figures 25, 26 and 27).

The results obtained for the kidney functions show no significant differences between the control group and the treatment group's creatinine and urea levels (Figures 28 and 29).

Comparison of liver, spleen and kidney between the control and experimental groups, shows no significant differences in weights at the end of the study period (Figures 30, 31 and 32).

Phase VI: 6-week chronic toxicity study

There were no deaths or any signs of toxicity observed after application or at any point during the study.

Weight gain patterns of both groups were similar (Figure 33).

Comparison of red blood cell count, hematocrit, haemoglobin and white cell count shows no significant differences between the control and treatment groups (Table 3).

Examination of the liver enzymes reveals no significant differences in AST, ALT and GGT between the untreated group and the treatment group (Figures 34, 35 and 36).

The results obtained for the kidney functions show no significant differences between the control group and the treatment groups' creatinine and urea levels (Figures 37 and 38).

Comparison of liver, spleen and kidney between the control and experimental groups, shows a significant increase in weight at the end of the study period (Figures 39, 40 and 41).

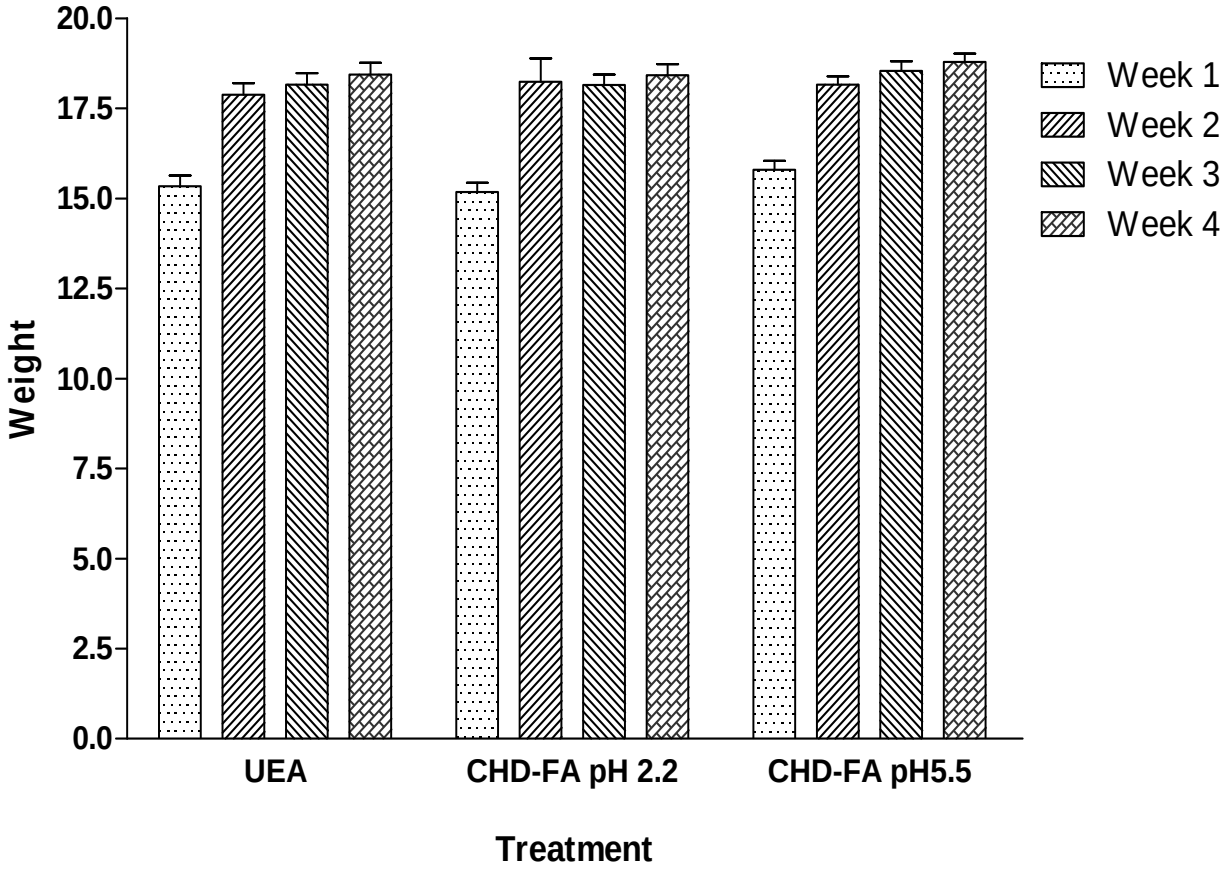

Figure 10: Weight gain pattern of mice over a one month period. Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

UEA: control group
CHD-FA: treated at either pH 2.2 or 5.5 with carbohydrate derived fulvic acid

Table 1: Comparison of the mean haematology values (±SD) of various groups of Balb-C mice after receiving 200mg UEA or CHD-FA (pH 2.2 or 5.5) applied topically to the left ear for a period of 1- month.

	UEA (n= 20) Mean ± s.d.	CHD-FA (n=20) Mean ± s.d.	CHD-FA (pH 5.5) (n=20) Mean ± s.d.
Hb (g/l)	147.68 (± 20.05)	155.75 (0.94)	151.85 (0.68)
RCC (x 10^{12} /l)	9.79 (± 0.44)	9.44 (± 1.99)	8.155(±0.81)***,##
HT (l/l)	0.460 (± 0.46)	0.466 (± 0.013)	0.465 (± 0.01)
MCV (fl)	46.76 (±3.2)	47.16 (± 0.46)	47.06 (± 0.88)
MCHC (g/dl)	32.03 (±3.5)	33.47 (± 0.34)	32.64 (± 0.23)
WCC (x 10^{9} /l)	7.44 (± 2.42)	7.95 (±2.45)	5.05 (± 0.81)**,###
Thr C (x 10^{9} /l)	624.89(±141.13)	695.40 (±102.65)	1036.40 (±74.62)***,###

Data recorded from blood samples taken from the mice via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Indicating a significant difference when compared to the UEA control group (***p<0.001, **p<0.01).
Indicating a significant difference when compared to the CHD-FA group (###p<0.001, ##p<0.01).

Control; untreated group
CHD-FA; treated with carbohydrate derived fulvic acid (150 mg/kg body mass).

Hb-Hemoglobin, Ht-Hematocrit ,MCHC-Mean Cell Hemoglobin Concentration, MCV-Mean Cell Volume, RCC-Red Cell Count, RDW-Red blood cell distribution width, THR C-Thrombocytes, WCC-White Cell Count

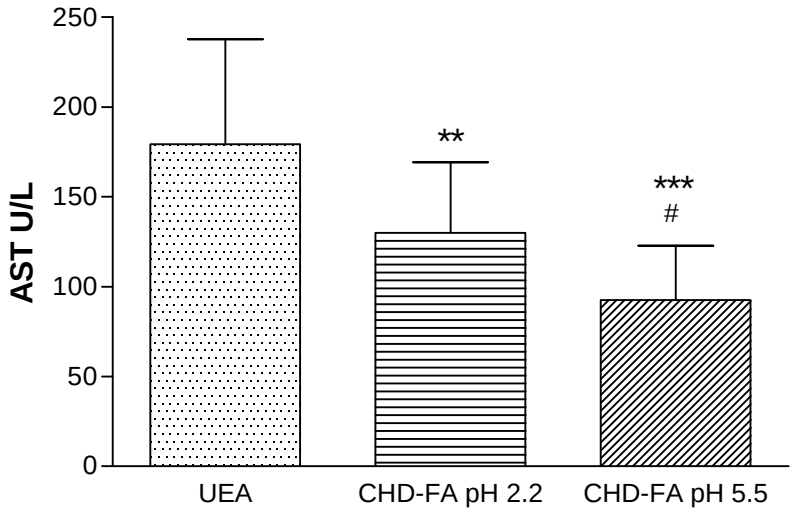

Figure 11: Liver AST levels of mice receiving 200mg UEA or CHD-FA (pH 2.2 or 5.5) applied topically to the left ear. Data recorded from blood samples taken from the mice at the end of 1-month and is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

UEA: control group
CHD-FA: treated at either pH 2.2 or 5.5 with carbohydrate derived fulvic acid

Indicating a significant difference between CHD-FA pH 2.2 and the UEA control group (** $p<0.01$).
Indicating a significant difference between CHD-FA pH5.5 and the UEA control group (***$p<0.001$).
Indicating a significant difference between CHD-FA pH 2.2 and the pH 5.5 group (# $p<0.05$).

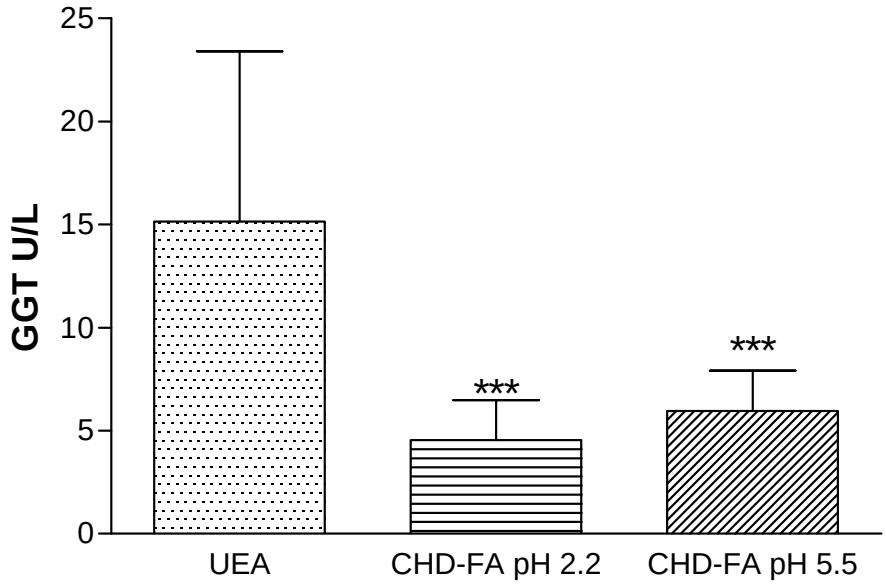

Figure 12: Liver GGT levels of mice receiving 200mg UEA or CHD-FA (pH 2.2 or 5.5) applied topically to the left ear. Data recorded from blood samples taken from the mice at the end of 1-month and is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

UEA: control group
CHD-FA: treated at either pH 2.2 or 5.5 with carbohydrate derived fulvic acid

Indicating a significant difference between the treatment group and the UEA control group (*** $p<0.001$).

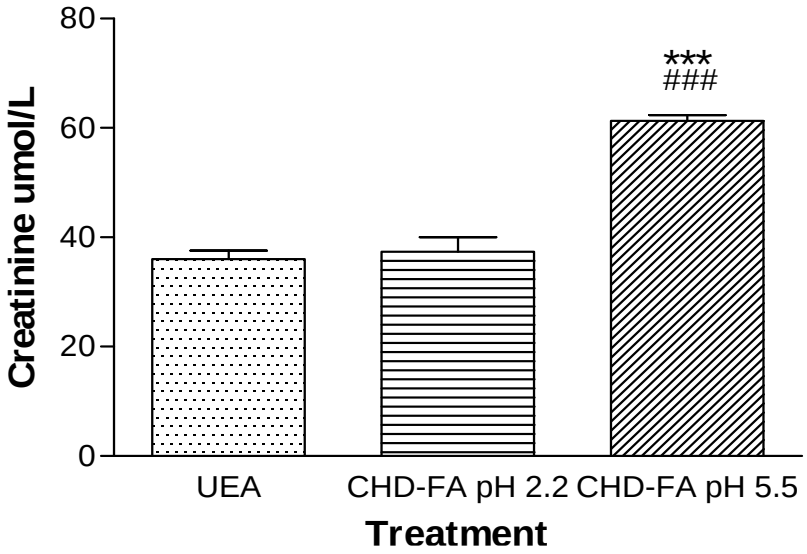

Figure 13: Kidney creatinine levels of mice receiving 200 mg UEA or CHD-FA (pH 2.2 or 5.5) applied topically to the left ear. Data recorded from blood samples taken from the mice at the end of 1-month and is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

UEA: control group
CHD-FA: treated at either pH 2.2 or 5.5 with carbohydrate derived fulvic acid

Indicating a significant difference between CHD-FA 5.5 and the UEA control group (*** $p<0.001$).
Indicating a significant difference between CHD-FA 5.5 and the CHD-FA 2.2 group (### $p<0.001$).

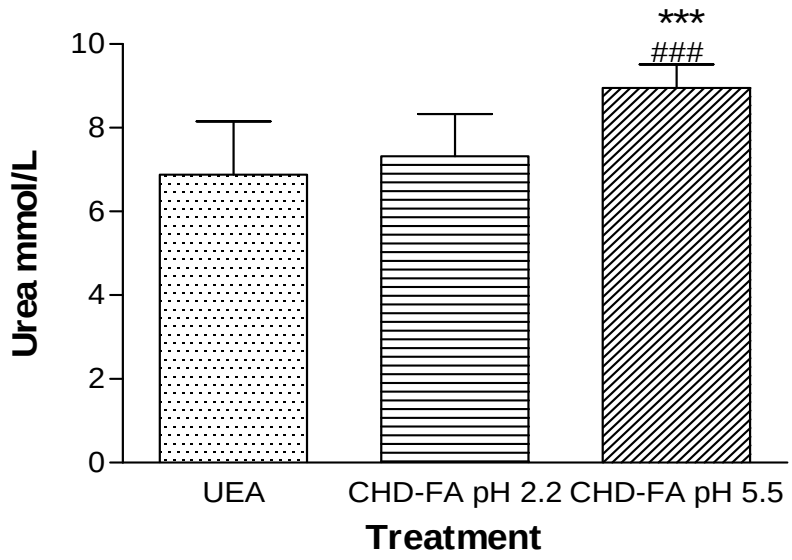

Figure 14: Kidney urea levels of mice receiving 200mg of UEA or CHD-FA (pH 2.2 or 5.5) applied topically to the left ear. Data recorded from blood samples taken from the mice at the end of 1-month and is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

UEA: control group
CHD-FA: treated at either pH 2.2 or 5.5 with carbohydrate derived fulvic acid

Indicating a significant difference between CHD-FA 5.5 and the UEA control group (*** $p<0.001$).
Indicating a significant difference between CHD-FA 5.5 and the CHD-FA 2.2 group (### $p<0.001$).

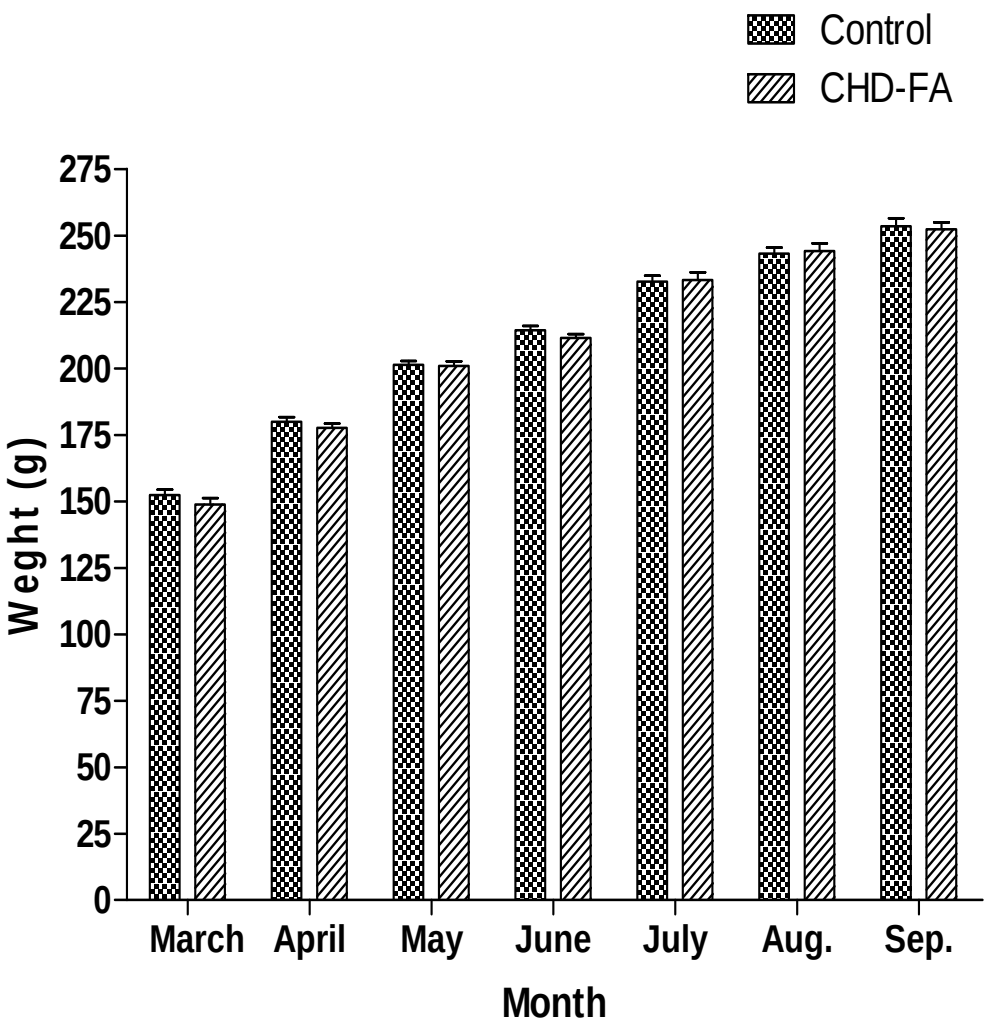

Figure 15: Weight gain pattern of animals receiving either 1ml CHD-FA (150 mg/kg body weight/day) or 1ml water for a 6-month period (including initial 6 weeks). Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group receiving water.
CHD-FA: treated with carbohydrate derived fulvic acid (150 mg/kg body mass)

Table 2: Comparison of mean haematology values (±SD) between groups of Sprague-Dawley rats after receiving either 1ml CHD-FA (150 mg/kg body weight/day) or 1ml water (control) for a 6-month period (including initial 6 weeks).

	Control (0 wks) (n=20)	Control (6wks) (n=20)	Control (6mnths) (n=20)	CHD-FA (initial) (n=20)	CHD-FA (6wks) (n=20)	CHD-FA (6mnths) (n=20)
Hb (g/l)	142.35 (±8.31)	151.55*** (±4.47)	.85* (±4.2)	148.2 (±8.24)	151.55$^{\#}$ (±6.436)	147.35 (±7.57)
RCC (x 10^{12} /l)	7.00 (± 0.4)	8.10*** (± 0.23)	7.99*** (±0.23)	7.32 (±0.43)	9.61$^{\#\#\#, xxx}$ (± 0.10)	7.85$^{\#\#\#}$ (±0.45)
HT (l/l)	0.361 (± 0.02)	0.47*** (±0.01)	0.44*** (±0.02)	0.38 (±0.023)	0.45$^{\#\#\#}$ (±0.02)	0.45$^{\#\#\#}$ (±0.02)
MCV (fl)	51.58 (±0.97)	57.98*** (±0.43)	57.55*** (±0.80)	51.97 (±0.87)	47.14$^{\#\#\#}$ (±0.45)	57.88$^{\#\#\#}$ (±1.6)
MCHC (g/dl)	39.42 (±0.53)	32.26*** (±0.39)	32.44*** (±0.41)	38.99 (±0.43)	34.19$^{\#\#\#}$ (±3.1)	32.44$^{\#\#\#}$ (±0.30)
WCC (x 10^{9} /l)	4.72 (±0.66)	5.31 (±0.97)	2.82*** (±0.76)	4.95 (±0.85)	5.19 (±1.49)	2.37$^{\#\#\#}$ (±0.83)
Thr C (x 10^{9} /l)	1133.20 (±59.27)	1064.90 (±82.44)	902.15*** (±46.67)	1085.00 (±193.5)	673.68 (±165.65)	881.5$^{\#\#\#}$ (±177.4)
RDW (%)	14.18 (±0.79)	15.62*** (±0.66)	16.00*** (±0.92)	14.13 (±0.62)	17.33$^{\#\#\#,xxx}$ (±0.99)	16.04$^{\#\#\#}$ (±0.96)

Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Indicating a significant difference when compared to CHD-FA initial (### $p<0.001$).
Indicating a significant difference between CHD-FA at 6-wks and the control at 6-weeks (xxx $p<0.001$).

Control; untreated group
CHD-FA; treated with carbohydrate derived fulvic acid (150 mg/kg body mass).

Table 3: Comparison of mean haematology values (±SD) between groups of Sprague-Dawley rats after receiving either 1ml CHD-FA (75 mg/kg body weight/day) or 1ml water (control) for a 6-month period (including initial 6 weeks).

	Control (initial) (n=10)	Control (6wks) (n=10)	CHD-FA (initial) (n=10)	CHD-FA (6wks) (n=10)
Hb (g/l)	156.70 (±4.30)	150.30 (±5.60)	158.90 (±5.90)	$145.90^{###}$ (±6.00)
RCC (x 10^{12} /l)	8.51 (±0.22)	8.02^{*} (±0.34)	8.70 (±0.37)	$7.81^{###}$ (±0.41)
HT (l/l)	0.48 (±0.004)	0.47 (±0.0056)	0.49 (±0.16)	$0.46^{##}$ (±0.0064)
MCV (fl)	56.50 (±0.20)	58.23^{***} (±0.29)	56.20 (±0.16)	$58.60^{###}$ (±0.25)
MCHC (g/dl)	32.64 (±0.0.09)	32.21^{*} (±0.15)	32.67 (±0.0.06)	$31.91^{###}$ (±0.11)
WCC (x 10^{9} /l)	3.62 (±0.69)	2.93 (±1.01)	3.40 (±0.49)	$2.29^{#}$ (±0.81)
Thr C (x 10^{9} /l)	1040.1 (±46.20)	906.7^{**} (±91.50)	1036.1 (±55.02)	$818.8^{##}$ (±232.80)
RDW (%)	16.44 (±0.73)	15.90 (±0.68)	15.85 (±0.55)	15.91 (±0.45)

Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Indicating a significant difference compared to the control initial (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)
Indicating a significant difference compared to the CHD-FA initial (# $p<0.05$, ### $p<0.001$)

Control; untreated group
CHD-FA; treated with carbohydrate derived fulvic acid (75 mg/kg body mass).

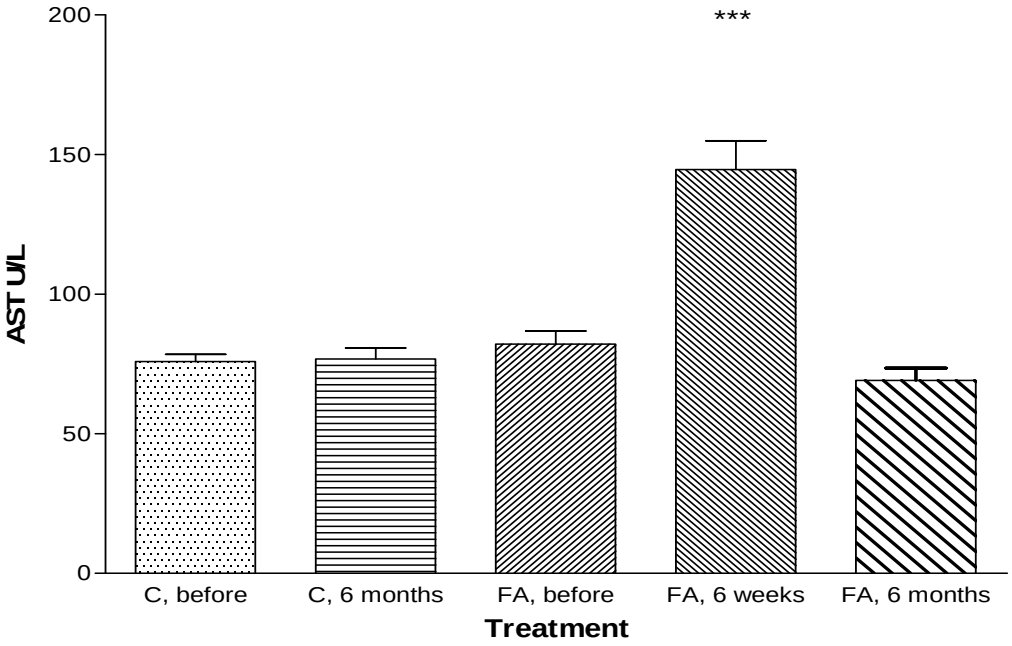

Figure 16: AST levels of rats at the beginning, after 6 weeks and at the end of a 6-month period. Animals received either 1ml of CHD-FA (150mg/kg/body mass/day) or water via gavage for the duration of the study. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

C: before; control group, beginning of the trial
C: 6 months; control group after 6 months
FA: before, carbohydrate derived fulvic acid (CHD-FA) treated group at beginning of trial
FA: 6 weeks; carbohydrate derived fulvic acid (CHD-FA) treated group after 6 weeks treatment
FA: 6 months; carbohydrate derived fulvic acid (CHD-FA) treated group after 6 weeks treatment

Indicating a significant difference compared to FA before (*** $p<0.001$)

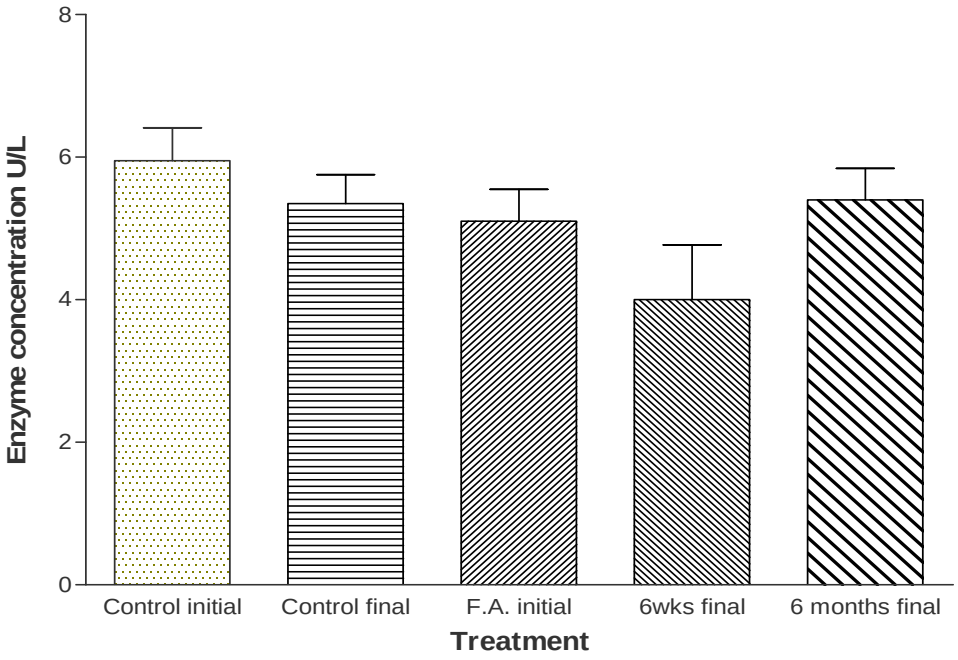

Figure 17: GGT levels of rats at the beginning, after 6 weeks and at the end of a 6-month period. Depending on group, animals received either 1ml of CHD-FA (150mg/kg/body mass/day) or water via gavage for the duration of the study. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

C, before; Control group, beginning of the trial
C, 6 months; Control group after 6 months on placebo
FA, before, carbohydrate derived fulvic acid (CHD-FA) treated group at beginning of trial
FA, 6 weeks; carbohydrate derived fulvic acid (CHD-FA) treated group after 6 weeks treatment
FA, 6 months; carbohydrate derived fulvic acid (CHD-FA) treated group after 6 weeks treatment

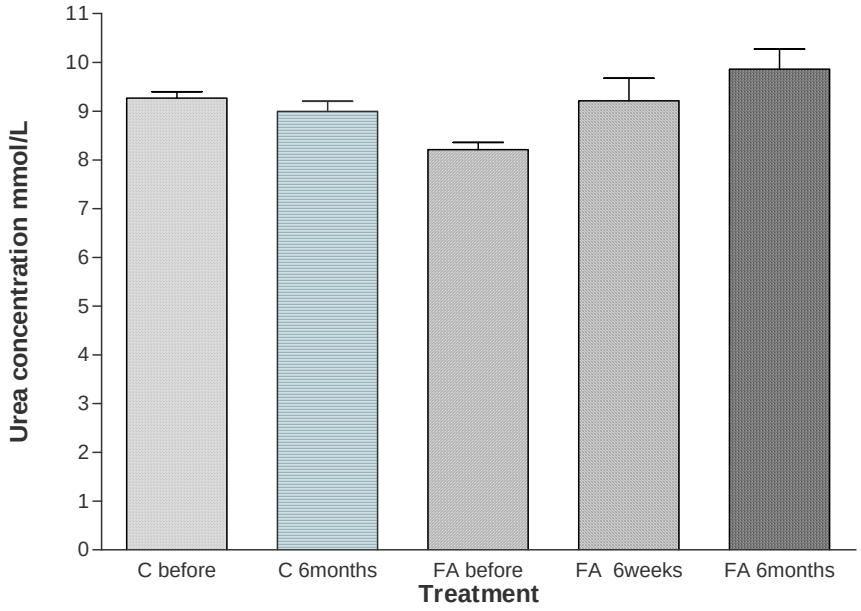

Figure 18: Urea levels of rats at the beginning, after 6 weeks and at the end of a 6-month period. Depending on group, animals received either 1ml of CHD-FA (150mg/kg/body mass/day) or water via gavage for the duration of the study. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

C, before; Control group, beginning of the trial
C, 6 months; Control group after 6 months on placebo
FA, before, carbohydrate derived fulvic acid (CHD-FA) treated group at beginning of trial
FA, 6 weeks; carbohydrate derived fulvic acid (CHD-FA) treated group after 6 weeks treatment
FA, 6 months; carbohydrate derived fulvic acid (CHD-FA) treated group after 6 weeks treatment

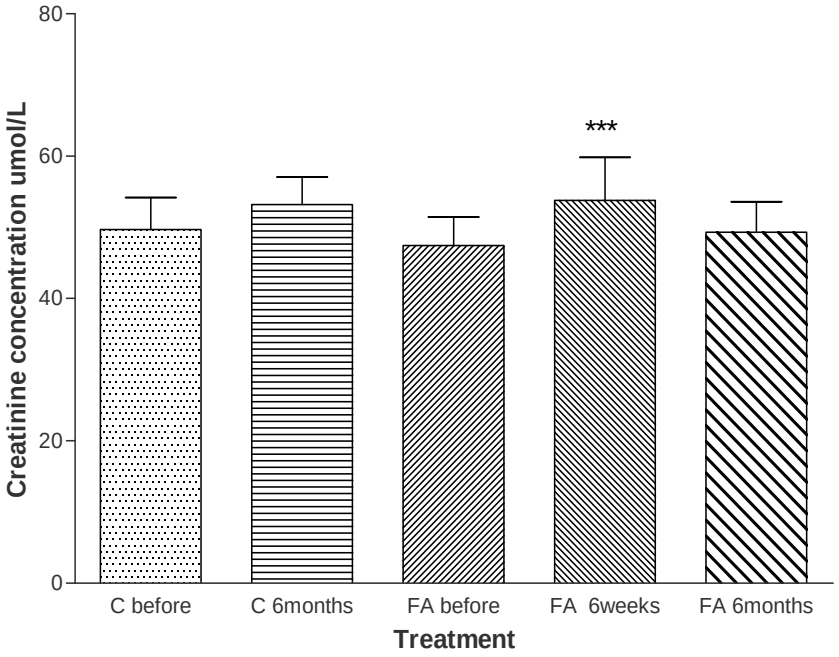

Figure 19: Creatinine levels of rats at the beginning, after 6 weeks and at the end of a 6-month period. Depending on group, animals received either 1ml of CHD-FA (150mg/kg/body mass/day) or water via gavage for the duration of the study. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

C, before; Control group, beginning of the trial
C, 6 months; Control group after 6 months on placebo
FA, before, carbohydrate derived fulvic acid (CHD-FA) treated group at beginning of trial
FA, 6 weeks; carbohydrate derived fulvic acid (CHD-FA) treated group after 6 weeks treatment
FA, 6 months; carbohydrate derived fulvic acid (CHD-FA) treated group after 6 weeks treatment

Indicating a significant difference when compared to the FA before (*** $p<0.001$)

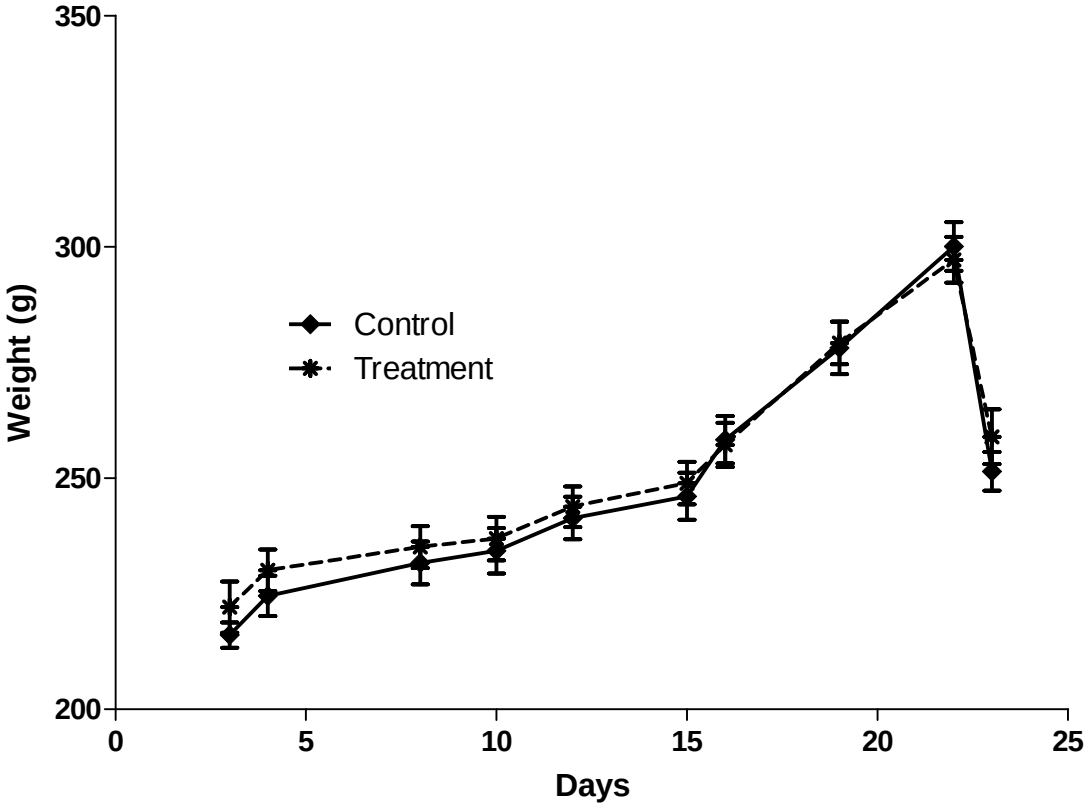

Figure 20: Comparison of the weight patterns of female rats receiving either 1ml CHD-FA (150 mg/kg/body weight/day) or water via gavage for the duration of the pregnancy period. Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: 1ml water via gavage.
Treatment: 1ml carbohydrate derived fulvic acid (CHD-FA) via gavage.

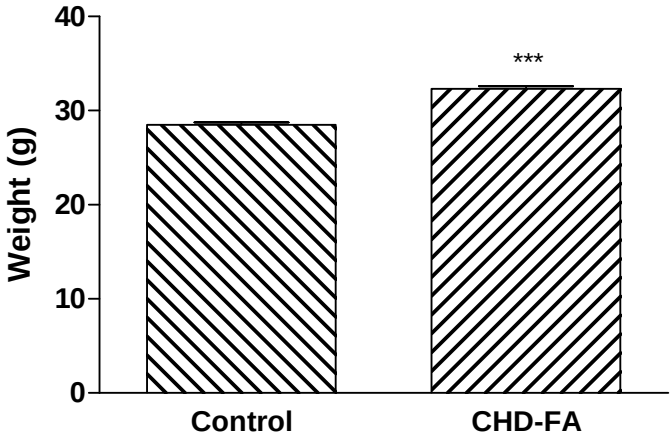

Figure 21: Comparison of the average weights (g) of pups of rats receiving either 1ml CHD-FA (150 mg/kg body mass/day) or water, depending on group. Data recorded at termination of pups and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: Pups of rats receiving 1ml water via gavage.
Treatment: Pups of rats receiving 1ml carbohydrate derived fulvic acid (CHD-FA) via gavage.

Indicating a significant difference of the CHD-FA group when compared to the control group (*** $p<0.001$).

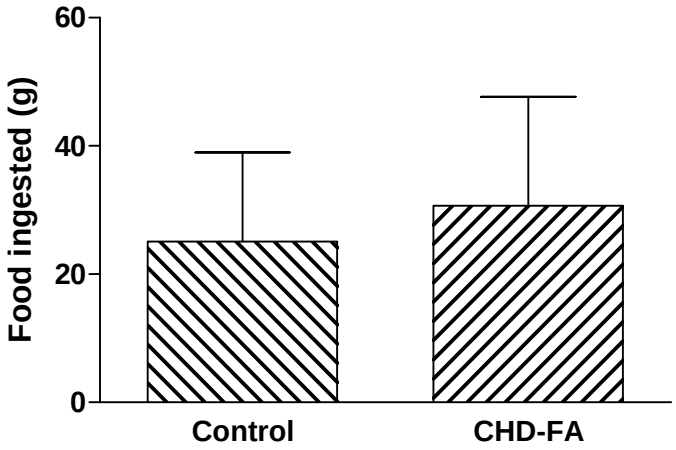

Figure 22: Comparison of the average amount (g) of food ingested by the pregnant females receiving either 1ml CHD-FA (150 mg/kg body weight/day) or water via gavage over the pregnancy period. Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: Pups of rats receiving 1ml water via gavage.
Treatment: Pups of rats receiving 1ml carbohydrate derived fulvic acid (CHD-FA) via gavage.

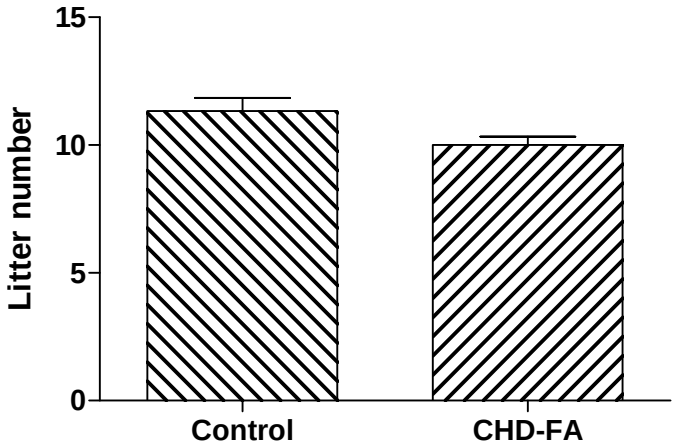

Figure 23: Comparison of the average number of pups per litter of pregnant females receiving either 1ml CHD-FA (150 mg/kg body weight/day) or water via gavage over the pregnancy period. Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: Pups of rats receiving 1ml water via gavage.
Treatment: Pups of rats receiving 1ml carbohydrate derived fulvic acid (CHD-FA) via gavage.

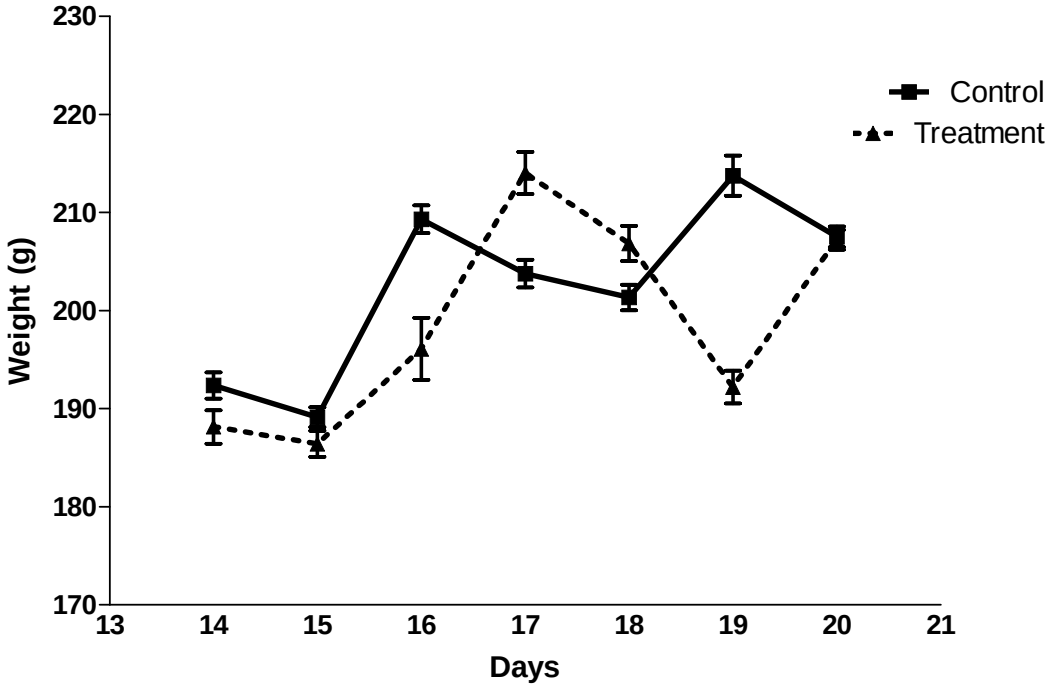

Figure 24: Comparison of the weight patterns of rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage. Data was recorded every day for a period of 7 days and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control group: 1ml Water
Treatment: 1 ml CHD-FA (150 mg/kg body mass)

Table 4: Comparison of mean haematology values (±SD) between Sprague-Dawley rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage.

	Control (n=20) Mean ± s.d.	CHD-FA (n=20) Mean ± s.d.
Hb (g/l)	148.40 (±9.58)	148.80 (± 11.47)
RCC (x 10^{12} /l)	7.71 (± 0.27)	7.72 (± 0.28)
HT (l/l)	0.44 (± 0.018)	0.44 (± 0.015)
MCV (fl)	56.98 (± 0.52)	56.80 (± (0.41)
MCHC (g/dl)	33.80 (± 2.12)	33.83(± 1.76)
WCC (x 10^{9} /l)	3.40 (± 1.17)	3.66 (± (0.48)
Thr C (x 10^{9} /l)	758.20 (± 156)	868.90 (± 63.22)

Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control; untreated group
CHD-FA; treated with carbohydrate derived fulvic acid (150 mg/kg body mass).

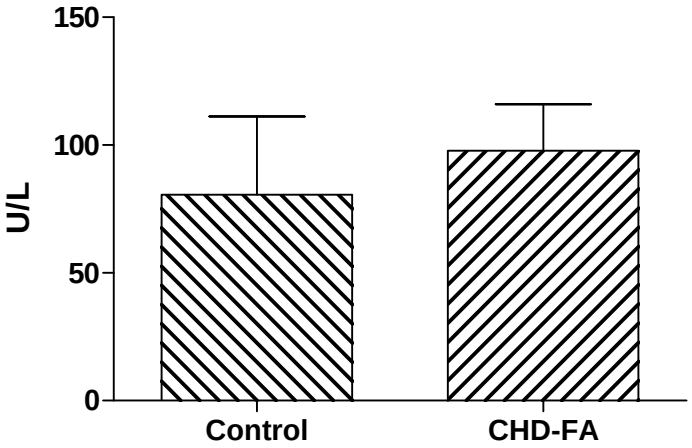

Figure 25: Liver AST levels of rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control group: 1ml Water
Treatment: 1 ml CHD-FA (150 mg/kg body mass)

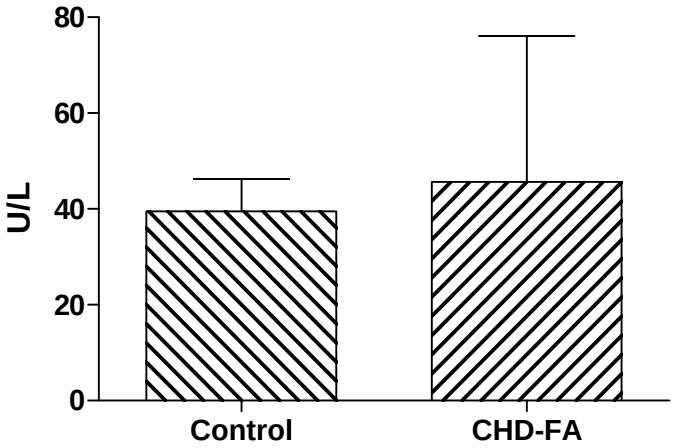

Figure 26: Liver ALT levels of rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control group: 1ml Water
Treatment: 1 ml CHD-FA (150 mg/kg body mass)

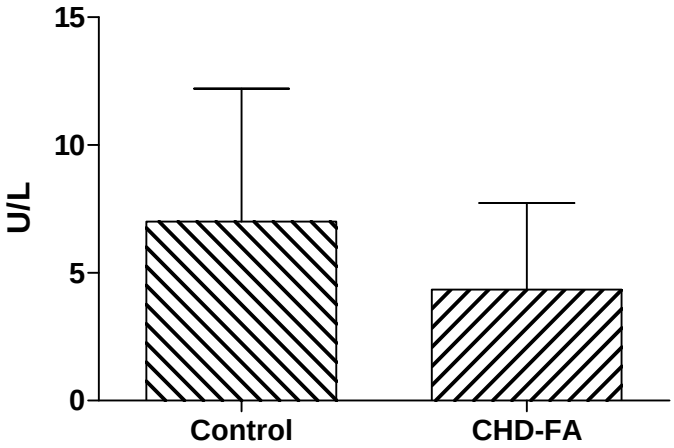

Figure 27: Liver GGT levels of rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control group: 1ml Water
Treatment: 1 ml CHD-FA (150 mg/kg body mass)

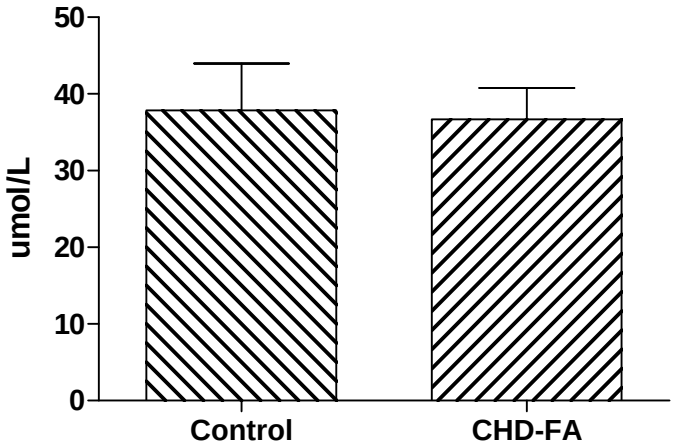

Figure 28: Kidney creatinine levels of rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control group: 1ml Water
Treatment: 1 ml CHD-FA (150 mg/kg body mass)

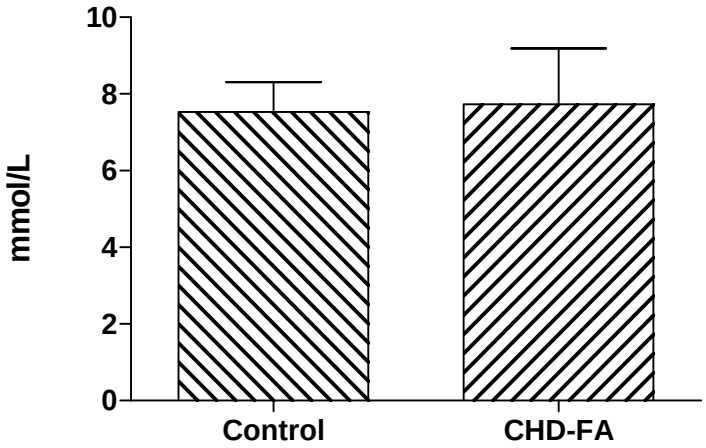

Figure 29: Kidney Urea levels of rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control group: Recieved1ml Water
Treatment: Received 1ml CHD-FA (150 mg/kg body mass)

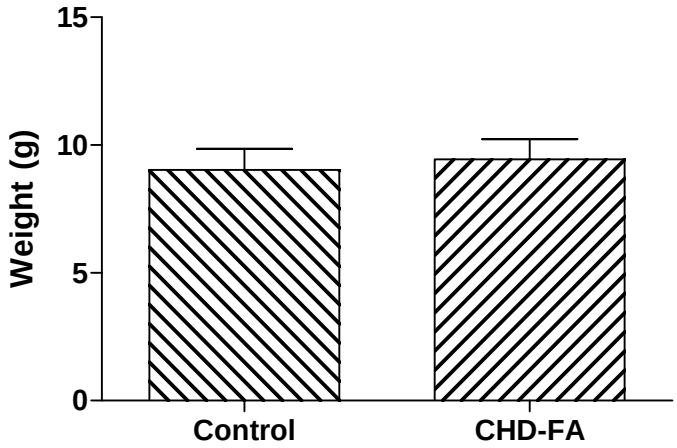

Figure 30: Liver weights of rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage. Animals were dissected and organs weighed at the end of a 7-day period. Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control group: Received 1ml water
Treatment group: Received 1ml CHD-FA (150 mg/kg body mass)

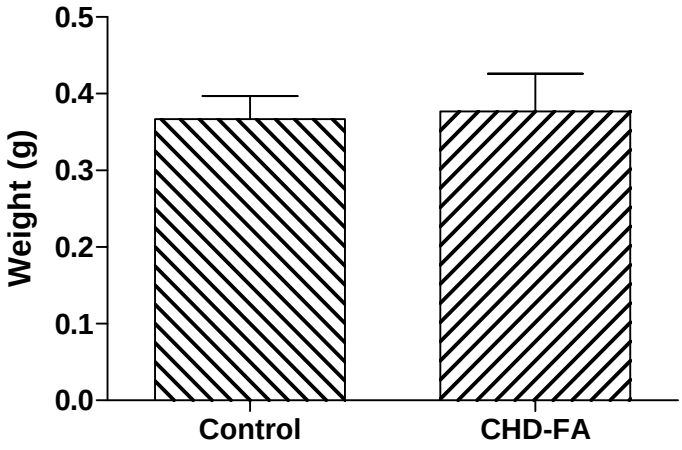

Figure 31: Spleen weights of rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage. Animals were dissected and organs weighed at the end of a 7-day period. Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control group: Received 1ml water
Treatment group: Received 1ml CHD-FA (150 mg/kg body mass)

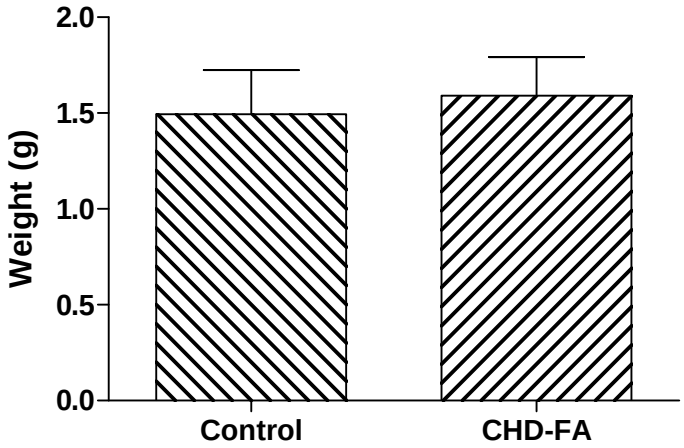

Figure 32: Kidney weights of rats which received a 1ml single dose of either CHD-FA (150 mg/kg body weight/day) or water via gavage. Animals were dissected and organs weighed at the end of a 7-day period. Data expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control group: Received 1ml water
Treatment group: Received 1ml CHD-FA (150 mg/kg body mass)

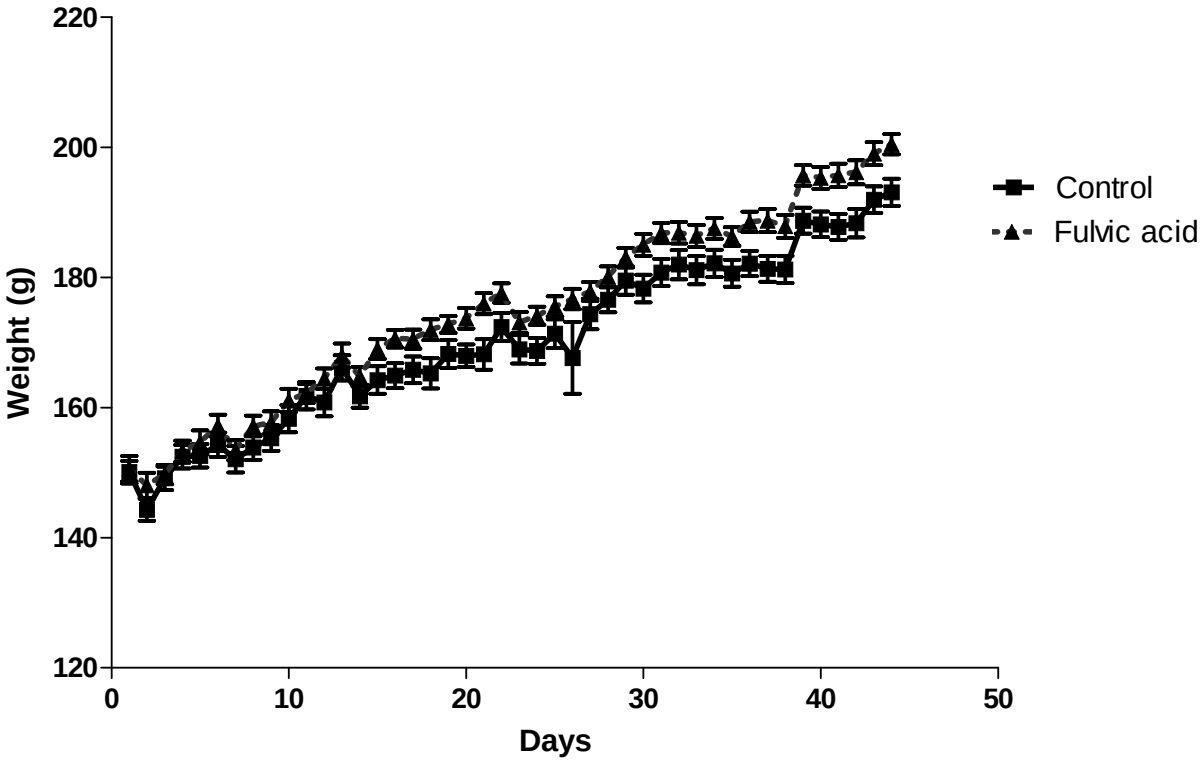

Figure 33: Weight patterns of Sprague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks. Weights expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group
CHD-FA: treated with carbohydrate derived fulvic acid (100 mg/kg body mass)

Table 5: Comparison of mean haematology values (± SD) of Sprague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks.

	Control (n=20) Mean ± s.d.	CHD-FA (n=20) Mean ± s.d.
Hb (g/l)	140.4 (± 3.573)	137.7 (±5.536)
RCC (x 10^{12} /l)	7.54 (± 0.17)	7.29 (± 0.31) **
HT (l/l)	0.43 ± 0.01	0.42 ± 0.03*
MCV (fl)	57.10 ± 0.46	57.17 ± 0.78***
MCHC (g/dl)	32.41 ± 0.37	32.49 ± 0.28
WCC (x 10^{9} /l)	4.28 ± 1.23	4.10 ± 0.72
Thr C (x 10^{9} /l)	833.40 ± 86.61	882.10 ± 74.03

Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Indicating a significant difference compared to the control (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Control; untreated group
CHD-FA; treated with carbohydrate derived fulvic acid (150 mg/kg body mass).

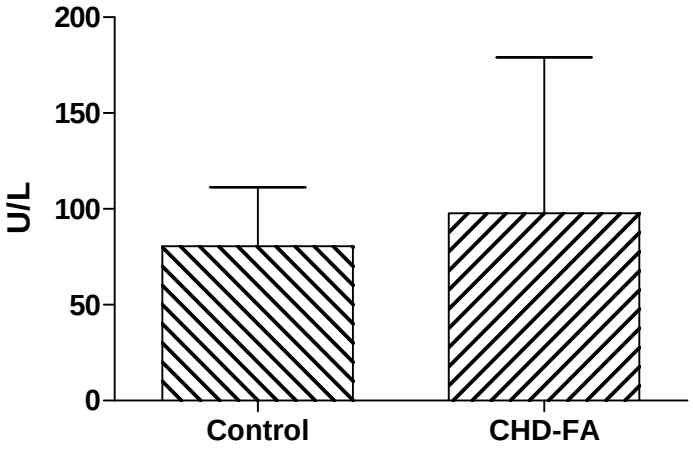

Figure 34: Liver AST levels of Spague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group
CHD-FA: treated with carbohydrate derived fulvic acid (100 mg/kg body mass)

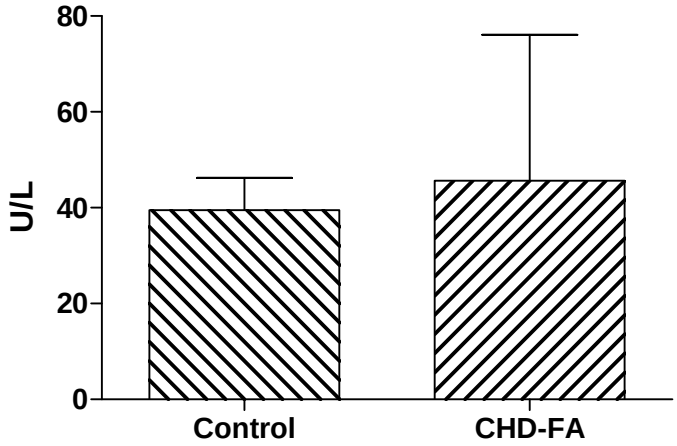

Figure 35: Liver ALT levels of Spague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group
CHD-FA: treated with carbohydrate derived fulvic acid (100 mg/kg body mass)

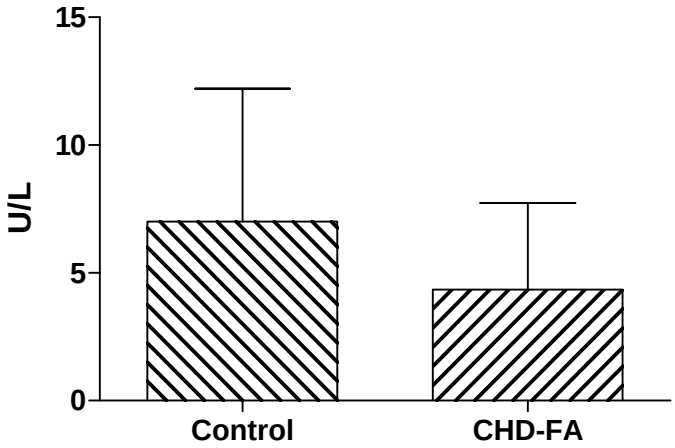

Figure 36: Liver GGT levels of Spague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group
CHD-FA: treated with carbohydrate derived fulvic acid (100 mg/kg body mass)

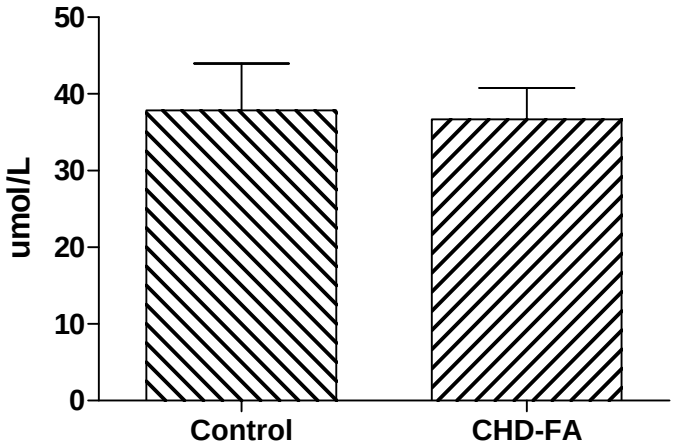

Figure 37: Kidney creatinine levels of Spague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group
CHD-FA: treated with carbohydrate derived fulvic acid (100 mg/kg body mass)

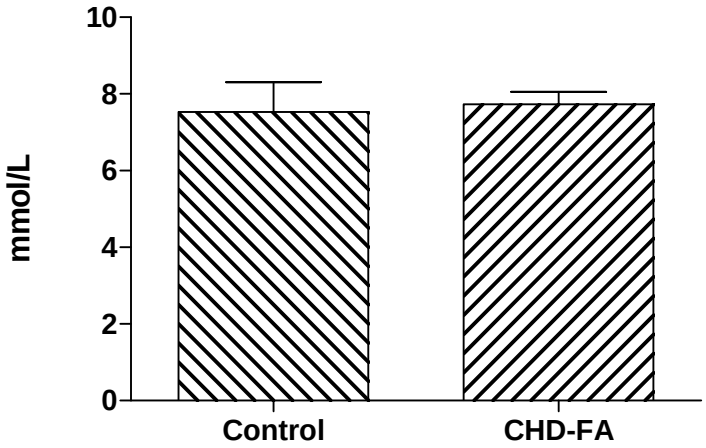

Figure 38: Kidney urea levels of Spague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks. Data recorded from blood samples taken from the rats via cardiac puncture and expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group
CHD-FA: treated with carbohydrate derived fulvic acid (100 mg/kg body mass)

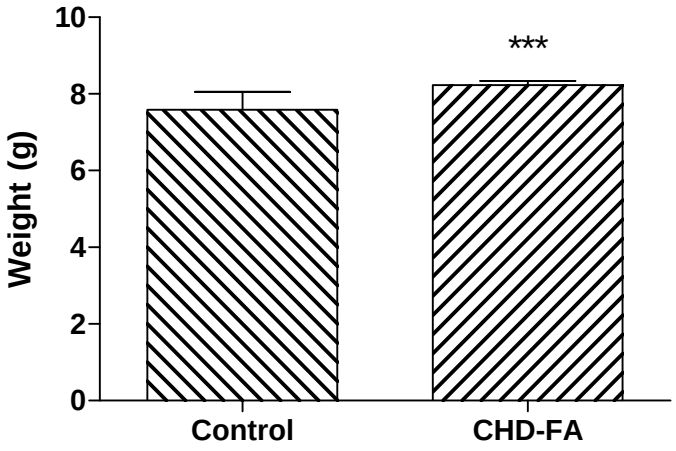

Figure 39: Liver weights of Spague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks. Weights expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group

CHD-FA: treated with carbohydrate derived fulvic acid (100 mg/kg body mass)

Indicating a significant difference compared to the control (*** $P < 0.001$).

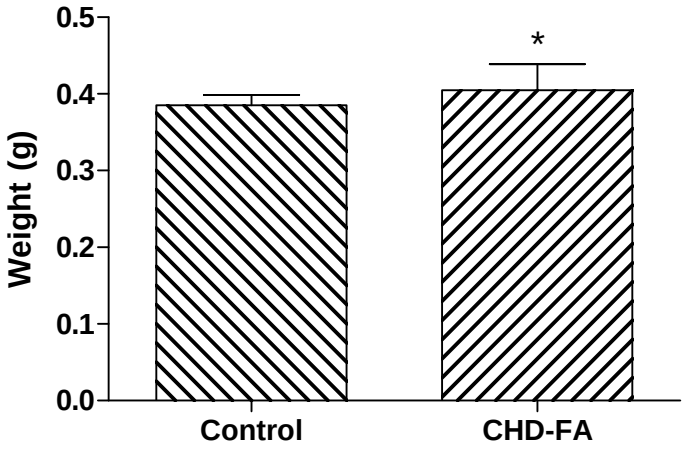

Figure 40: Spleen weights of Spague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks. Weights expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group

CHD-FA: treated with carbohydrate derived fulvic acid (100 mg/kg body mass)

Indicating a significant difference compared to the control (* $p<0.05$)

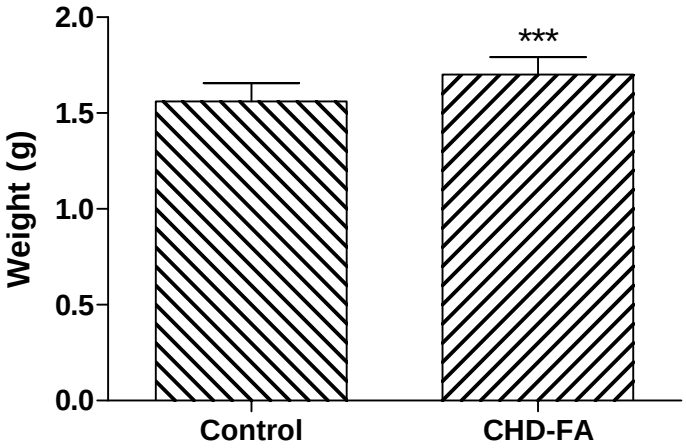

Figure 41: Kidney weights of Sprague-Dawley rats which received a 1ml dose of either CHD-FA (100 mg/kg body weight/day) or water via gavage for a period of 6-weeks. Weights expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control: untreated group
CHD-FA: treated with carbohydrate derived fulvic acid (100 mg/kg body mass)

Indicating a significant difference compared to the control (*** $P < 0.001$)

DISCUSSION

Fulvic acids have long been used for their medicinal properties and have undergone numerous safety studies in the past. The fulvic acid (CHD-FA) used in this study however was produced in a novel manner and safety therefore, needed to be re-established. During the numerous studies undertaken no changes in animal behaviour were observed nor did any deaths result from the drug therapy.

Changes in body and internal organ weights can indicate adverse side effects. Generally, weight loss is a simple and sensitive index of toxicity after exposure to toxic substances[34, 35]. In all the studies undertaken, the animals gained weight and there were no significant differences in the weight growth patterns of the treated groups compared to that of the control groups. Organ weights (liver, spleen and kidney) recorded from the 6-week chronic study did however, show a slight increase compared to that of the control. This was an observation not found in the 1-week acute study with animals taking a higher dose (150 mg/kg body mass). Taken in conjunction with the biochemistry, haematology and the general behaviour however, the increase was considered to be of non-clinical relevance.

Blood is an important index of physiological and pathological changes in an organism and can really only be considered valuable if the normal values are known [36]. In the absence of known normal values, the treatment groups were compared to the control. Examination of the haematology results obtained from the sensitivity to topical application study (phase I) indicates significant decreases in the red cell, white cell and the thrombocyte counts of the buffered CHD-FA compared to that of the control and un-buffered CHD-FA groups.

Although significantly different, the values were still well within the known normal values for mice. The haematology results obtained in the remaining oral dosage studies (phases II, III, V, VI) show significant differences in a number of the variables. These are however, well within the published normal ranges and the patterns observed were similar in both the control and treatment groups indicating that the changes were not specific to the treatment being applied and was therefore not considered of any clinical significance[36].

The degree of damage to the tissue or whole body, respectively, can be assessed by specific tests of enzymes. In clinical diagnostics, determination of transaminases, creatinine and urea are of great importance as they are excellent indicators of liver and kidney function [37]. As an intracellular enzyme, AST is released into the blood stream in proportion to the number of damaged cells. AST is however, located in the cytosol and mitochondria of different tissues (heart, skeletal muscle, liver, kidneys, pancreas and erythrocytes). ALT is therefore important in identifying specific liver-function disorders as it is primarily located in the cytosol of hepatocytes. GGT is specific to the hepatobiliary system and is therefore needed in identifying the exact location of hepatic damage [34, 37].

In phase I of the study, there was a significant decrease in AST and GGT compared to the control in both the treatment groups indicating no liver toxicity. Although there was a significant increase in creatinine and urea concentrations compared to the control, these were within the normal range of 26.52-88.40 µmol/L and 4.96-10.10 µmol/L respectively.

At the end of the sub-chronic 6-week study (phase II) blood samples analysed showed that all the values recorded were similar for the control and the treatment groups. Exceptions to this were the AST and creatinine

concentrations. The AST concentration at the end of the study was significantly increased compared to that at the beginning and was higher than the known normal range for Sprague-Dawley rats of 45.7-123.9 U/L. Creatinine was only slightly increased and as the animals showed no behavioural changes as well as all the other parameters being normal, a decision was made to upgrade the study to a 6-month chronic study (phase III). As an element of safety, a third group receiving half the original dose (75mg/kg body mass) was added and received treatment for a 6-week period. Samples taken from these animals (receiving 75mg/kg body mass) at the end of the 6-week period showed no significant differences to the control group in any of the parameters analysed and the AST levels were within the normal range. This result suggested that the initial increase in AST was perhaps dose related and a further two studies were initiated to examine this possibility. These studies included a 7-day acute study with animals receiving 150 mg/kg body mass CHD-FA (phase V) and a 6-week chronic study with animals receiving 100 mg/kg body mass CHD-FA. Results at the end of all three studies i.e. phase III, V, VI, showed no significant differences between the control and treatment groups in any of the parameters studied, including the AST levels which had returned to normal in the 6-month study.

Phase III was initiated in an attempt to explore the possibility of any teratogenic properties the product might have. All pregnant females carried to term and there were no differences in behaviour between the control and treatment groups. All pups were morphologically normal and histological examination of the organs was all normal. All other parameters examined, i.e. litter number and pup weights, were comparable to the control and indicate therefore that CHD-FA is safe for use during pregnancy

Looking at all the data presented in the above studies, the deduction can be made that CHD-FA is safe for use topically and can be applied safely at the doses used and for the periods tested. The product is also safe to consume during pregnancy.

CHAPTER IV: *IN VIVO* WOUND HEALING EFFICACY STUDY

INTRODUCTION

Wounds are physical injuries that result in an opening or breaking of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin [38]. Wound healing is a highly dynamic process and involves complex interactions of extracellular matrix (ECM) molecules, soluble mediators, various resident cells, and infiltrating leukocyte subtypes. The immediate goal in repair is to achieve tissue integrity and homeostasis. The healing process involves four phases that overlap in time and space: haemostasis, inflammation, tissue formation, and tissue remodelling [39].

Injury causes leakage of blood constituents into the wound site as well as release of vasoactive factors resulting in the activation of the clotting cascade and hemostats is. Hemostasis caused by coagulation then provides the basic architecture to initiate the inflammatory phase and tissue formation[40, 41]. The development of a wound infection depends on the complex interplay of many factors. The potential for infection depends on a number of patient variables such as the state of hydration, nutrition and existing medical conditions as well as extrinsic factors, for example related to pre-, intra-, and post-operative care if the patient has undergone surgery. This often makes it difficult to predict which wounds will become infected [42]. Consequently the prevention of wound infection should be a primary management objective for all healthcare practitioners. Infections of the surgical wound are one of the most common causes of morbidity and mortality in hospitals. The delay in recovery and subsequent increased length of hospital stay also has economic consequences. It

has been estimated that each patient with a surgical site infection will require an additional 6.5 days in hospital, which results in the doubling of hospital costs associated with that patient.

Wound infection is usually the most common reason for poor wound healing. It is important to note that all wounds are contaminated with bacteria. Whether a wound becomes infected is determined by the host's immune competence and the size of the bacterial inoculum. With normal host defences and adequate debridement, a wound may bear a level of 100,000 (10^5) microorganisms per gram of tissue and still heal successfully. Beyond this number, a wound may become infected [43].

Staphylococcus aureus is considered one of the most common causes of nosocomial infections worldwide [44]. Staphylococci are Gram-positive cocci colonising the epithelial surfaces in the majority of humans. S. *aureus* is usually regarded as a transient, pathogenic organism in the skin with about 20% of the population always harbouring it on the nasal mucosa without any pathogenic effects [45, 46]. In certain situations, however, *S. aureus* can express a wide variety of virulence factors. These include wall teichoic acid and surface proteins that promote adherence to damaged tissue, and can diminish neutrophil functions as well as antibody and cell-mediated immune responses [47, 48]. In addition, the organism secretes exotoxins and enzymes that can cause a variety of cutaneous and systemic infections. These include, furuncles, subcutaneous abscesses, staphylococcal scalded skin syndrome (SSSS), toxic shock syndrome (TSS) and neonatal toxic shock syndrome-like exanthematous disease (NTED) [49, 50].

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in the UK in the 1980s and remains a cause of concern for all healthcare practitioners

[51, 52]. Severe infections with MRSA and multiresistant S. *aureus* are associated with an increased rate of mortality, prolonged hospitalization and increased health care costs compared to infections with susceptible isolates [53, 54]. There are now many different strains of MRSA affecting a large number of individuals in many different healthcare settings. The degree to which people are affected ranges in severity from simple wound colonisation, which does not need to be treated aggressively, to systemic infection such as bronchopneumonia, which may prove to be fatal. Anecdotal evidence suggests that MRSA is no more pathogenic in a wound than the non-resistant version. However, it is accepted that if a wound is infected with MRSA it is difficult to manage with antibiotics [55].

AIM

The aim of this study was to test the antimicrobial efficacy and wound healing properties of CHD-FA.

MATERIALS AND METHODS

All chemicals unless specifically noted, were sourced from Sigma-Aldrich (Pty) Ltd., Aston Manor 1630, South Africa. Isoflurane sourced from Abbott Laboratories South Africa. Buprenorphine sourced from Schering-Plough, Woodmead, South Africa and cyclophosphamide from Aventis, South Africa.

Formulation development

In order to make the product more tolerable for use in humans, the pH of the neat fulvic acid (pH 1.9) needed to be adjusted. Various bases were used in an attempt to adjust the CHD-FA without losing the antimicrobial efficacy of the product. The following chemicals: i.e. Potassium hydroxide, sodium hydroxide, sodium acetate, tetra-methyl ammonium hydroxide, tetra ethylamine and citrate were used to adjust the pH. Dry powders of the bases were added to the fulvic acid product until the desired pH was obtained.

The CHD-FA, adjusted to a pH of 5.5 using the above alkaline chemicals were then tested for anti-microbial efficacy using the radial diffusion inhibition method, as described in chapter II. The CHD-FA/alkaline chemical combination which showed the most anti-microbial efficacy was used in all further studies.

UEA aqueous creams with 8-20% (w/w) solutions of propylene glycol dissolved into the creams were prepared (Sourced from Van Dyk Pharmaceutical Products (Pty) Ltd, Melville, South Africa). CHD-FA (3.9% w/v, pH 5.5) was added in 50:50, 30:70 and 35:65 (CHD-FA: aqueous) ratios to obtain homogenous

aqueous creams with CHD-FA dissolved in it. Homogenous mixtures were prepared by stirring each mixture for 30 minutes using a magnetic stirrer. The combinations were then left for a period of a day and thereafter checked for consistency. All analysis and tests were conducted at room temperature. Samples of these creams were then tested for anti-microbial efficacy using the radial diffusion method, whereby, 50µl of the aqueous cream mixture were placed into wells punched into agar plates containing *S. aureus* (ATCC 12600), as described previously.

Wound healing study: In vivo efficacy

This study was conducted in two phases. Phase one was a pilot study which was followed by the main study (phase 2).

Phase 1: Pilot study

The purpose of the pilot study was to determine the size of the inoculum needed to induce a suitable infection. The concentration determined in this study was then used in the main study.

Bacteria used were cultured on plates, prepared with MacConkey agar (with 0.85% salt). Plates were then incubated at 37 ºC where after, colonies were harvested from the plate and placed into 2ml of saline. This suspension was then read on a calorimeter with a 520nm filter to determine optical density (O.D). Colonies were added to the suspension until an O.D. of 0.7 was obtained. Using serial dilutions and viable plate counts, this O.D. was previously calculated to correspond to $1x10^8$ C.F.U/ml. A one in two dilution of this sample was then made to obtain a $5x10^7$ C.F.U/ml suspension. These two concentrations of *Staphylococcus aureus* (ATCC 12600) were then used in the study.

20 animals were divided into four groups of 5. The first group received an inoculum corresponding to $1x10^8$ CFU/ml whilst the second received $5x10^7$ CFU/ml. Groups 3 and 4 both received the same inoculum as groups 1 and 2, but

were immunocompromised with an i.v. injection of cyclophosphamide (200 mg/kg body weight dissolved in 200 µl distilled water-as described in a previous protocol H12-04 and Hou *et al*)[56].

Animals were first anaesthetized using isoflurane after which a painkiller was administered intramuscularly (Buprenorphine, 0.3mg/kg, repeated every 12 hrs as needed). Thereafter, the hair was removed from the test area using the clippers and two full skin thickness wounds, approximately 4mm in diameter, was created on the backs of the animals by cutting a circular area of skin from the test area.

One of the wounds was then inoculated, by pipetting 200µl of the prepared bacterial suspension directly into the wound. The wounds were then covered with an occlusive dressing. The other wound served as a control and was not inoculated. Animals were then observed for a period of a week, during which time the dressing was removed every second day to ascertain if an infection was occurring. The concentration, which produced a notable infection, was then used in the main study. Animals were euthanized by CO_2 asphyxiation at the end of the study.

Phase II: Main study

For this phase, 30 rats were divided into 2 main groups-:

Group 1: Topical treatment of CHD-FA

Group 1 comprised of 10 rats and received a topical application of the fulvic acid (1.75%) in the form of a cream/lotion. Animals were immunocompromised 4 days prior to the beginning of the study (200 mg/kg body weight dissolved in 200 µl distilled water-as described in the pilot study above)

The rats were sedated using isoflurane and the hair from the test area removed using the clippers.

The area was then sterilized with a 70% alcohol solution after which, four lesions were produced on the backs of each rat by cutting a circular area, approximately 4mm, of skin from the test area. The selected bacterial concentration was then introduced into the wound as described in the pilot study. The lesions were treated as follows-:

A.Negative control (Cetomacrogol (aqueous) cream without fulvic acid)

B.Positive control (0.05g Fusidic acid based cream, 10mg/g)

C.Fulvic acid cream, pH 2.3 (CHD-FA 1.75%)

D.Fulvic acid cream, pH 5.5 (CHD-FA 1.75%)

Wounds were treated by applying approximately 2ml of cream using a graduated pipette. CHD-FA/Cetomacrogol creams were formulated to contain 1.75% fulvic acid.

The entire area was then covered with an occlusive dressing (Transpore-sourced from 3M South Africa, Pty, Ltd). The animals were returned to their cages for

the next 48-hours. At the end of this period, the dressing was removed and the initial measurements of the wounds recorded.

Once the initial recordings were completed, the treatments were applied in accordance with the above list (A-D). The area was once again covered and the animals were returned to their cages for another 24 hours. Wound size measurements were thereafter taken every 24 hours for the next 6 days. Treatment was reapplied before the wound was re-dressed with the occlusive covering. At the end of the eighth day, the animals were terminated by CO_2 asphyxiation.

Wound area and degree of healing was used as an indication of antimicrobial efficacy. Percentage wound closure was calculated as [(initial-final)/initial] x 100.

Group 2-: Oral treatment of CHD-FA

Group 2 was further divided into two sub-groups (2A and 2B) and received an oral treatment of fulvic acid (adjusted to pH 5.5 with sodium acetate) or water depending on the group. Each sub-group was comprised of 10 rats each and underwent the same procedures as that of group 1, with the exception of receiving only two wounds on their backs instead of four. One wound was infected with the same concentration as that used in the above main study (i.e. $1x10^8$ CFU/ml) whilst the other served as a control.

Group 2A received 1ml of a 1.75% solution of a fulvic acid with a pH of 5.5 (100mg/kg/day body mass) by gavage whilst group 2B received 1ml water (negative control).

RESULTS

Formulation development

When the pH of the CHD-FA was adjusted using sodium acetate, a z.i. measuring 11.80mm ± 0.33mm was obtained. Sodium acetate adjusted the pH to the required acidity as well (table 6).

The mixture of CHD-FA to Aqueous cream that produced the most homogeneous and stable cream was that of a 50:50 (CHD-FA: Aqueous) ratio. It was therefore decided that the 50:50 (CHD-FA: Aqueous) mixture would be used in subsequent studies.

Wound healing study: In vivo efficacy

Phase 1: Pilot study

The weights of all the groups decreased with the weight growth pattern of the immunocompromised groups falling more steeply (fig 42).

Figures 43-46 represents the wound closure patterns over a period of 5 days. Results indicate that the infected wounds tend to heal better than the control wounds.

Phase II: Main study

The results show that all the groups decreased in weight indicating that the cyclophosphamide had a negative effect on the overall health of the animals used (fig 47).

Figure 48 shows that CHD-FA adjusted to a pH of 5.5 (1.75%) was not effective when compared to the positive control. However, CHD-FA at a pH of 1.98 (1.75%) was comparable to the positive control (fig 48). Compared to the

negative control, all the groups were more effective (fig 48). The animals treated by gavage with the CHD-FA buffered to pH 5.5 showed no difference in wound closure patterns when compared to those gavaged with water (fig 49).

Table 6: Mean Inhibition zones (±SD) around the wells (containing the various solutions of CHD-FA in which the pH was adjusted using the listed chemicals) indicating sensitivity of *S. aureus* (ATCC 12600*) in vitro*.

Chemical used	**pH**	**Zone of Inhibition (mm)**
None	2.2	17.86 ± 0.26
Citrate	3.39	27.78 ± 0.52
Sodium acetate	5.5	11.80 ± 0.33
Tetramethyl ammonium OH	5.5	0.00
Triethylamine	5.5	0.00
Potassium OH	5.5	0.00
Sodium OH	5.5	0.00

Statistical significance was calculated using ANOVA.

Chemical used: Chemical used to adjust the pH.
pH: The pH obtained using the chemical
Zone of Inhibition: Measured Z.I. using the combination of CHD-FA and the pH adjuster.

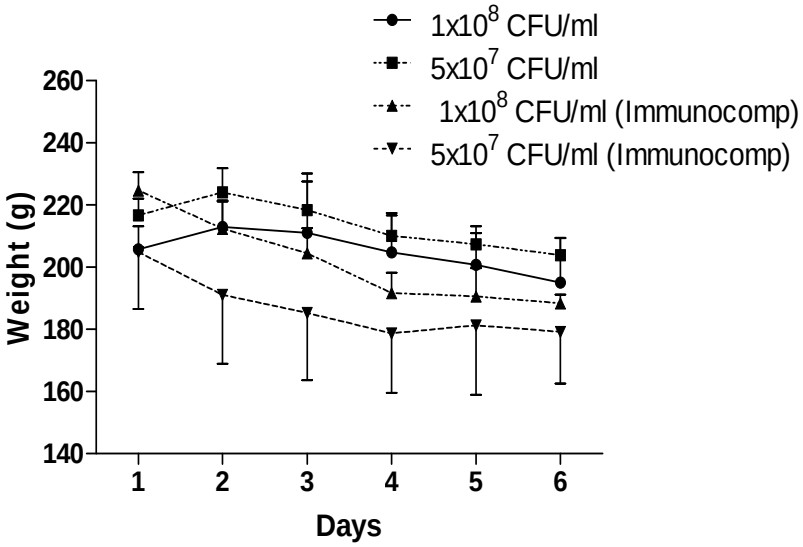

Figure 42: Weight patterns of Sprague-Dawley rats on which 2 full skin thickness wounds were cut on the backs on day 1. Animals were divided into 4 groups, in which only groups 2 and 3 were immunocompromised using cyclophosphamide (200mg/kg body mass injected i.v.). The wounds of groups 1 and 3 were inoculated with a *S. aureus* (ATCC 12600) concentration of $1x10^8$ C.F.U/ml whilst the wounds of groups 3 and 4 were inoculated with $5x10^7$ C.F.U/ml of the same organism. Weights are expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

$1x10^8$ CFU/ml-: Group of rats with immune system intact receiving an inoculum concentration of $1x10^8$ CFU/ml of S. aureus (ATCC 12600).
$5x1^7$ CFU/ml-: Group of rats with immune system intact receiving an inoculum concentration of $5x10^7$ CFU/ml of S. aureus (ATCC 12600).
$1x10^8$ CFU/ml (Immunocomp)-: Group of rats with immune system compromised receiving an inoculum concentration of $1x10^8$ CFU/ml OF S. aureus (ATCC 12600).
$5x10^7$ CFU/ml (Immunocomp)-: Group of rats with immune system compromised receiving an inoculum concentration of $5x10^7$ CFU/ml OF S. Aureus (ATCC 12600).

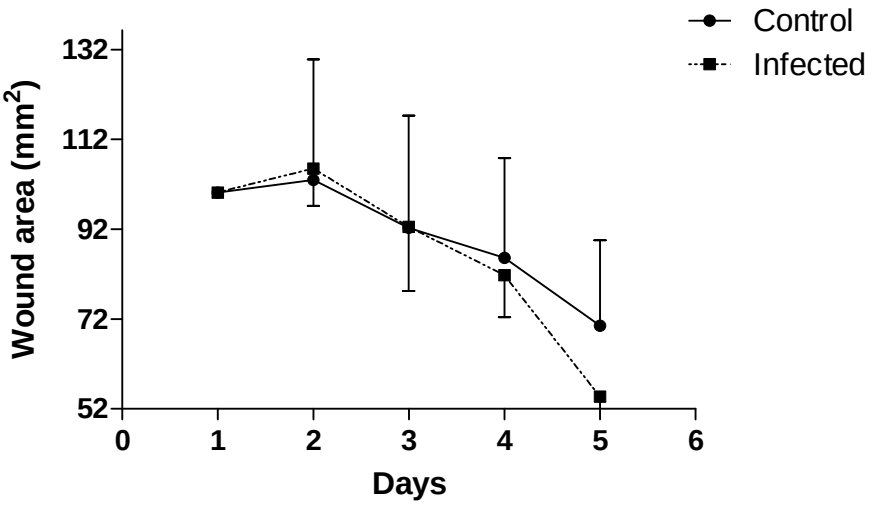

Figure 43: Wound closure pattern of Sprague-Dawley rats over a period of 5 days on which 2 full skin thickness wounds were cut on the backs. Animals received an inoculum of *S. aureus* (ATCC 12600 with a concentration of $1x10^8$ CFU/ml), placed into one of the wounds using a syringe. The data is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control-: Wound receiving no inoculum.
Infected-: Wound receiving an inoculum concentration of $1x10^8$ CFU/ml of S. aureus (ATCC 12600).

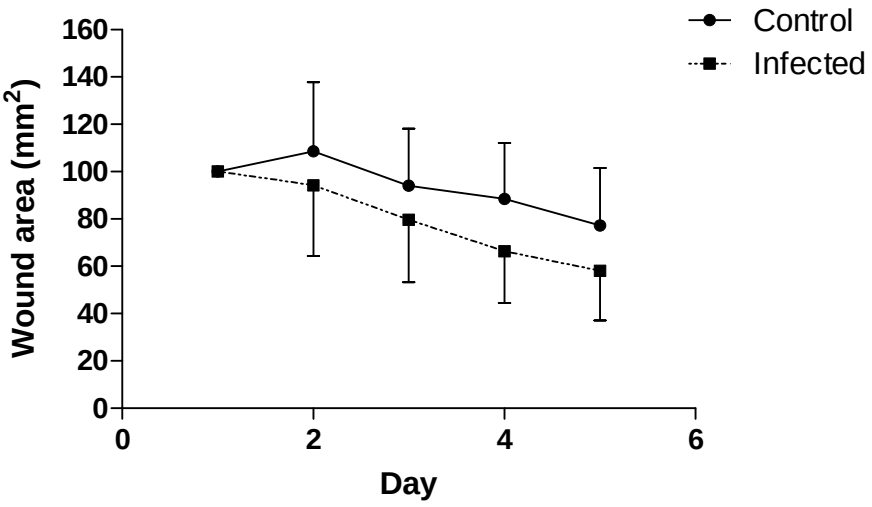

Figure 44: Wound closure pattern of Sprague-Dawley rats over a period of 5 days on which 2 full skin thickness wounds were cut on the backs. Animals received an inoculum of *S. aureus* (ATCC 12600 with a concentration of $5x10^7$ CFU/ml), placed into one of the wounds using a syringe. The data is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control-: Wound receiving no inoculum.
Infected-: Wound receiving an inoculum concentration of $5x10^7$ CFU/ml of S. aureus (ATCC 12600)

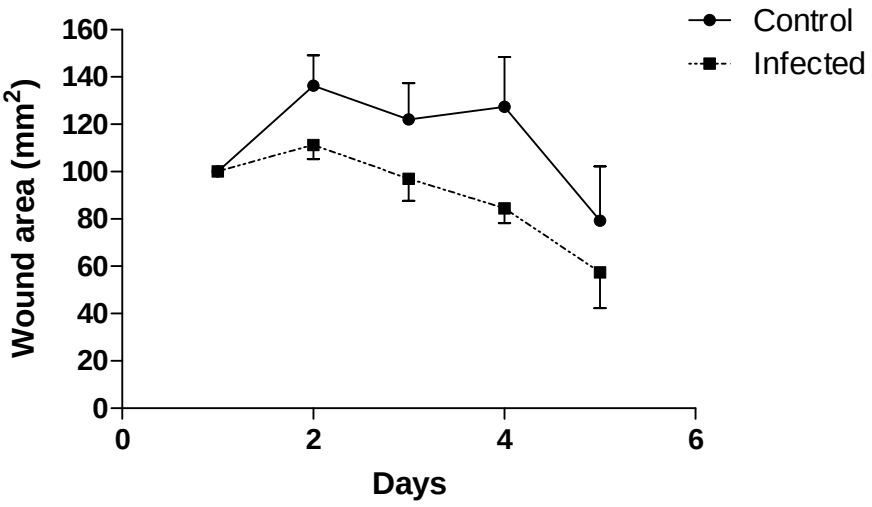

Figure 45: Wound closure pattern of immunocompromised Sprague-Dawley rats over a period of 5 days on which 2 full skin thickness wounds were cut on their backs. Animals received an inoculum of *S. aureus* (ATCC 12600 with a concentration of $1x10^8$ CFU/ml), placed into one of the wounds using a syringe. The data is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control-: Wound receiving no inoculum.
Infected-: Wound receiving an inoculum concentration of $1x10^8$ CFU/ml of S. aureus (ATCC 12600)

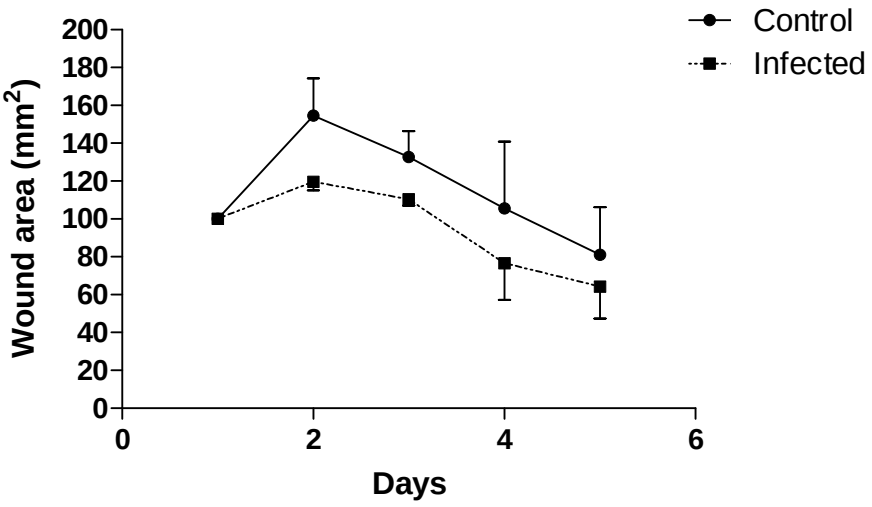

Figure 46: Wound closure pattern of immunocompromised Sprague-Dawley rats over a period of 5 days on which 2 full skin thickness wounds were cut on the backs. Animals received an inoculum of *S. aureus* (ATCC 12600 with a concentration of $5x10^7$ CFU/ml), placed into one of the wounds using a syringe. The data is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control-: Wound receiving no inoculum.
Infected-: Wound receiving an inoculum concentration of $5x10^7$ CFU/ml of S. aureus (ATCC 12600)

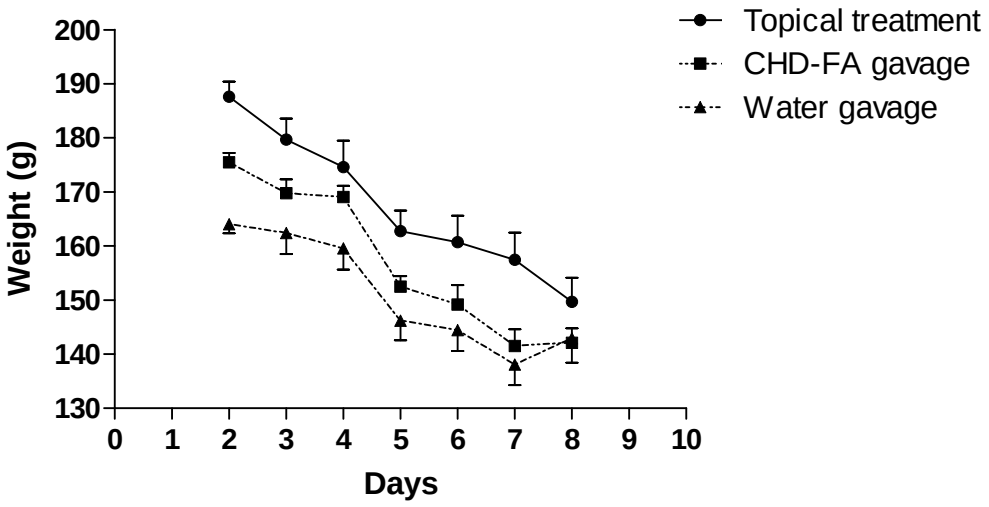

Figure 47: Weight patterns of immunocompromised Sprague-Dawley rats on which full skin thickness wounds were cut on the backs on day 1. Animals were divided into 3 groups, according to treatment route (oral or topical) of CHD-FA. Weights are expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Topical treatment-: Group of immunocompromised Sprague-Dawley rats with two wounds created on the backs. Animals received 1ml of a 1.75% CHD-FA bolus via gavage daily for the duration of the study.

CHD-FA-: Group of immunocompromised Sprague-Dawley rats with four wounds created on the backs. The wounds were treated with Cetomacrogol (negative control), fusidic acid (positive control), CHD-FA at pH 2.3 (1.75%) and CHD-FA pH 5.5 (1.75%).

Water gavage-: Group of immunocompromised Sprague-Dawley rats with two wounds created on the backs. Animals received a 1ml bolus of water via gavage daily for the duration of the study.

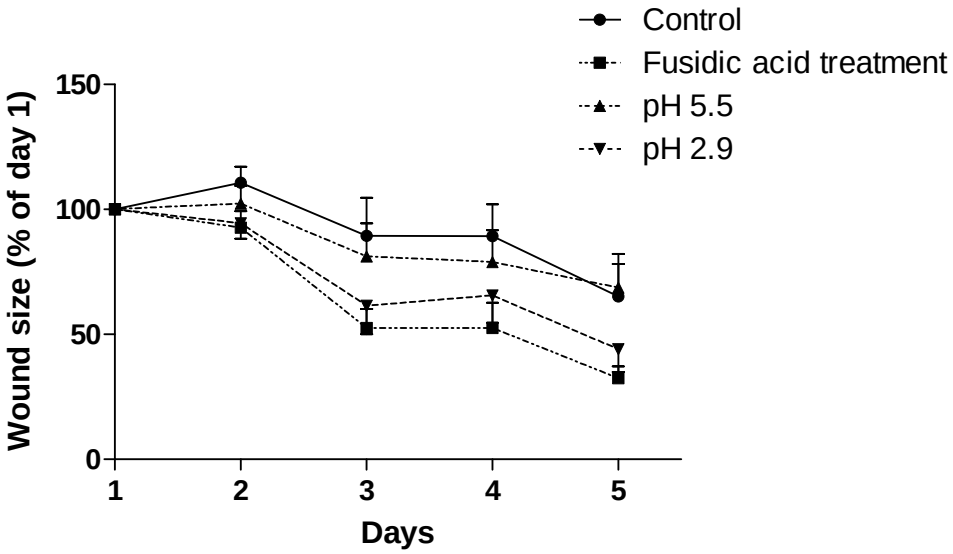

Figure 48: Wound closure pattern of immunocompromised Sprague-Dawley rats over a period of 5 days on which 4 full skin thickness wounds were cut on the backs. Animals received an inoculum of *S. aureus* (ATCC 12600 with a concentration of $1x10^8$ CFU/ml), placed into each of the wounds using a syringe. Wounds were labelled according to the treatment used on each. The data is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control-: Wound treated with Cetomacrogol aqueous cream (negative control)
Fusidic acid-: Wound treated with 0.05g Fusidic acid based cream, 10mg/g (positive cream)
pH 5.5-: Wound treated with a 1.75% CHD-FA based aqueous cream, pH adjusted to 5.5 with sodium acetate.
pH 2.9-: Wound treated with 1.75% CHD-FA based aqueous cream, pH left at 2.3.

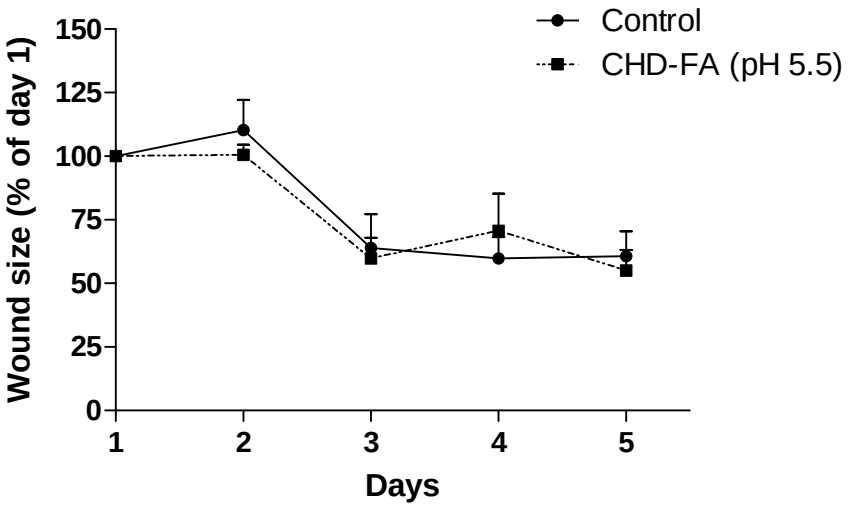

Figure 49: Wound closure pattern of a group of immunocompromised Sprague-Dawley rats over a period of 5 days on which 2 full skin thickness wounds were cut on the backs. Animals were divided into two groups of ten depending on the oral treatment each received. Animals received an inoculum of *S. aureus* (ATCC 12600 with a concentration of $1x10^8$ CFU/ml), placed into each of the wounds using a syringe. The data is expressed as means ±SD. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

Control-: Animals receiving 1ml water each day for the duration of the study (negative control).
CHD-FA (pH 5.5)-: Animals receiving 1ml of a 1.75% solution of CHD-FA with the pH adjusted to 5.5 using sodium acetate. Treatment was given daily for the duration of the study.

DISCUSSION

Wound healing in the human has many unique aspects that depend on the physiology, aging and regional characteristics of the species, but the opportunity to carry out controlled experimentation on the nature and therapy of wounds is limited. There is also enormous variability in the standard of care amongst institutions and clinicians, making it often difficult to compare treatments or outcomes. To overcome these problems, investigators have turned to other organisms in hopes of analyzing the complexities of wound healing in circumstances having less genetic and environmental variability. Although there is considerable biological variation of the wound healing response even amongst inbred animal strains, variance falls to levels that allow limited numbers of invasive procedures to yield significant conclusions. In general, animal models attempt to reflect the human wound healing problems: dehiscence, ischemia, ulceration, infection and scarring [57]. The model used in this study was of the full thickness variety and involves the complete removal of the epidermis to the depth of fascial planes or subcutaneous fat. The wounds were then inoculated with a strain of MRSA in order to test the antimicrobial and wound healing properties of CHD-FA.

The objective in wound management is to heal the wound in the shortest time possible, with minimal pain, discomfort, and scarring to the patient [58]. In the past, there have been many studies evaluating the wound healing properties of certain substances. Plant extracts including ginkgo biloba, echinacea, ginseng, grape seed, green tea, lemon, lavender, rosemary, thuja, sarsaparilla, soy, prickly pear, sagebrush, jojoba, *Aloe vera*, allantoin, feverwort, bloodroot, apache plume, and papaya, has long been used as topical applications for wound healing treatments. The compounds in these studies are similar to CHD-FA in that in many of these studies, the actual active compound is not known.

Results from the pilot study showed that the immunocompromised animals inoculated with a $1x10^{10}$ CFU/ml concentration of *S. aureus* produced a notable wound infection. It was decided, therefore, that this setup would be used for the main study.

Results from the main study show that both fuscidic acid and CHD-FA (pH 1.98) were effective in this model with CHD-FA (pH 1.98) however, being slightly less effective than the positive control. CHD-FA (pH 5.5), on the other hand, was the least effective in terms of promoting wound healing. These results compare favourably with a similar study conducted on a plant *Arrabidaea chica* in which the plant was shown to be effective in reducing the healing time of wounds created on the backs of Wistar rats [59]. A 96% wound closure was observed in the group treated topically with *Arrabidae chica* (A.C.) at the end of 10 days. The control group could only manage a 13% closure of the created wounds over the same period. Although the current study showed a wound closure of only 55% for the CHD-FA (pH 1.98) treated group, the test period of 5 days was half of the A.C. study. In addition, the above mentioned study did not take into account infection which is known to delay healing. In contrast to the topical CHD-FA treatment however, the oral treatment with CHD-FA (pH 5.5) showed no significant improvement over placebo.

In conclusion, it is clear from the above results that CHD-FA could serve as a viable alternative in the topical treatment of full thickness wounds. A further advantage is that the product is effective against infection, which is a typical problem in wounds such as those caused by burns.

CHAPTER V: DISCUSSION AND CONCLUSION

DISCUSSION

Traditional fulvic acids are obtained from soils and water surfaces, and as a result, are products of the environment from which they were obtained. This means that metals and other compounds located at the site are likely incorporated into these fulvic acid products. The result is fulvic acids are potentially composed of toxic heavy metals. In contrast, the fulvic acid examined in this study was obtained using a patented method of wet oxidation on a metal free carbohydrate source. The resulting product is thus free of heavy metals and is therefore potentially less toxic than traditional fulvic acids. The primary objective of this study was therefore to examine the anti-microbial efficacy and safety of this novel, carbohydrate derived fulvic acid (CHD-FA).

The initial *in vitro* screening studies using the radial diffusion inhibition method showed that the product was effective against *S. aureus*. CHD-FA obtained a Z.I. of greater than 13mm for both a laboratory and patient strain. Using CLSI published criteria to ascertain susceptibility; a Z.I. of 13mm or more is considered susceptible when testing against *S. aureus*. The results obtained from the radial diffusion inhibition study indicate that the product is as effective as oxacillin in the case of a laboratory strain of *S. aureus*. Furthermore, CHD-FA showed a greater efficacy towards a patient strain of *S. aureus*. This result is confirmation of the outcome obtained in a study conducted by van Rensburg *et al*, in which oxifulvic acid was shown to be effective against a strain of *S. aureus* [60]. In addition, CHD-FA compared favourably to *Aloe vera* which in a similar study, obtained a Z.I. of 18 mm. The volume (100µl) of *aloe vera* used in the study was double the volume used for the CHD-FA (50µl) though and this could be an indication that the CHD-FA is more active against *S. aureus* than

aloe vera [22]. In addition to being effective against *S. aureus*, an added advantage was uncovered by the Franz-type diffusion cell studies. CHA-FA at a pH of 2.9 was able to penetrate though the skin barrier and as such, could be an ideal candidate for subcutaneous infections such as boils and folliculitis.

The safety of the product was extensively examined in animals during the course of the study. Results indicated that the product did not induce any sensitivity or toxicity upon topical application at a dose of 800mg/kg bw/day and continuous oral application for periods lasting up to six months and at doses of up to 150mg/kg bw/day, produced no signs of toxicity. Furthermore, the product proved safe for use in pregnant rats at a dose of 150mg/kg bw/day, with both mother and pups showing no signs of toxicity or teratogenicity.

To further demonstrate the effectiveness of CHD-FA, the product was tested in an *in vivo* infected wound healing animal model. The study consisted of animals on which full thickness wounds were created. The wounds were then infected with strains of *S. aureus* before treatment was applied. Results obtained from this study show that the topical CHD-FA (pH 1.98) was effective in terms of promoting wound healing. These results compare favourably with a similar study conducted on a plant *Arrabidaea chica* in which the plant was shown to be effective in reducing the healing time of wounds created on the backs of Wistar rats [59]. This study did not however, take infection into account.

CONCLUSION

The research contained in this document clearly demonstrates that CHD-FA possesses anti-microbial activity against *S. aureus in vitro*. In addition, the product used in this study has the added benefit of not containing possible hazardous heavy metals. The benefit of which was confirmed in the toxicity

studies conducted on Sprague-Dawley rats in which no negative side effects were reported.

The wound healing study demonstrated clearly as well, that the product is effective in reducing the healing period of infected wounds.

Although the active compound is not known as yet and as such, a mechanism of action is not clearly apparent. It could be postulated however, that the low pH has some influence on the mode of action. This could serve as the bases of a new study designed to examine the mechanism of action of the product.

The CHD-FA product could prove to be a highly effective and safe alternative to the current treatment options used in the treatment of cuts and burns in human patients. The importance of this alternative is made more evident in the light of the ever increasing microbial resistance to mainline treatment. The results obtained from this study thus lends credence to the logic that initial human studies should be conducted on the product.

REFERENCES

1. Brundtland, G.H.2000. *Overcoming Antimicrobial Resistance*, in *in World Health Report on Infectious Diseases*World Health Organization.
2. Souhami, R.L. and Moxham, J., eds. *Textbook of Medicine*. 2nd ed. 1994, Churchill Livingstone.
3. Mandell, G.L., Douglas, R.G., and Bennett, J.E., eds.1990. *Principals and Practices of Infectious Diseases*. New York: Churchill Livingstone.
4. Bennett, J.C. and Plum, F., eds. *Cecil Textbook of Medicine*. 1996, W.B. Saunders Company: London.
5. Prior, L.S.O.S.2005. *Resistance to Antimicrobials in Humans and Animals.* British Journal of Medicine. **331**: p. 1219-1220.
6. Beović, B.2006. *The issue of antimicrobial resistance in human medicine.* International Journal of Food Microbiology. **112**: p. 280-287.
7. Stevenson, F.J.1982. *Reactive Functional Groups of Humic Substances Humus Chemistry*, in *Genesis, Composition, Reactions*Wiley-Interscience: New York.
8. Peña-Méndez, E.M., Havel, J., and Patočka, J. 2005. *Humic substances-Compounds of still unknown structure: applications in agriculture, industry, environment, and biomedicine.* Journal of Applied Biomedicine. **3**: p. 13-24.
9. Buffle, J.A.E. *Les substances humiques et leurs interactions avec les ions mineraux*. in *Conference Proceedings de la Commission d`Hydrologie Appliquee de A.G.H.T.M.* 1977. Universte d`Orsay.
10. Jooné, G.K. and Van Rensburg, C.E.J.2004. *An in vitro investigation of the anti-inflammatory properties of potassium humate.* Inflammation. **28**: p. 169-174.

11. Bergh, J.J., Cronje, I.J., and Dekker, J.1997. *Non-catalytic oxidation of water-slurried coal with oxygen: Identification of fulvic acids and acute toxicity.* Fuel(76): p. 149-154.
12. VanRensburg, C.E.J., Dekker, J., Weis, R., Smith, T.L., and Schneider, J.2002. *Investiation of the anti-HIV properties of oxihumate.* Chemotherapy(48): p. 138-143.
13. Van Rensburg, C.E.J., Van Straten, A., and Dekker, J.,2000. *An in vitro investigation of the antimicrobial activity of oxifulvic acid.* Antimicrobial Chemotherapy(46): p. 835-854.
14. Dekker, J. and Medlen, C.E., 1999. *Fulvic acid and its use in the treatment of various conditions*, P. Corporation, Editor.
15. Snyman, J.R., Dekker, J., Malfeld, S.C.K., and Van Rensburg, C.E.J.,2002. *Pilot study to evaluate the safety and therapeutic efficacy of topical oxifulvic acid in atopic volunteers.* Drug Development Research. **51**: p. 1-4.
16. McAllister, C.F., 2003. *Manual for Microbiology Laboratory*. Georgia: Morton Publishing.
17. Mandell, G.L., Douglas, R.G., and Bennett, J.E., eds. *Principals and Practices of Infectious Diseases*. third ed. 1990, Churchill Livingstone: New York.
18. 1997. *Guidance for Industry: M3 nonclinical safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals*, F.A.D.A. U.S. Department of Health and Human Services, Editor, Center for Drug Evaluation and Research.
19. Chah, K.F., Eze, C.A., Emuelosi, C.E., and Esimone, C.O., 2006. *Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants.* Journal of Enthnopharmacology. **104**: p. 164-167.

20. Fernandes, A.C., Pauw, E., and Van Rensburg, C.E.J., 2006. *An in vitro investigation of the antimicrobial activity of a novel, carbohydrate derived fulvic acid*, Department of Pharmacology, University of Pretoria.
21. Mathew A.W., Franklin R.C., William A.C., and N.D., M., 2006. *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard*. Clinical and Laboratory Standards Institute document, ed. M.J. F. Vol. 26. Pennsylvania: Clinical and Laboratory Standards Institute.
22. Agarry O.O., Olaleye M.T., and C.O., B.-M., 2005. *Comparative antimicrobial activities of aloe vera gel and leaf.* African Journal of Biotechnology. **4**: p. 1413-1414.
23. Chien, Y.W.,1987. *Drug Developement.* Industrial Pharmacy(13): p. 589-654.
24. Katz, M. and Poulsen, B.J., eds. *Handbook of Experimental Pharmacology*. ed. B.B. Broie and J.R. Gillette. Vol. 27. 1971, Springer-Verlag: Berlin. 103-174.
25. Degim, I.T.,2005. *Understanding Skin Penetration: Computer Aided Modeling and Data Interpretation.* Current Computer-Aided Drug Design(1): p. 11-19.
26. Holbrook, K.A. and Odland, G.E.,1974. *Reginal differences in the thickness (cell layers) of the human stratum corneum: an ultrastructural analysis.* . Journal of Investigational Dermatology. **62**: p. 415-422.
27. Arellano, A., Santoyo, S., Martin, C., and Ygartua, P.,1998. *Influence of propylene glycol and isopropyl myristate on the in vitro percutaneous penetration of diclofenac sodium from carbopol gels.* European Journal of Pharmaceutical Science. **7**: p. 129-135.
28. Gummer, C.L., 1989. *Developmental Issues and Research Initiatives*, in *Transdermal drug delivery*, J. Hadgraft and R.H. Guy, Editors, Marcel Dekker inc.: New York. p. 177-196.

29. Tanojo, H., Roemele, P.E.H., Veen, G.H.v., Stieltjies, H., Junginger, H.E., and Bodde, H.E.,1997. *New design of flow-through permeation cell for studying in vitro permeation studies across biological barriers.* Journal of Controlled Release. **45**(1): p. 41-47.

30. Degim, I.T.,2006. *New tools and approaches for predicting skin permeability.* Drug Discovery Today. **11**: p. 517-523.

31. Valenta, C., Auner, B.G., and Loibl, I.,2005. *Skin permeation and stability studies of 5-aminolevulinic acid in a gel and patch preparation.* Journal of Controlled Release. **107**: p. 495-501.

32. Swarbrick, J. and Boylan, J.C.,2002. *Encyclopedia of Pharmaceutical Technology*. 2nd ed. Vol. 1: Informa Health Care.

33. Schacter, B.,2006. *The New Medicines. How drugs are created, approved, marketed and sold.*, N.Y.: Praeger Publishing.

34. Obici, S., Otobone, F.J., Ramos da Silva Sela, V., Carlos da Silva, J., Nakamura, C.V., and Audi, E.A.,2007. *Preliminary toxicity study of dichloromethane extract of Kielmeyera coriacea stems in mice and rats.* Journal of Enthnopharmacology. **115**: p. 131-139.

35. Raza, M., Al-Shabanah, O.A., El-Hadiya, T.M., and Al-Majed, A.A.,2002. *Effect of prolonged vigabatrin treatment on hematological and biochemical parameters in plasma, liver and kidney of Swiss albino mice.* Scientia Pharmaceutica. **70**: p. 135-145.

36. Mitruka, B.M. and Rawnsley, H.M.,1981. *Clinical biochemistry and hematological reference values in normal experimental animals.*, New York: Masons Publishing.

37. Conkova, E., LaciaKova, A., Pastrova, B., Seidel, H., and Kovac, G.,2001. *The effect of zearalenone on some enzymatic parameters in rabits.* Toxicology Letters. **121**: p. 145-149.

38. Chattopadhyay, D., Arunachalam, G., Mandal, A.B., Sur, T.K., Mandal, S.C., and Bhattacharya, S.K.,2002. *Antimicrobial and anti-inflammatory*

activity of folklore: Mellotus peltatus leaf extract. . Journal of Ethnopharmacology. **82** p. 229–237.

39. Singer, A.J. and Clark, R.A.F.,1999. *Cutaneous Wound Healing.* New England Journal of Medicine. **341**: p. 738-746.
40. Eming, S.A., Brachvogelb, B., Odorisiod, T., and Koch, M.,2007. *Regulation of angiogenesis: Wound healing as a model.* Progress in Histochemistry and Cytochemistry **42**: p. 115–170.
41. Singer, A.J. and Clark, R.A.F.,1999. *Cutaneous wound healing.* . New England Journal of Medicine (341): p. 738–746.
42. Heinzelmann, M., Scott, M., and Lam, T.,2002. *Factors Predisposing to Bacterial Invasion and Infection.* American Journal of Surgery. **183**: p. 179-190.
43. Robson, M.C., Stenberg, B.D., and Heggers, J.P.,1990. *Wound healing alterations caused by infection.* Clinical Plastic Surgery. **17**((3)): p. 485-492.
44. Strommenger, B., Schmidt, C., Werner, G., Beate, R.L., Bachmann, T.T., and Witte, W.,2007. *DNA microarray for the detection of therapeutically relevant antibiotic resistance determinants in clinical isolates of Staphylococcus aureus.* Molecular and Cellular Probes. **21**: p. 161-170.
45. Iwatsuki, K., Yamaski, O., Morizane, S., and Oono, T.,2006. *Staphylococcal cutaneous infections: Invasion, evasion and aggression* Journal of Dermatological Science. **42**: p. 203-214.
46. Peacock, S.J., de Silva, L., and F.D., L.,2001. *What determines nasal carriage of Staphylococcus aureus?* Trends in Microbiology. **9**: p. 605-610.
47. Weidenmaier, C., Kokai-Kun, J.F., and Kristian, S.A.,2004. *Role of teichoic acid in Staphylococcus aureus nasal colonization: A major risk factor in nosocomial infections.* Nature Medicine. **10**: p. 243-245.

48. Foster, T.J.2005. *Immune evasion by staphylococci.* Nature Reviews Microbiology. **3**: p. 943-958.

49. Dinges, M.M., Orwin, P.M., and Schlievert, P.M.,2000. *Exotoxins of Staphylococcus aureus.* Clinical Microbiology Review. **13**: p. 16-34.

50. Ladhani, S., Joannou, C.L., Lochrie, D.P., Evans, R.W., and S.M., P.,1999. *Clinical, microbial and biochemical aspects of exfoliative toxins causing staphyloccal scalded-skin syndrome.* Clinical Microbiology Review. **12**: p. 224-242.

51. Witte, W.,1999. *Antibiotic resistance in Gram-positive bacteria: epidemiological aspects.* Journal of Antimicrobial Chemotherapy. **44**: p. 1-9.

52. Cooper, R., Kingsley, A., and White, R.2003. *Wound Infection and Microbiology.* Medical Communications (UK) Ltd for Johnson & Johnson Medical.

53. Cosgrove, S.E., Sakoulas, G., Perencevich, E.N., Schwaber, M.J., Karchmer, A.W., and Carmeli, Y.2003. *Comparison of mortality associated with methicillinresistant and methicillin-susceptible Staphylococcus aureus bacteremia: a meta-analysis.* Clinical Infectious Disease. **36**: p. 53-59.

54. Engemann, J.J., Carmeli, Y., Cosgrove, S.E., Fowler, V.G., Bronstein, M.Z., and Trivette, S.L.,2003. *Adverse clinical and economic outcomes attributable to methicillin resistance among patients with Staphylococcus aureus surgical site infection.* Clinical Infectious Disease. **36**: p. 592–598.

55. Bowler, P., Duerden, B., and Armstrong, D.,2001. *Wound microbiology and associated approaches to wound management.* Clinical Microbiology Review **14**(2): p. 244-269.

56. Hou, F.X., Yang, H.F., Yu, T., and Chen, C.,2007. *The immunosuppresive effects of 10 mg/kg cyclophosphamide in Wistar rats.* Environmental Toxicology and Pharmacology. **24**: p. 30-36.

57. Davidson, J.M.,1998. *Animal Models for Wound Repair.* Archives of Dermatological Research. **290 (Suppl)**: p. S1-S11.

58. MacKay, D. and Miller, A.L.,2003. *Nutritional Support for Wound Healing.* Alternative Medicine Review. **8**: p. 359-377.

59. Jorge, M.P., Madjarof, C., Ruiz, A.L.T.G., Fernandes, A.T., Rodrigues, R.A.F., de Oliveira Sousa, I.M., Foglio, M.A., and de Carvalho, J.E.,2007. *Evaluation of wound healing properties of Arrabidaea chica Verlot extract.* Journal of Ethnopharmacology(1016).

60. Van Rensburg, C.E.J., Van Straten, A., and Dekker, J.,2000. *An in Vitro Investigation of the Antimicrobial Activity of Oxifulvic Acid.* Antimicrobial Chemotherapy. **46**(835-854).