JEFFREY HAROLD PING

Identification of new drug compounds active against *Pediculus humanus capitis,* and incorporation of the compounds into a safe and potentially effective drug product formulation

(Under the direction of JAMES C. PRICE)

Individual citronella oil constituents were screened for *in vitro* activity against lice and their ova using ASTM methods. The screening studies revealed that all compounds tested possessed pediculicidal activity, but none possessed ovicidal activity. Citronellyl acetate emerged as the target drug compound based on its preferred physiochemical and efficacy properties.

Initial vehicle development efforts produced a vehicle (2-component, "add-mix" system) that maximized the *in vitro* efficacy profiles of citronellyl acetate. An *in vitro* dose response study identified the requirements of 3.94% and 8.38% citronellyl acetate for 50% and 100% activity, respectively. Increasing exposure time from 10 to 20 or 30 minutes did not increase activity. Pre-clinical toxicity was assessed using a 12.5% strength product. *In vitro* assessment of possible dermal penetration revealed that no transdermal absorption of citronellyl acetate occurred after a 10-miunte topical exposure. Acute oral and dermal toxicity testing demonstrated extremely low toxicity (LD_{50} in mice greater than 5000 mg/kg and between 2000 to 5000mg/kg in rats and dermal LD_{50} greater than 2000 mg/kg in rabbits). The add-mix formulation was a severe irritant to both the skin (PII = 6.75) and eyes of rabbits. Subsequent human clinical trials failed to identify a product that possessed any ovicidal activity or a 95% or greater clinical cure rate, documented instances of contact site irritation, and demonstrated poor correlation with *in vitro* results.

Additional efforts to optimize the delivery system resulted in a clear gel formulation and modified *in vitro* assessment methods with improved correlation and prediction. An *in vitro* dose response study with the gel identified the requirement of 3.3% and 9.0% citronellyl acetate for 50% and 100% pediculicidal activity, respectively,

and 3.0% and 8.3% for 50% and 100% ovicidal activity, respectively. Also, a minimum of 11.1% was required to inhibit all embryonic development. A 12.5% strength gel was only moderately irritating to the skin (PII = 4.54) but severely irritating to eyes.

Extensive literature research uncovered two possible modes of action, isoprenylation inhibition and cuticle destruction. Results of an efficient and cost effective experiment with chirally pure actives and SEM inspection indicated a combination of both possible modes.

INDEX WORDS: Head lice, Lice, Pediculosis, Citronella oil, Citronellol, Citronellal, Citronellyl acetate, Terpene, Pediculicidal, Ovicidal, Dose response *in vitro*, Mode of action, Inhibition of G-protein isoprenylation, Surface effect, Disruption of cuticle, Anhydrous gel vehicle, Pre-clinical toxicology, Insecticide, Scanning Electron Microscopy, SEM, GMP, GLP, ICH

IDENTIFICATION OF NEW DRUG COMPOUNDS ACTIVE AGAINST *PEDICULUS HUMANUS CAPITIS*, AND INCORPORATION OF THE COMPOUNDS INTO A SAFE AND POTENTIALLY EFFECTIVE DRUG PRODUCT FORMULATION

by

JEFFREY HAROLD PING

B.S. Chemistry, Otterbein College, 1991

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSHPHY

ATHENS, GEORGIA

2001

IDENTIFICATION OF NEW DRUG COMPOUNDS ACTIVE AGAINST *PEDICULUS HUMANUS CAPITIS*, AND INCORPORATION OF THE COMPOUNDS INTO A SAFE AND POTENTIALLY EFFECTIVE DRUG PRODUCT FORMULATION

by

JEFFREY HAROLD PING

Approved:

Major Professor: James Price

Committee: Anthony Capomacchia
Stuart Feldman
Robert Lu
Donald Champagne

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The Univerity of Georgia
May 2001

DEDICATION

Dedicated to my best friend, Kimberly, who just happens to also be my wife. Without your inspiration, patience, and support, I could never have achieved this dream. Without you, my life would be incomplete and meaningless. Thank you for putting your needs on hold for so long while I pursued one of mine.

ACKNOWLEDGEMENTS

I would like to thank Dr. James Price for his constant, positive support through the peaks and valleys of my endeavors. While his scientific expertise guided me to think critically and overcome difficult obstacles, his actions taught me valuable, lifelong lessons. I also thank Dr. Stewart Feldman and Dr. Tony Capomacchia for taking the time to discuss the opportunities of the program and convincing me to enroll. I owe much to them, Dr. Price, Dr. Robert Lu, Dr. Don Champagne, and all other faculty in the pharmaceutics group for having the insight and flexibility to establish a program for "non-traditional" students currently employed in industry.

I must express the utmost gratitude to Ed, Bob, and Mel Burklow, owners of Effcon Laboratories, Inc., for funding the research. Even in the deepest valleys of the project, their support was unconditional. Without their support, I would have never been able to achieve my goals. I must also thank other colleagues who added tremendously to the research such as Ian Burgess of Medical Entomology Centre (expertise and work involved with *in vitro* assessments and resistance issues), LiceSource, Inc. (use of their facilities as a "testing ground"), and Terri Meinking (University of Miami, expertise on resistance and clinical aspects).

I also especially thank my wife (for her extreme patience), Randy Warner (for always being there as a sounding board), P3 Laboratories (for financial support), and Emily Ware (for assisting me with a tremendous amount of work). Finally, I must ultimately thank my parents for their unconditional love and fostering my quest to learn and always improve myself.

TABLE OF CONTENTS

Page

CHAPTER I

INTRODUCTION

The recent emergence of human head lice (*Pediculus Humanus Capitis*) resistant to currently available treatments has caused great alarm within the pharmaceutical industry and with clinicians. If a child is found to possess an infestation, he or she is removed from school and not allowed to return until the infestation is completely cured. In many cases, conventional treatments available "over the counter" (OTC) such as Rid™ (pyrethrum extract with piperonyl butoxide) or Nix™ (permethrin) are no longer curing infestations. Due to the lack of efficacy of the OTC products, other traditional prescription products and "non-traditional" treatments are being used much more frequently. Prescription products include topical application of lindane and malathion both of which have much greater human toxicity and a greater chance of adverse side effects than the OTC products. The non-traditional treatments include systemic dosing of antibiotics including ivermectin, topical application of potentially toxic substances such as kerosene, turpentine, DDT, and Raid™ insect killer, and topical application of non-toxic substances such as mayonnaise and Vaseline™.

For more than a decade, no new drugs have been approved with an indication for curing head lice infestations. The most recent new drug approval for curing head lice in the U.S. was the switch of Nix™ from prescription to OTC status in May 1990. Lack of research and approval of new drugs for head lice is most likely due to two issues. First, head lice infestations are not considered to be a major health hazard. Second, the total dollar volume of the head lice market is small in relation to other disease states.

However, a great need exists for a new treatment to cure head lice infestations with minimal human toxicity. The goal of the research was to identify a new target drug compound for treatment of head lice infestations and to incorporate it into a safe and potentially effective finished drug product. The specific objectives were to:

1. Screen individual components of citronella oil for lethal activity against head lice and their eggs *in vitro*.
2. Incorporate the identified active components from the screening studies into a suitable pharmaceutical vehicle resulting in a potentially effective drug product formulation.
3. Identify a target drug product from the initial screening and formulation studies from objectives 1 and 2 and assess the potential safety and toxicity of its use.
4. Optimize the pharmaceutical vehicle incorporating the target compound to maximize its safety and efficacy profiles.
5. Identify the critical dose of the target compound required in the optimized vehicle for 100% pediculicidal and 100% ovicidal activity *in vitro*.
6. Elucidate the possible mode(s) of action for the target compound.

The research documents the methods and difficulties associated with screening, identifying, assessing, and developing a new drug product for curing head lice infestations.

CHAPTER II

REVIEW OF LITERATURE

BACKGROUND OF HUMAN LICE INFESTATION

Pediculus humanus capitis, the human head louse, has most likely existed since the existence of man and has never been eradicated. Head lice and eggs have been recovered from ancient combs dated to as far back as 100 B.C. and have been found on Egyptian mummies dating back approximately 5000 years.[1-4] While lice infestations are not generally recognized as a major health risk, they are still known to cause impetigo, pyroderma, and can be vectors for classical typhus caused by *Rickettsia prowazeki* and louse-borne relapsing fever caused by *Borrelia recurrentis*.[2,5,6]

Today, head lice infestations are most commonly found in children 4 to 11 years old but can also occur in older children and adults.[7] Infestations are not seasonal, but variations can occur and are typically attributable to social behavior.[7,8] While no major health risks are posed, infestations can be irritating and eventually lead to secondary infection caused by excessive scratching. Arguably the greatest detriments of today's infestations are the removal of children from school and social attitudes towards those infected. Schools in the U.S. have adopted "no nit policies" in attempt to minimize the transmission of the disease. According to the policy, any child with nits (eggs) is immediately removed from school and not allowed to return until passing an inspection proving the removal all nits regardless of their viability. Only viable nits are indicative of an active infestation, but nonviable or hatched nits can remain on the hair for a considerable length of time.[2] The misconception that merely the presence of nits indicates an active infestation has led to many inappropriate dismissals. Children can miss a tremendous

amount of school until all nits are removed even though they may not even have active infestations. In addition, infested children and their parents often endure considerable emotional distress due to social pressures. In the U.S. especially, society has unjustifiably deemed that infestation is due to poor hygiene and poverty, and those infested are often ridiculed by their peers based upon the misconceptions. However, it should be noted that infestations are socially acceptable in other areas of the world.

Maunder considers most infections to be symptom free.[8] Bites cannot be felt as the saliva of lice contains an anticoagulant to keep blood flowing freely thus reducing the need for multiple or deep incisions. Subsequent itching is a result of an allergic reaction to the injection of the saliva. Typically it can take up to three months for the itching to develop meaning the infestations can be active for an extended period of time unbeknownst to the infected person, parents, friends, or schools.[5]

BIOLOGY OF HUMAN LICE

Ian Burgess has extensively described the biology of human lice.[2] Basically, three different types of lice inhabit humans; however, debates are common over actual taxonomy classification. Generically they are categorized as the head louse, body or clothing louse, and pubic louse. While the three lice vary in body size, shape, habitat and feeding patterns, they do share similar features such as the length of claws, length of joints, and relative size to each other.

The anatomy of human lice was extensively characterized in the early 1900's.[9-12] Buxton summarized these and other studies in 1947[13], and Burgess used these for his anatomical description. The anatomical differences and differences in habitat resulted in the categorization of the three types of lice into separate, specific subspecies. The human pubic or crab louse is known as *Pthirus pubis*, the human body or clothing louse as *Pediculus humanus humanus* (*P. h. humanus*), and the human head louse as *Pediculus humanus capitis* (*P. h. capitis*).

Figure 1 contains a magnified picture of a human head louse, the species of focus, of this research project. However, it is important to understand the general attributes that differentiate the three subspecies. The head, body, and pubic louse are basically similar anatomically, but some variations do exist. Both head and body lice have a highly sclerotized thorax and an elongated abdomen with lateral plates that surround the spiracles while the pubic louse has a triangular abdomen with three, closely spaced spiracles contained on segment 5, angled anteromediately. Each has seven abdominal segments, but the cuticle of only head and body lice can be pigmented based upon host coloring at the time of the first feeding.[14] Generally, body lice are larger than head and pubic lice but typically have a thinner exoskeleton. The most drastic difference that separates pubic lice from head and body lice is the pubic louse's huge pairs of claws on the middle and rear legs, which is responsible for its common name, the "crab louse." This anatomical difference allows the crab louse to move freely between widely spaced hairs and lie flat against the host while holding the hairs making it ideally suited for its habitat in the pubic areas of humans.

Body and head louse eggs are structurally different from the crab louse specifically in the opercular cap. In both eggs, the opercular cap serves as a "lid" on top of the egg and possesses pores allowing for the transfer of vital moisture and oxygen to the embryo. However, the head louse egg operculum contains 7 to 11 pores while the body louse egg operculum contains 12 to 21. The crab louse egg opercular cap differs in shape, rounded compared to egg shaped, in number of pores, 14 to 19, and they have a reticular matrix separating the pores while the eggs of body and head lice do not.[2,15-17]

Nutall first documented the life cycle of human lice in 1917, and no recent research has documented any significant deviations from that originally published.[10,18] Eggs are laid onto human hair with a glue-like substance. The most common misconception is that the eggs are actually "glued" via a surface-surface bond with the hair. Figure 2 shows an actual example of the bond to hair. In reality, the glue like substance encases the hair shaft and

Figure 1. Magnified image of a human head louse (nymph).

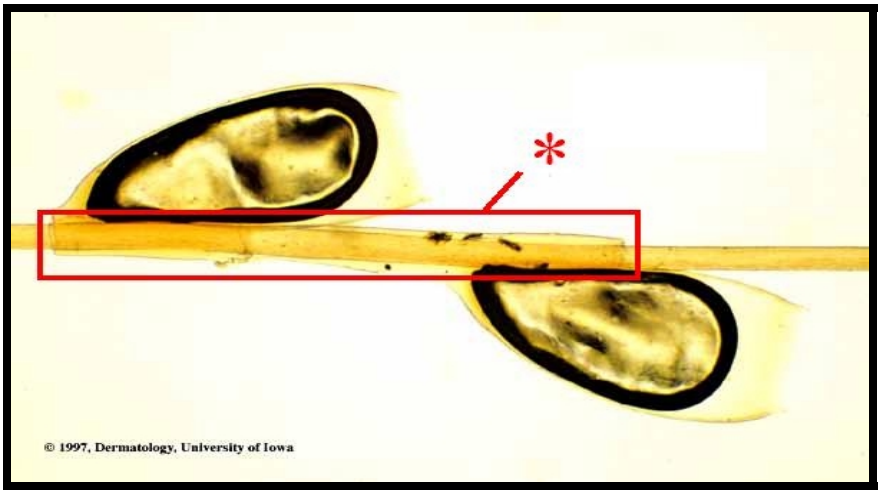


Figure 2. Example of nit cylindrical bond to hair shaft.

shrinks upon drying thereby forming a cylindrical bond around the hair through pressure created from the shrinking process. Hatching of the eggs is temperature dependent but typically occurs within 6 to 9 days after being laid. Shortly after hatching, the emerging nymphs have their first blood meal and continue to do so many times each day.[14] Nymphs molt three times, termed "instars," each of which occurs every 3 to 5 days depending on temperature fluctuations and food availability. The sex of the nymph is not determinable until after the third and final molt when the adult emerges. The female adults begin to mate within 1 to 2 days after the final molt, lay eggs shortly after mating, and the entire process is repeated with another generation. In captivity, adults have lived to 30 days, but in reality the life span is probably shortened by the rigors of daily exposure to the harsh habitat of a human head.

An interesting physiological attribute of human lice is that they are extremely moisture sensitive. They can rapidly dehydrate to a point of lethality. Studies have shown that head lice removed from the host become immobile in 21.3 (+/- 12.1) hours.[19,20] While the cuticle of the louse is composed of lipids to minimize water loss, its composition does not completely eliminate loss of moisture. The lice can only re-hydrate through a blood feeding and, therefore, they obtain 4 to 5 smaller feedings per day rather than one large one.

DISEASE STATE (HUMAN HEAD LICE INFESTATION)

Again, Burgess has previously described the issues of diagnosis, transmission, and epidemiology of human lice.[2] Locating a louse is the only true diagnosis, and it is the most difficult for human head lice. Typically, patches of hair are inspected systematically, but many cases are missed due to inexperience of the observer. One survey documented that 50% of pediatric nurses and physicians had never previously even seen a louse.[21] Lice detection combs have proven to be the most useful tool for inspection since they can be combed through the hair removing the smallest of nymphs.[8,21-26]

Most experts agree that the majority of infestations are transmitted through personal contact and specifically from head to head contact with an infected child or person. Much disagreement exists over the role of other instruments of transmission including hats, combs, towels, and linens mainly due to the lack of research into these possibilities. While a few studies have been performed, they have not been able to confirm any other means of transmission other than through direct bodily contact with one who is infested.[14,27] Therefore, some experts dismiss the belief that shared personal items are vectors for disease transmission attributing the belief to be based merely on social attitudes.[14,27,28] One expert, Maunder, argued that even though lice have been found on these types of items, they are too weak, sick, or injured to be capable of re-infesting a host.[14,29] Still others argue that louse behavior is dependent on environmental issues and can differ based on the specific area of the world in which the host lives.[6] Burgess and his other colleagues have recently performed field experiments and surveys in the United Kingdom in attempt to assess transmission. They found evidence that transmission is not as easy as previously thought thus discounting the concept that transmission is rampant throughout the schools. However, it is worth noting that no studies have been conducted on the actual vector(s) of lice transfer, but much research has been conducted in attempt to establish the roles that race, age, gender, hair length and hygiene possibly play in lice infestation and transmission.

Physical differences, such as claw size and abdomen shape, between lice have been documented based upon geographical location and race of the host thereby making transmission between races difficult in a mixed population even to the extent that Blacks within populations comprised largely of other races have been found to be almost completely free from infestations.[12,30-36] However, a large incidence of infestation has been documented in all Black African populations.[37-45] While most research indicates that race plays a role in mixed populations, one researcher found no such evidence.[46]

Mellanby conducted a survey of about 60,000 hospital patients in England and found that head lice are mainly prevalent in children but also were found to infect adults at

a lower rate.[47-49] Studies since that of Mellanby have almost entirely been focused on children, but none has really been able to identify a specific age range during which children are most susceptible.[43,50-54] Some have documented the majority of infestation to occur in children from 6 to 11, while others have found most to occur above the age of 9.[19,32,38,40-45,55-66] Prevalence in children is most likely due to higher incidence of personal contact with other infested children.

Gender roles have been well researched, and Mellanby discovered a higher prevalence in females, especially female children.[47] Almost all subsequent research has confirmed Mellanby's findings with the exception of one.[33] As with age, the prevalence in females is most likely due to higher incidence of personal, head-to-head contact.[60] Studies have shown that boys tend to quickly move away from close contact activities while girls tend to continue close contact activities in pairs and small groups.[67,68]

Most research on the role that hair length plays on infestation has been unable to identify a statistically significant correlation. The few studies that have identified a correlation negate each other since about half indicate a role while the other half do not.[8,38,44,59,63,69,70] Others have suggested that different attributes such as hair thickness, styling, and grooming play a more important role than length.[13,36,38,40]

Social attitudes have long attributed head lice to poor hygiene and lower socioeconomic status. Studies on the possible relationship of economic stature on infestation are somewhat mixed in findings. While some researchers have found a correlation due to conditions such as family size, overcrowding, and bed sharing, others have found the exact opposite relationship or that infestation was more prevalent in the upper socioeconomic classes.[34,36,38,40,45,50,60,71,72] Studies have not demonstrated a statistically significant relationship between hygiene and incidence of head lice. Despite the lack of proof, society still associates infestations with a lack of cleanliness and parents go to utmost extremes to assure infested children's scalp and hair are extremely clean. Maunder stated this ridiculous nature best when he wrote, "The shampoo is apparently used as much

to remove a ritual taboo as to remove the lice. Otherwise, who but an idiot would wish to wash an insecticide off an insect before it was dead?"[14]

TREATMENTS FOR HUMAN HEAD LICE

As should be expected with a disease that has existed over many millennia, many different treatments have been used in attempt to treat head lice infestations. These treatments include topical application of active compounds both of natural and synthetic origin, and recently some systemic treatments have been used in extreme cases. The oldest treatments date back to Chinese medicine where formulations containing botanical extracts, such as *Stemosa tuberosa* (pai pu) extract, seem to have been effective.[73] Other natural or botanical compounds have been used in the past including stavesacre extract, sassafras oil, eucalyptus oil, piperonal, coconut oil, mustard oil, and benzyl benzoate, but many had limited efficacy or severe human toxicity associated with their use.[35,73-82] Two of the most successful natural treatments to be discovered are natural pyrethrins and rotenone.[2]

Initial chemical treatment incorporated mineral oil, produced from petroleum or kerosene distillates, which blocked the louse's tracheal system.[2,13,79,83-86] Other chemicals have been used with solvents or mineral oil including cresol, naphthalene, creosote, cresylic acid, phenolics, and mercury compounds, but these obviously have inherent toxicity issues associated with human use.[2,13,87-91] Numerous chemicals have been evaluated by military groups during the World Wars including diphenylamine, bis(ethyl)xanthogen, methyl formate, ethyl formate, methyl bromide, chloropicrin, hydrogen cyanide, and sulphur dioxide, but again human toxicity issues were inherent with their use.[74,92-95] Thiocyanates were the first synthetic chemicals successfully used when incorporated into a vehicle (trade name Lethane®). However, these compounds were aesthetically unpleasant and irritating. The most widely used synthetic thiocyanate was and is DDT, which at the time of invention was thought to be the greatest achievement for ectoparasitic control and for prevention of the spread of typhus.[13,96]

A variety of treatments are used today to treat head lice infestations. DDT powder and lotions are still used in some countries, but the emergence of new treatments and resistant lice has nearly eliminated DDT as an effective treatment.[97-100] Mainstream current treatments incorporate active ingredients such as pyrethrins synergized with piperonyl butoxide, permethrin, malathion, carbaryl, and lindane, and more aggressive, radical treatments incorporating actives such as antibiotics (sulfa and avermectins) and NSAIDs (Non-steroidal anti-inflammatory drugs).

The most common modern active ingredient for head lice treatment is natural pyrethrins even though the products are almost useless against lice in the current formulations. Products containing natural pyrethrins are available "over the counter" (OTC) in the U.S. Natural pyrethrins are obtained from flowers of chrysanthemum and consist of acid and alcohol moieties and are classified as "Type 1 pyrethroids" based upon the group substituted for the alcohol moiety.[101] Natural pyrethrins have been used safely for some time, but people allergic to *Chrysanthemums* are predisposed to allergic reactions to the product.[102] Overall, these products are not very stable and no longer demonstrate significant effectiveness or ovicidal activity, but new formulation approaches are being explored in attempt to optimize the effectiveness of the natural pyrethrins. These new formulation approaches have included the addition of an "activator" such as piperonyl butoxide. Figure 3 shows the structure of piperonyl butoxide. Piperonyl butoxide inhibits cytochrome P-450 activity through competitive binding preventing the degradation of the pyrethrins.[103] Additional formulation approaches have been focused on vehicle development such as incorporating the pyrethrins in mousses and gels.

Due to the instability of the natural pyrethrins, much research was performed to develop a synthetic pyrethroid that would exhibit greater stability and efficacy. The most widely used synthetic product developed was and still is permethrin, the active ingredient in Nix™ that is also available OTC in the U.S. Permethrin is classified as a "Type 2 pyrethroid" based on a substituted phenoxybenzyl alcohol moiety that increases insecticidal

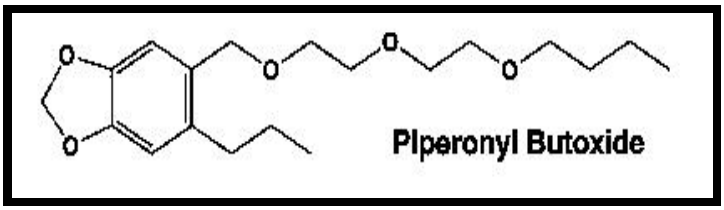


Figure 3. Chemical structure of piperonyl butoxide.[103]

activity by about a factor of 10 over natural pyrethrins.[101] Permethrin was incorporated into a creme rinse formulation at 1% (Nix™) and initially demonstrated 100% cure with a single application despite a lack of complete ovicidal activity.[104-109] Permethrin's ability to cure with one treatment in spite of a lack of 100% ovicidal activity is due to residual, lethal traces remaining on the hair even after treatment and rinsing. Treatment with 1% permethrin also resulted in minimal side effects as one study demonstrated that only 2.2 reports of adverse events per 1000 patients treated were observed.[110] Figure 4 displays some examples of both Type 1 and 2 pyrethroids.[101] Type 1 and 2 pyrethoids possess different modes of insecticidal action, but both affect the central nervous system (CNS). Type 1 pyrethoids, such as the natural pyrethrins, cause excitability and convulsions whereas the Type 2 pyrethroids result in ataxia and loss of coordination. While both types of pyrethroids cause death from their ability to slow or prevent the closing of the sodium channel involved with nerve impulse transmission, they differ in the duration of the effect resulting in the different symptoms. Type 1 effects only last for less than a second while Type 2 effects last for minutes or longer. Since nerve impulses are transmitted as a wave whose upstroke is regulated by an increase in sodium ions and down stroke is regulated by potassium ions, the upstroke of the impulse is propagated and multiple spikes are induced by Type 1 pyrethroids resulting in convulsive type symptoms. However, the extended duration of effect by Type 2 pyrethroids results in depolarization of the axon membrane potential, which actually decreases the amplitude of the transmission and results in a loss of excitability.

Lindane, the active ingredient in Kwell™, is the organochlorine compound, γ–Hexachlorocyclohexane. While available prior to the discovery of DDT, it was not until afterwards that lindane was assessed for possible use against human ectoparasites. Figure 5 shows the chemical structure of lindane. Until the last 5-10 years, lindane was a fairly successful treatment, and even though transdermal absorption has been demonstrated for the shampoo formulation, many prescriptions for lindane are still written.[2] Lindane is

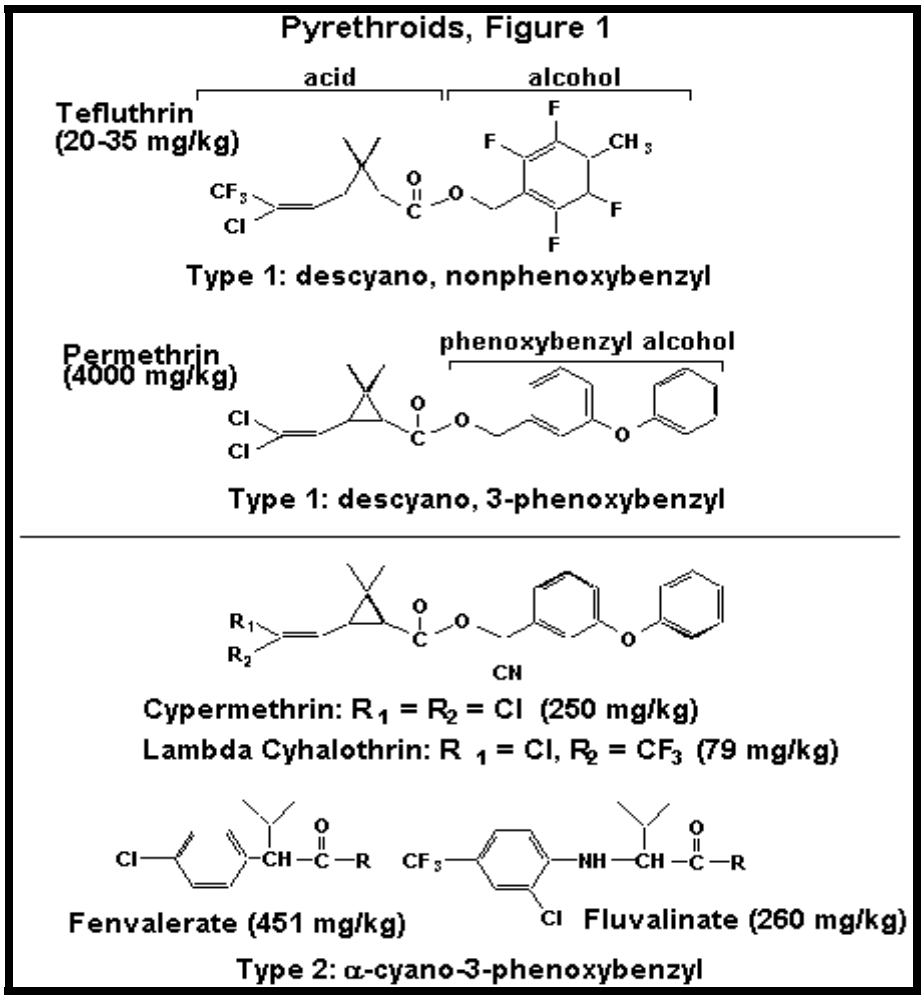


Figure 4. Examples of pyrethrins and pyrethroids.[101]

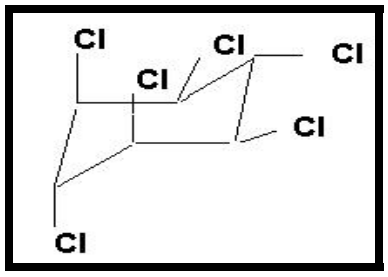


Figure 5. The chemical structure of lindane.[101]

classified as a "chloride channel blocking convulsant" and results in similar symptoms as the pyrethroids, excitability and convulsions, since it also affects the CNS.[101] Specifically, lindane acts through competitive inhibition of the neurotransmitter g-aminobutyric acid's (GABA) receptor. Typically, GABA is released from the presynaptic nerve terminal, binds with its postsynaptic receptor protein, and opens a chloride channel that ultimately acts to diminish nerve impulse firing. Therefore, lindane prevents the receptor from GABA activation blocking the chloride channel, which in turn eliminates the ability to terminate the impulses leading to excitability and convulsions. Unfortunately, significant resistance of head lice to lindane has been documented since 1971.[111]

Malathion, the active ingredient in Ovide™, is an organophosphate (OP) compound that was first investigated for lice treatment on DDT-resistant lice and proved to possess better efficacy than lindane.[112,113] While many different formulations incorporating malathion have been assessed, the only one demonstrating 100% ovicidal and pediculicidal activity was malathion in alcohol with a 12-hour exposure period.[2,44,45,50,56,111,114] Figure 6 shows the chemical structure of malathion. Malathion contains a sulfur atom directly attached to the phosphorus by a double bond (phosphorothionate). The lethal properties of the compound require oxidative desulfuration that occurs via cytochrome P450 monooxygenases, and the resulting oxidized metabolite possesses far greater toxicity than the original compound. These metabolites also affect the CNS causing excitability, tremors, convulsions, and paralysis, but the mechanism is much different that that of the pyrethroids or lindane. The mode of action for OPs is their inactivation of acetylcholinesterase. The metabolites competitively bind with the serine hydroxyl group of the enzyme and phosphorylate the group, inactivating the enzyme. Once the enzyme is inactivated, acetylcholine is not degraded back into choline, and as levels continue to rise CNS excitation occurs. Since all of the cholinergic nerves are within the CNS, CNS toxicity is the only lethal pathway for OPs. Malathion does not possess onset of action as rapid as that of the pyrethroids since it must be metabolized for activity.[103]

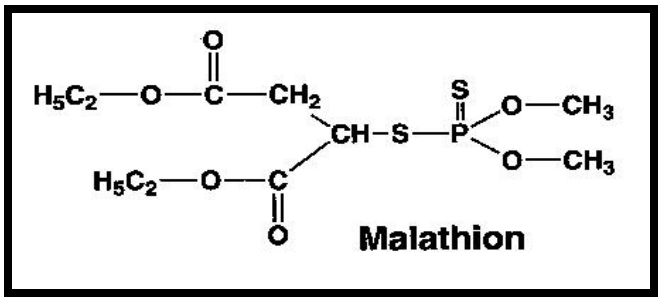


Figure 6. Chemical structure of malathion.[103]

While not approved for use in the U.S., carbaryl is used regularly in other countries. Carbaryl is classified as a carbamate and its structure is shown in Figure 7. Carbaryl toxicity symptoms are almost identical to those of malathion since it also acts as an acetylcholinesterase inhibitor. However, the difference is that carbaryl carbamylates the serine hydroxyl group resulting in a much less stable complex than that with the phosphorylated group. The unstable complex actually hydrolyzes within minutes rendering it inactive.[101] Unfortunately, carbaryl also possesses toxicity to mammals and has been found to easily select for a resistant lice strain, which begins to explain it limited use.[2,101,115]

While traditional treatments incorporate pesticides, the evolution of nontraditional treatments also has occurred. One such approach is the use of antibiotics currently approved for human use, and non-traditional ones not approved for human use such as the avermectins. Avermectins have been used extensively as antihelminthics and antiparasitics in veterinary medicine. The structure of a specific avermectin homologue is shown in Figure 8. Avermectins like lindane act as GABA inhibitors, but act specifically on the neuromuscular junctions. Symptoms of toxicity differ somewhat as exposure to the avermectins causes almost instant inability to feed or lay eggs.[103] Avermectins are dosed systemically and are currently under evaluation for treatment of human head lice infestations, but toxicity issues are inherent with its potential use, which causes a concern when considering its use with a predominantly pediatric population.

EVALUATION OF TREATMENTS

Realistic assessment of current and new product performance remains a great challenge. While both *in vitro* and *in vivo* methods exist, both have inherent errors that can greatly skew results and can be subjective in nature. In general, the *in vitro* methods are used for initial screening techniques, and *in vivo* studies are used for ultimate clinical and regulatory assessment.

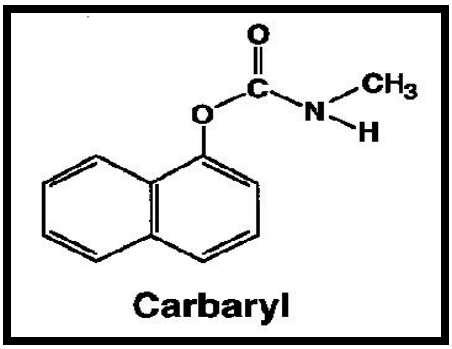


Figure 7. Chemical structure of carbaryl.[101]

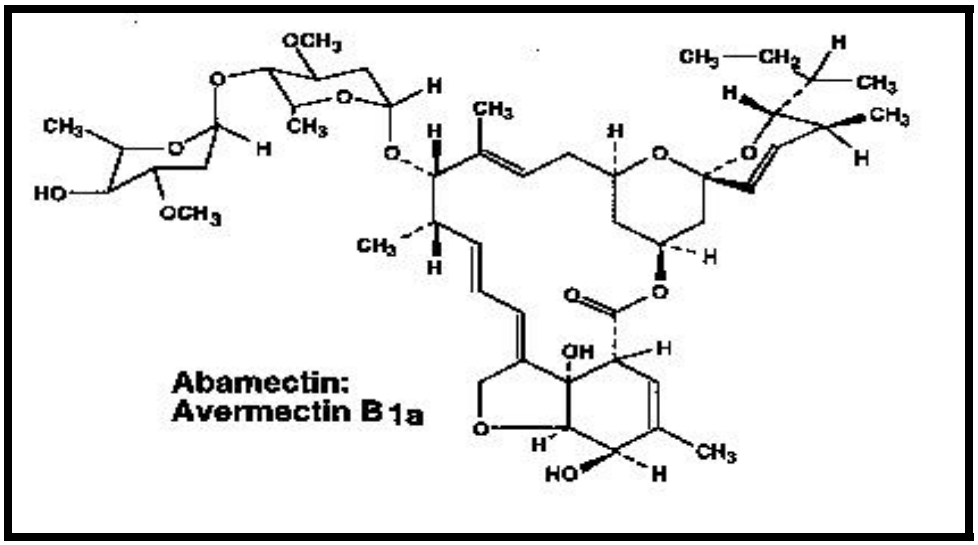


Figure 8. Chemical structure of avermectin B_{1a} homologue.[103]

Standardized *in vitro* test methods have been adopted on a worldwide scale in attempt to minimize the subjectivity of the testing. *In vitro* studies are possible due to the "culturing" of lice in a laboratory setting. The laboratory lice originated as body lice that were adapted to feed once a day on volunteers.[2,116-122] Eventually, the laboratory colony was selectively adapted to feed on rabbit blood instead of human blood. This adapted strain of lice is perpetually bred and used for *in vitro* evaluation of products. American Society for Testing and Materials (ASTM) methods exist that outline standardized procedures for *in vitro* pediculicidal and ovicidal assessments.[123,124]

ASTM method E938-83, "Standard Testing Methods for Determining the Effectiveness of Liquid, Gel, Cream or Shampoo Insecticides Against Human Lice," describes the appropriate techniques and materials for performing *in vitro* pediculicidal assessments.[123] Basically, for each replicate, ten lice are collected shortly after a blood feeding, and five replicates are evaluated per test product. Approximately 5 mL of the test product (or water control) are added to a test tube, and the lice are submerged and held in the test product for the identified residence time. After the residence time has expired, the lice are removed from the solution, rinsed with water, and blotted dry. The lice are then transferred to a piece of corduroy cloth, placed in a petri dish, and are incubated under life sustaining conditions (31.7°C and 60% relative humidity). Assessments are made at 1 hour and 24 hours by placing the corduroy patch with the lice on a slide warmer. The lice that are not dead or moribund will crawl away from the heat source to the cooler parts of the patch. Lice classified as dead or moribund at 24 hours are added together, and pediculicidal activity is corrected for the control as calculated through Abbott's formula. Abbott's formula for calculating the corrected mortality to lice is shown in Figure 9.

ASTM method E1517-93, "Standard Testing Methods for Determining the Effectiveness of Liquid, Gel, Cream or Shampoo Insecticides Against Human Louse Eggs," describes the appropriate techniques and materials for performing *in vitro* ovicidal assessments.[124] In summary, each replicate consists of ten eggs that are collected within 2

$$\text{Corrected Mortality} = \frac{(\%\ \text{Lice alive in control} - \%\ \text{Lice Alive in test})}{\%\ \text{Lice Alive in control}} \times 100$$

Figure 9. Abbott's formula for corrected pediculicidal activity calculation.

days after being laid on human hair (1 egg per each hair), and five replicates are evaluated per test product. The ten hairs are taped to an applicator stick that is submerged in the test product (or water control) for the identified residence time. After the residence time has expired, the hairs with eggs are removed from the solution, rinsed with water and blotted dry. The hairs with the eggs are then placed in a petri dish and incubated at life sustaining conditions (31.7°C and 60% relative humidity). After a 14-day incubation period, the petri dishes are examined for number of eggs that hatched. The total number of hatched eggs is used to determine ovicidal activity through Abbott's formula that corrects for the control. Abbott's formula for calculation of corrected mortality to eggs is shown in Figure 10.

While the ASTM techniques have evolved to minimize subjectivity, the techniques obviously do not closely simulate *in vivo* treatment of head lice. This has resulted in poor correlation between the *in vitro* and *in vivo* methods. Two critical differences exist between the methods that lead to the limitations. First, the *in vitro* test incorporates human body lice that have been adapted to feed on rabbit blood not human head lice. It is plausible that a difference in efficacy could be observed based on the difference of lice being tested, especially when considering that human head lice must endure and overcome a much harsher environment. Second, in the *in vitro* tests, the lice and eggs are completely submerged in the test solution for the entire identified residence time. This method of product "application" differs drastically from *in vivo* topical application of products that are applied like traditional shampoos or creme rinses. The only positive correlation between the methods is that when a product does not possess *in vitro* efficacy, it almost never is efficacious *in vivo*.

Just as certain aspects of the *in vitro* methods lend to poor correlation, so too do the *in vivo* techniques. Most variations with *in vivo* studies also involve product application. The amount of product applied can vary drastically depending on the one applying it.[2,12,5] If the person applying the product is trained and skilled in the art of louse and egg removal, artificially high success may be achieved with the product. Because most

$$\text{Corrected Mortality} = \frac{(\%\ \text{Eggs hatched in control} - \%\ \text{Eggs hatched in test})}{\%\ \text{Eggs hatched in control}} \times 100$$

Figure 10. Abbott's formula for corrected ovicidal activity calculation.

treatments require a second application, the period of time required to assess success is fairly long (typically 2 weeks). Re-infestation is very common within the assessment period and renders it difficult to distinguish it from clinical failure resulting in many incorrect product evaluations. Another source of assessment error involves the use of nit comb after treatment with a product. Nit combing is indicated for most products and can yield artificially high success based merely on the removal of viable eggs. Recently, in attempt to overcome the subjective issues with *in vivo* methods, lice and eggs are collected prior to and after application of the test product. The lice and eggs are incubated and observed for assessment of lethality. The additional collection and evaluation technique has proven to provide more meaningful insight into a product's true pediculicidal and ovicidal attributes that could be masked from mere "pass or fail" clinical assessments.

RESISTANCE TO CURRENT AND FUTURE TREATMENTS

Thomas Miller described general insecticide resistance well in his lecture notes.[126] Insects developed resistance to many insecticides through genetic selection for survival after direct contact with them or their residues. Since no real proof exists that the insecticides cause resistance via a direct mechanism, experts believe it occurs by the natural process of "normal" mutation and selection. Once a mutation is expressed, it is selected since the mutated cell is more likely to survive the presence of the toxin(s). Typical mutation rates occur one in every 10^{4}-10^{6} cell divisions and typically result in a loss of function rather than a gain of function since there are many more mechanisms for a loss of function mutation to occur. For example, bacteria have a 2×10^{-6} chance to develop a dependence on arabinose as a loss of function, and have a 4×10^{-10} chance to develop streptomycin resistance as a gain of function. Once a mutation has occurred, the natural selection process begins allowing for the selection of the mutant organism.

Actual resistance occurs through four different pathways: behavior, penetration, metabolism, and/or altered site reaction. Mosquitoes exhibited the first example of behavior resistance during the malaria eradication program. When workers sprayed the

walls of huts with a DDT solution for protection, it was discovered that the mosquitoes would enter the huts and only land on the untreated ceiling thus avoiding the treated areas. An interesting aspect of behavior resistance is that it may be significantly reduced through the use of insecticides with repellant qualities since it would hinder the selection process.

Penetration resistance is most prominent for decreasing the toxicity of unstable, easily degradable compounds. As penetration is limited, more degradation occurs prior to entering the insect minimizing its lethality. While this pathway does not account for a large amount of resistance, it greatly enhances overall resistance when combined with other pathways. Metabolic resistance is achieved through an increase in the typical enzymatic activity responsible for the metabolism of toxins naturally occurring in plants or the environment ("xenobiotics"). Insects need a mechanism for survival after ingesting or exposure to the toxins. Therefore, only an increase in metabolism of the toxin can result in survival, and the natural selection process begins from those who have generated this metabolic resistance and lived.

Finally, altered cholinesterase and *kdr*-gene resistance exemplifies altered site of action resistance the best. Altered cholinesterases target carbamate and OP compounds reducing their binding ability resulting in the ability to resist their actions. The "knockdown resistance" (*kdr*) gene has been identified as the mode of DDT resistance. DDT affects the Na^+/K^+ channel involved with nerve impulse transition preventing normal transmission of nerve impulses. The *kdr* gene affects the same Na^+/K^+ channel reducing nerve sensitivity to compounds that act via the ion channel such as DDT. Identification of the *kdr* mechanism has subsequently lead to the demonstration of cross-resistance in other insects between DDT, other organochlorine compounds, and other compounds that act on the ion channel such as pyrethroids.[127-130]

Human head lice, like other insects, will and have developed resistance to the chemical treatments and will continue to do so.[2] Head lice resistance to DDT was first documented in the late 1960's, and resistance to malathion and permethrin was first

documented in the mid 1990's. Perhaps the most alarming aspect of head lice resistance is the speed at which head lice have developed resistance to permethrin. After only 2.5 years (about forty generations of lice) of treatment with permethrin in Israel, resistance began to appear.[127] The ability of head lice to develop resistance to permethrin is undoubtedly due to its "residual effect." Permethrin will remain on the hair long after the product is washed off. This results in the ability of adult lice, nymphs, hatchlings, and eggs to be exposed to sub-lethal amounts of the compound thus propagating the selection process of resistant insects.

The evolution of head lice resistant to permethrin and other current treatments has caused a major concern with those developing new products and with many appropriate regulatory bodies responsible for ultimate approval of new products. New treatments are needed due to the lack of efficacy of current treatments, but their success may be limited and brief if they act via a path of known resistance. For complete evaluation of new compounds, researchers need to identify a new compound's mode of action or develop new compounds that target different avenues than those currently associated with resistance. Obviously, compounds that act on physiological processes different from those of existing treatments with known resistance are less likely to be subject to cross-resistance. In addition, new compounds that exhibit lethal toxicity through multiple, different mechanisms would be much more difficult for resistance development. Consequently, researchers and regulatory committees must not merely be interested in assessing clinical outcome but also in identifying mode of action to predict the future possibility of cross-resistance.

CHAPTER III

EXPERIMENTAL

IN VITRO SCREENING OF CITRONELLA OIL CONSITIUENTS FOR LETHAL QUALITIES TO HUMAN LICE

BACKGROUND:

The insect repellent qualities of citronella oil have long been documented and exploited. The same is true for some of the constituents of citronella oil such as geraniol, nerol, and citronellal. However, Juvin and Moreau explored citronella oil's possible insecticidal qualities and documented their findings in U.S. Patent Number 4,518,593 (1985).[131] The patent divulges data indicating *in vitro* pediculicidal qualities of citronella oil when formulated with an anionic surfactant and acetic acid. A specific formulation encompassed in the patent is currently marketed internationally under the trade name SH-206 but is not available in the U.S. Based on the information divulged in the patent, the SH-206 product and its vehicle were used as a basis for the selection and *in vitro* screening of pure constituents of citronella oil.

Citronella oil's variability in purity and composition presents major obstacles for its use as an active drug compound. Because java citronella oil is extracted from a specific type of grass, the resulting product can contain many different constituents at highly variable levels amongst different batches. Regulatory agencies around the world have consistently voiced concerns on the use of impure, natural plant extracts as drug substances, and minimal chances of approval exist for them. Preferred drug substances are "pure" chemical entities, not of a mixture of various ones.

Java citronella oil is comprised mostly of geraniol and nerol but also contains some other similar compounds with different functional groups in far less concentration such as geranyl acetate, neryl acetate, citronellol, citronellal, and citronellyl acetate. A very intriguing aspect of citronellol, citronellal, and citronellyl acetate is their "Generally Recognized As Safe" (GRAS) status for use in food, cosmetic, and perfume products. Because of the GRAS status, potential toxicity testing and issues would be minimal due to historically safe human exposure to the compounds both topically and systemically. Therefore, these experiments focused on *in vitro* efficacy screening of the three GRAS status compounds, citronellol, citronellal, and citronellyl acetate.

MATERIALS:

Table 1 summarizes the original SH-206 formulation and sources of materials for the screening studies. Food grade citronellol, citronellal, and citronellyl acetate were obtained either from Technology Flavors and Fragrances (TFF) or Penta Chemicals. NF grade methylparaben and propylparaben were ultimately incorporated into the formulation and were obtained from Ashland Chemical.

METHODS:

All formulations were produced by the same method as the patented product. The SH-206 formulation was reproduced in the laboratory along with the same vehicle incorporating the individual components for screening. Table 2 summarizes the method of production used.

Since access to the laboratory lice and their eggs is severely limited (possessed by only 3 laboratories in the world), the *in vitro* studies were conducted at a contract laboratory. Insect Control & Research (IC&R) in Baltimore, Maryland, performed the *in vitro* pediculicidal activity and ovicidal assessments according to ASTM protocols E938-83 and E1517-93, respectively.[123,124] All assessments incorporated a 10-minute exposure time since it is the common time indicated for most currently marketed products.

Table 1. Summary of SH-206 formulation used for screening studies

Ingredient	% (w/w)	Source	Grade
Glacial Acetic Acid	3.65	JT Baker	USP
Synthetic Camphor	0.76	Penta	NF
Citronella Oil	0.76	Berjé	Java
Sodium Chloride	4.87	AKZO	USP
Sodium laureth sulfate (Steol CS-230)	36.55	Stepan	USP
Purified water	diluent	In house	USP

(*Note: Steol CS-230 is the trade name of 100% sodium laureth sulfate with 25.5% actives ethoxylated to an average of 2 moles.)

Table 2. Method of production for supplies used in screening studies.

Step #	Description
1	Add acetic acid and about 7.5% of the total amount purified water required for the entire batch to vessel #1
2	Add citronella oil (citronellal, citronellol, or citronellyl acetate as applicable) to acetic acid solution in vessel #1 and mix for 10 minutes
3	Add sodium chloride in about 25% of the total amount purified water required for the entire batch in a separate vessel, #2, and mix for 30 minutes
4	Add camphor to sodium chloride solution in vessel #2 and heat to 45°C
5	Add the warm sodium chloride/camphor solution in vessel #2 to the acetic acid/citronella oil solution in vessel #1
6	Add sodium laureth sulfate to total solution in vessel #1 and mix for 30 min.
7	Dilute the solution in vessel #1 with purified water to the final weight and mix for 10 minutes
8	Allow to stand for 1 hour

RESULTS AND DISCUSSION:

Initially, the SH-206 product was formulated in the laboratory along with its vehicle incorporating the three initial target compounds (at same concentration as citronella oil in SH-206) produced according to the procedure previously described. Table 3 summarizes the 4 initial formulations produced. Basic physical observations such as appearance, clarity, and color were noted for the formulations, and an interesting characteristic differentiated them. All formulations possessed shampoo like viscosity, but the formulations incorporating citronella oil and citronellol resulted in oil-in-water (o/w) emulsions while the citronellal and citronellyl acetate formulations were clear solutions. After formulation, each product was packaged in 60 mL high-density polyethylene (HDPE) bottles and sealed with a polypropylene (PP) cap. The samples were shipped to IC&R for *in vitro* pediculicidal activity assessment according to the ASTM protocol referenced previously. Table 4 summaries the *in vitro* pediculicidal activity of each formulation. The fact that all formulations possessed little or no activity was surprising at the least. Interestingly, the results contradicted the patented product's claims of lethality.

In attempt to elucidate any possible activity for the compounds, additional formulations were prepared using the same production procedure and a similar vehicle with higher levels of active. Based upon the preferred solubility characteristics in the vehicle and preferred aesthetic qualities of citronellal and citronellyl acetate, citronellol was eliminated from the screening process. Due to cost restraints and immediate availability of the compounds, citronellal was selected and incorporated into the vehicle at various levels. Additionally, one formulation was prepared with a higher concentration of citronella oil, one with a higher concentration of citronellyl acetate, and one "placebo" (vehicle without an active) formulation was prepared to serve as a vehicle control. The only change to the vehicle was the incorporation of a preservative system in attempt to prevent possible antimicrobial contamination common with unpreserved liquids. Methylparaben and propylparaben were used for the system and were added to the warm camphor/sodium

Table 3. Summary of 4 initial formulations for *in vitro* screening studies.

Ingredient	% (w/w)			
Java Citronella Oil	0.75	0	0	0
Citronellol	0	0.75	0	0
Citronellal	0	0	0.75	
Citronellyl Acetate	0	0	0	0.75
Synthetic Camphor	0.75	0.75	0.75	0.75
Glacial Acetic Acid	3.65	3.65	3.65	3.65
Sodium Chloride	4.87	4.87	4.87	4.87
Steol CS-230	36.55	36.55	36.55	36.55
Purified water	diluent	diluent	diluent	diluent

Table 4. Results of *in vitro* pediculicidal assessment.

Compound	*In vitro* Pediculicidal Activity
Java Citronella oil	1.2%
Citronellol	1.2%
Citronellal	3.4%
Citronellyl acetate	0.3%
Water control	1.2%

chloride solution in vessel #2 directly after step #4 in the procedure. Tables 5 and 6 summarize the citronellal, citronellyl acetate, citronella oil, and placebo formulations along with their respective *in vitro* pediculicidal activity.

Basic physical observations were noted for these formulations too, and an interesting characteristic differentiated these also. The formulations with 2.5% or greater citronellal and citronella oil resulted in resulted in o/w emulsions while the formulation with 8.9% citronellyl acetate was a clear solution. All formulations possessed a fairly strong aroma, but the citronellyl acetate formulation's was less pungent by far. All formulations with greater than 2.5% active, regardless of the type of compound, possessed water-like viscosities. Interestingly again, the patented formulation proved to be ineffective even with drastically more citronella oil present. However, the 8.9% citronellyl acetate and 10% citronellal formulations exhibited 95% or greater pediculicidal activity.

Because of the successful findings and citronellyl acetate's preferred solubility and aesthetic qualities, additional formulations were prepared with 10% and 12.5% citronellyl acetate for basic physical observation and confirmation of *in vitro* pediculicidal activity. Table 7 summarizes the additional citronellyl acetate formulations and their *in vitro* pediculicidal activity (IC&R). Both formulations resulted in clear solutions with water-like viscosity, and 100% *in vitro* pediculicidal activity was confirmed with both strengths.

Based on the 10% and 12.5% citronellyl acetate formulations' extreme lethality to laboratory lice, *in vitro* ovicidal assessments were initiated. In addition, identical formulations were prepared with 10% and 12.5% citronellal for comparison. Table 8 summarizes the lack of *in vitro* ovicidal activity demonstrated by all formulations.

SUMMARY:

These studies demonstrated the inability of the patented SH-206 formulation to kill lice even with a five-fold increase in the concentration of its active ingredient, citronella oil. However, when the SH-206 vehicle incorporated citronellal and citronellyl acetate at 10% or greater concentration, they exhibited the ability to kill lice *in vitro* and in fact possessed

Table 5. Summary of citronellal formulations and *in vitro* assessments.

Ingredient	% (w/w)				
Citronellal	0.75	2.5	5.0	7.5	10.0
Methylparaben	0.2	0.2	0.2	0.2	0.2
Propylparaben	0.1	0.1	0.1	0.1	0.1
Synthetic Camphor	0.75	0.75	0.75	0.75	0.75
Glacial Acetic Acid	3.65	3.65	3.65	3.65	3.65
Sodium Chloride	4.87	4.87	4.87	4.87	4.87
Steol CS-230	36.55	36.55	36.55	36.55	36.55
Purified water	diluent	diluent	diluent	diluent	diluent
In vitro Pediculicidal Activity (water control = 3.8%)	1.5%	15.7%	10.9%	17.0%	100.0%

Table 6. Formulation and activity summary of additional formulations and vehicle control.

Ingredient	% (w/w)		
Citronella oil	4.2	0	0
Citronellyl acetate	0	8.9	0
Methylparaben	0.2	0.2	0.2
Propylparaben	0.1	0.1	0.1
Synthetic Camphor	0.75	0.75	0.75
Glacial Acetic Acid	3.65	3.65	3.65
Sodium Chloride	4.87	4.87	4.87
Steol CS-230	36.55	36.55	36.55
Purified water	diluent	diluent	diluent
In vitro Pediculicidal Activity (water control = 3.8%)	0.0%	97.0%	0.0%

Table 7. Summary of citronellyl acetate formulations and activity.

Ingredient	% (w/w)	% (w/w)
Citronellyl acetate	10.0	12.5
Methylparaben	0.2	0.2
Propylparaben	0.1	0.1
Synthetic Camphor	0.75	0.75
Glacial Acetic Acid	3.65	3.65
Sodium Chloride	4.87	4.87
Steol CS-230	36.55	36.55
Purified water	diluent	diluent
In vitro Pediculicidal Activity (water control = 6.0%)	100.0%	100.0%

Table 8. Summary of *in vitro* ovicidal activity of citronellyl acetate formulations.

Formulation	*In vitro* Ovicidal Activity
10.0% Citronellyl acetate	0.0%
12.5% Citronellyl acetate	0.0%
10.0% Citronellal	3.5%
12.5% Citronellal	0.0%
Water control	11.3%

100% or greater pediculicidal activity. However, neither citronellyl acetate nor citronellal formulations possessed even the slightest *in vitro* ovicidal activity.

Based on the results of this experiment, the two GRAS certified compounds citronellal and citronellyl acetate were identified as possible new future drug compounds for treatment of head lice. Citronellyl acetate was used as the target compound for future studies due to its lethal characteristics to live lice, preferred solubility within the vehicle, and less pungent odor. At this point, many additional studies were needed to truly evaluate its potential for success. This research included optimization of the vehicle to maximize *in vitro* ovicidal activity, assessing potential toxicity to humans, and attempting to elucidate of the compound's possible modes of lethal action.

INITIAL VEHICLE OPTIMIZATION FOR CITRONELLYL ACETATE DELIVERY

BACKGROUND:

Previous experiments demonstrated the feasibility of using citronellyl acetate for possible treatment of human head lice infestations. *In vitro* studies documented that citronellyl acetate can kill all lice exposed when incorporated at 10% or greater into a vehicle comprised of acetic acid, sodium laureth sulfate, purified water, sodium chloride, methylparaben and propylparaben. The resulting product is a clear solution with water-like viscosity, and Table 9 summarizes the formulation. However, the formulation possessed no ability to kill lice eggs. Therefore, additional research and experimentation was initiated to optimize the vehicle for delivery of citronellyl acetate across the egg barriers.

The composition of many currently marketed products was reviewed in an attempt to identify common vehicles or excipients. The review uncovered that isopropanol (IPA or 2-propanol) is incorporated in the most products. Many of the products also contained a surfactant and an organic acid, both of with have been incorporated into the formulation previously. While most products are shampoo formulations, Nix™ is a creme rinse and Ovide™ is merely the active, malathion, dissolved in IPA.

Table 9. Summary of pediculicidal citronellyl acetate formulation.

Ingredient	% (w/w)
Citronellyl acetate	≥ 10.0
Methylparaben	0.2
Propylparaben	0.1
Synthetic Camphor	0.75
Glacial Acetic Acid	3.65
Sodium Chloride	4.87
Steol CS-230	36.55
Purified Water	diluent

Experiments were designed to alter the original vehicle used for the delivery of citronellyl acetate in attempt to maximize its *in vitro* pediculicidal properties and elicit *in vitro* ovicidal activity. Vehicle modification included the elimination of sodium chloride, inclusion of an alcohol, alteration of the levels of acetic acid and sodium laureth sulfate, and the development of creme rinse formulations. *In vitro* efficacy assessments were performed to quantitate activity, assess the impact of vehicle changes, and identify the minimum concentration of citronellyl acetate required for efficacy.

MATERIALS:

Table 10 outlines the materials, grades, and sources used to prepare the shampoo formulations. Table 11 outlines the materials, grades, and sources used to prepare the creme rinse formulations.

METHODS:

All shampoo formulations were produced by a similar method as outlined in the previous screening experiment. However, some steps were altered to incorporate the alcohol into the formulation. Two creme rinse formulations were prepared using techniques different than those for the shampoo formulations. One creme rinse was formulated as a "concentrate" to be diluted with an equal amount of water prior to use. The other creme rinse product was formulated to exclude the incorporation or use of water completely. Table 12 outlines the modified procedure for the preparation of the alcoholic shampoo formulations. Tables 13 and 14 outline the procedure used to prepare the "concentrate" and "anhydrous" creme rinse formulations, respectively.

In vitro assessments with a 10-minute exposure period were again conducted at IC&R according to the ASTM protocols. Additionally, because the dose response studies included numerous samples, Ian Burgess at Medical Entomology Center in Cambridge, England performed them due to time and cost restraints. Two modifications to the ASTM pediculicidal assessment protocol were implemented for these assessments. First, three replicates of testing were performed on each sample with 20 lice per replicate. Secondly,

Table 10. Materials for shampoo formulations.

Ingredient	Source	Grade
Glacial Acetic Acid	JT Baker	USP
Synthetic Camphor	Penta	NF
Citronellyl acetate	Penta	Java
Steol CS-230 (25% sodium laureth sulfate)	Stepan	USP
Methylparaben	Ashland Chemical	NF
Propylparaben	Ashland Chemical	NF
IPA	Aaper	USP
Ethanol, 95% (EtOH)	Aaper	USP
Purified water	In house	USP

Table 11. Materials for creme rinse formulations.

Ingredient	Source	Grade
Glacial Acetic Acid	JT Baker	USP
Synthetic Camphor	Penta	NF
Citronellyl acetate	Penta	Java
Amidox L5 (PEG-6 Lauramide)	Stepan	Cosmetic
Ammonyx 4002 (Stearalkonium chloride)	Stepan	Cosmetic
Cedepal HC (proprietary emulsifying formulation)	Stepan	Cosmetic
Glycerin	VW&R	USP
Propylene glycol	VW&R	USP
Methylparaben	Ashland Chemical	NF
Propylparaben	Ashland Chemical	NF
IPA	Aaper	USP

Table 12. Preparation of alcoholic shampoo vehicle with citronellyl acetate.

Step #	Description
1	Add alcohol to an adequately sized mixing vessel and begin mixing
2	Add camphor to the solution from step #1 and mix until completely dissolved
3	Add methylparaben and propylparaben to the solution from step #2 and mix until completely dissolved
4	Add glacial acetic acid to alcohol from step #3 and mix for 2 minutes
5	Add citronellyl acetate to the solution from step #4 and mix for 2 minutes
6	Add the sodium laureth sulfate (Steol CS-230) to the solution from step #5 and mix for 2 minutes
7	Dilute the solution in vessel #1 with purified water to the final weight and mix for 10 minutes

Table 13. Preparation of "concentrate" creme rinse formulation with citronellyl acetate.

Step #	Description
1	Add alcohol to an adequately sized mixing vessel and begin mixing
2	Add Ammonyx 4002 to alcohol from step #1 and mix until completely dissolved
3	Add glacial acetic acid to alcohol from step #2 and mix for 2 minutes
4	Add methylparaben and propylparaben to the solution from step #3 and mix until completely dissolved
5	Add citronellyl acetate to the solution from step #4 and mix for 2 minutes
6	Add the Amidox L5 to the solution from step #5 and mix until completely dissolved
7	Dilute the solution in vessel #1 (from step #6) with Cedepal HC to the final weight and mix for 10 minutes

Table 14. Preparation of "anhydrous" creme rinse with citronellyl acetate.

Step #	Description
1	Add alcohol to an adequately sized mixing vessel and begin mixing
2	Add glacial acetic acid to alcohol from step #1 and mix for 2 minutes
3	Add methylparaben and propylparaben to the solution from step #2 and mix until completely dissolved
4	Add citronellyl acetate to the solution from step #3 and mix for 2 minutes
5	Add Cedepal HC to the solution from step #4 and mix for 2 minutes
6	Add the propylene glycol to the solution from step #5 and mix for 2 minutes
7	Dilute the solution in vessel #1 (from step #6) with glycerin to the final weight and mix for 10 minutes

the application procedure was modified to submerge the lice in the test articles for only 5 seconds, then remove but not rinse them until the remainder of the 10-minute exposure period had elapsed. Also, possible exposure time effect on both pediculicidal and ovicidal activity was assessed at Medical Entomology Centre. These studies also incorporated modified methods. Pediculicidal studies used the modified method previously described. Ovicidal assessments were modified to collect eggs on tresses of human hair (drastically increasing the numbers of eggs collected and tested per sample) and incorporated the same modified "dip and wait" application method.

Stability studies were performed on the most promising formulation in attempt to discern the anticipated shelf life. Analysis consisted of assay for citronellyl acetate prior to and after 2 weeks of storage at 60°C and prior to and after 1 and 2 months of storage at room temperature conditions (RT, 25°C) and accelerated conditions (AST, 40°C). Performance of stability testing at RT and AST conditions complied with current Good Manufacturing Practice (cGMP) and International Committee for Harmonization (ICH) guidelines.

A reversed phase HPLC procedure was used to determine the concentration of citronellyl acetate in the formulated drug product. The reagents used for the assay included HPLC grade acetonitrile (Fisher), citronellyl acetate "reference standard" (food grade citronellyl acetate from Penta Chemicals with Certificate of Analysis identifying purity), reagent grade sodium phosphate monobasic (Fisher), and purified water (in house). Equipment comprised an HPLC system (Waters WISP Model 600E and Waters pump Model 700) with UV detection at 200 nm (Waters Model 481) and peak integration software (Shimadzu Class-VP). The column used for the separation was a Whatman Partisphere RTF C_{18}, 5µm, 15cm x 4.6mm with guard column. Table 15 outlines the conditions required for the assay.

For assessment of a product with a target citronellyl acetate concentration of 12.5%, standard solutions were prepared by weighing 125.0 mg of citronellyl acetate reference

Table 15. HPLC conditions for citronellyl acetate assay.

Ingredient	% (w/w)
Injection volume	10 µL
Flow rate	2.0 mL/min
Mobile phase	45% acetonitrile, 55% 0.018M sodium phosphate monobasic buffer
Run time	20 min
Column temperature	Ambient
Citronellyl acetate elution time	17.4 min
Solution for sample preparation	75% acetonitrile, 25% purified water

standard into a 100 mL volumetric flask. Approximately 20 mL 75% acetonitrile solution (in purified water) was added to dissolve the citronellyl acetate, and then the solution was diluted to the 100 mL volume with the 75% acetonitrile solution (in purified water). Two separate standards were prepared for performance of system suitability and calibration. Samples were prepared by weighing 1.0 gram of product into a 100 mL volumetric flask, which was dissolved and diluted with the same 75% acetonitrile solution (in purified water) used for the standard preparation. An aliquot was obtained from each sample preparation, filtered through a 0.45 μm PP filter, and injected directly onto the column.

Prior to running samples, system suitability and calibration of the system were performed. For system suitability, the first standard was injected 5 times and citronellyl acetate concentrations were calculated as percent label claim (%LC). The results were averaged and percent relative standard deviation (%RSD) between the 5 peak responses was calculated. Figure 11 displays the formula used to calculate citronellyl acetate concentration expressed as %LC. Acceptable performance criteria for system suitability was achieved with %RSD less than or equal to 3%. Calibration was performed through two separate injections of the second standard. Acceptable calibration was achieved when the difference in average %LC between the two standards was less than or equal to 3%. Once system suitability and calibration had passed, each sample was injected in duplicate with a standard injection after every two samples. Citronellyl acetate concentration (in %LC) was calculated for each sample and standard injection using the formula shown in Figure 11.

Statistical analyses were performed with appropriate computer software. Calculations of means, ANOVA analyses, and generation of basic graphs were performed with Microsoft Excel 2000. Additional statistical software, student version of JMP IN version 3.2.1 from the SAS Institute, was used to statistically identify the lowest dose of citronellyl acetate required to kill 50% (*in vitro* LD_{50}) and 100% (*in vitro* LD_{100}) of lice, to perform linear regression modeling of data, and to check the fit of the linear model.

$$\%LC = \frac{\text{Peak response of sample}}{\text{Peak response of standard}} \times \text{Calc. \%LC of standard} \times \text{Sample wt. factor}$$

$$\text{Sample wt factor} = \frac{\text{Target Sample Weight}}{\text{Actual Sample Weight}}$$

Figure 11. Calculation of average citronellyl acetate concentration.

RESULTS AND DISCUSSION:

All alcoholic shampoo formulations and the anhydrous creme rinse formulation were prepared with 12.5% citronellyl acetate while the "concentrate" creme rinse contained 25% citronellyl acetate (equivalent to 12.5% after 1 to 1 dilution with water). The shampoo formulations incorporated either IPA or ethanol at 20% or 30%. Also, the amount of acetic acid and Steol CS-230 in each shampoo formulation increased from 3.65% and 36.55% to 6.0% and 55.0%, respectively. Both creme rinse formulations excluded camphor and included 5.0% acetic acid and 20% IPA. The formulations were prepared as previously described and tested for *in vitro* ovicidal efficacy at IC&R with a 10-minute exposure period. The "concentrate" creme rinse was diluted 1 to 1 with tap water prior to assessment. Tables 16-18 summarize the initial shampoo and 2 creme rinse formulations and their resulting *in vitro* ovicidal activities.

Each shampoo formulation resulted in a clear solution with water-like viscosity. Both creme rinse formulations resulted in an opaque, white emulsion. All formulations possessed a strong aroma that more closely resembled alcohol and acetic acid rather than citronellyl acetate.

Inclusion of an alcohol and increasing the amounts of acetic acid and sodium laureth sulfate remarkably enhanced the *in vitro* ovicidal qualities of citronellyl acetate. In fact, the alterations transformed the results from a lack of any activity to complete lethality. No significant difference in *in vitro* activity existed between the formulations with IPA or the ones with ethanol. Also, no difference in efficacy resulted between the use of 20 or 30% of either alcohol. Interestingly, the 2 creme rinse vehicles did not enhance citronellyl acetate penetration into the eggs and possessed activity no different than the water control, which resulted in 11.9% activity. Obviously, the alcoholic shampoo vehicle optimized citronellyl acetate's lethal qualities, and the incorporation of 20% IPA was chosen for additional assessment.

Table 16. Alcoholic shampoo formulations and their *in vitro* ovicidal activity.

Ingredient	% (w/w)			
Citronellyl Acetate	12.5	12.5	12.5	12.5
IPA	20.0	30.0	0	0
EtOH	0	0	20.0	30.0
Synthetic Camphor	0.75	0.75	0.75	0.75
Glacial Acetic Acid	6.0	6.0	6.0	6.0
Steol CS-230	55.0	55.0	55.0	55.0
Methylparaben	0.2	0.2	0.2	0.2
Propylparaben	0.1	0.1	0.1	0.1
Purified water	diluent	diluent	diluent	diluent
In vitro Ovicidal Activity	100.0%	99.2%	100.0%	100.0%

Table 17. "Concentrate" creme rinse formulation and its *in vitro* ovicidal activity.

Ingredient	% (w/w)	% (w/w) after 1:1 dilution
Citronellyl Acetate	25.0	12.5
IPA	40.0	20.0
Amidox LS	4.0	2.0
Ammonyx 4002	8.4	4.2
Glacial Acetic Acid	10.0	5.0
Cedepal HC	diluent	diluent
Methylparaben	0.4	0.2
Propylparaben	0.2	0.1
In vitro Ovicidal Activity		12.0%

Table 18. "Anhydrous" creme rinse formulation and its *in vitro* ovicidal activity.

Ingredient	% (w/w)
Citronellyl Acetate	12.5
IPA	20.0
Glacial Acetic Acid	5.0
Cedepal HC	15.0
Methylparaben	0.2
Propylparaben	0.1
Propylene glycol	20.0
Glycerin	diluent
In vitro Ovicidal Activity	13.0%

A dose escalation study was designed, and a series of additional formulations were prepared with increasing amounts of citronellyl acetate incorporated into the preferred alcoholic shampoo vehicle. A placebo formulation was also prepared and included as a vehicle control. IC&R conducted *in vitro* pediculicidal assessments on each formulation in attempt to identify the lowest strength required for greater than 99% activity. Table 19 summarizes the formulations and activities. In addition, identical formulations were prepared with 0.1% acetic acid instead of 6.0% for comparison. Table 20 summarizes the additional formulations and activities.

Obviously, the higher acetic acid concentrations coupled with the alcohol greatly enhanced citronellyl acetate's penetration into the eggs but also into the live lice. According to the study, only 5.0% citronellyl acetate was required in the optimized vehicle to elicit 100% pediculicidal activity *in vitro* as opposed to 10.0% in the non-alcoholic vehicle with less acetic acid. The vehicle with 6.0% acetic acid, 20.0% IPA, and 55.0% Steol CS-230 revealed itself as the most promising carrier for citronellyl acetate. Therefore, the optimized formulation with 12.5% citronellyl acetate, summarized in Table 19, was selected for a physical stability assessment.

The formulation was filled into white, 2 oz. HDPE (high density polyethylene) bottles with 60 mL of product per bottle. The bottles were capped with a PP (polypropylene) flip-top cap. Bottles were placed inside of three stability chambers preset at 25°C, 40°C, and 60°C. The bottle stored at 60°C was inspected after 2 weeks of storage, and the bottles at 25°C and 40°C were inspected at 1 and 2 months of storage. Upon the first pull at 2 weeks, physical inspection revealed incompatibility with the packaging materials and degradation. The bottles had actually turned a faint brownish, yellow color and were disfigured with an appearance of "melting." The product itself had turned a brownish, yellow color similar to that which appeared on surface of the bottle. The product had degraded at the elevated temperature and not only leached through the HDPE but also caused the bottle to become severely disfigured resulting in many

Table 19. Summary of preferred vehicle formulations with increasing amounts of citronellyl acetate with their *in vitro* pediculicidal activities.

Ingredient	% (w/w)					
Citronellyl acetate	0.0	1.0	2.5	5.0	10.0	12.5
Methylparaben	0.2	0.2	0.2	0.2	0.2	0.2
Propylparaben	0.1	0.1	0.1	0.1	0.1	0.1
Synthetic Camphor	0.75	0.75	0.75	0.75	0.75	0.75
Glacial Acetic Acid	6.0	6.0	6.0	6.0	6.0	6.0
IPA	20.0	20.0	20.0	20.0	20.0	20.0
Steol CS-230	55.0	55.0	55.0	55.0	55.0	55.0
Purified water	diluent	diluent	diluent	diluent	diluent	diluent
In vitro Ped. Activity (water control = 2.3%)	2.1%	4.3%	40.7%	100.0%	100.0%	100.0%

Table 20. Optimized vehicle with 0.1% acetic acid and increasing concentrations of citronellyl acetate and their *in vitro* pediculicidal activities.

Ingredient	% (w/w)					
Citronellyl acetate	0.0	1.0	2.5	5.0	10.0	12.5
Methylparaben	0.2	0.2	0.2	0.2	0.2	0.2
Propylparaben	0.1	0.1	0.1	0.1	0.1	0.1
Synthetic Camphor	0.75	0.75	0.75	0.75	0.75	0.75
Glacial Acetic Acid	0.1	0.1	0.1	0.1	0.1	0.1
IPA	20.0	20.0	20.0	20.0	20.0	20.0
Steol CS-230	55.0	55.0	55.0	55.0	55.0	55.0
Purified water	diluent	diluent	diluent	diluent	diluent	diluent
In vitro Ped. Activity (water control = 10.4%)	6.3%	6.9%	8.4%	29.6%	41.6%	42.3%

indentations and "melted" look. Inspection of the 1 month 40°C sample revealed the same physical characteristics in a slightly less dramatic fashion.

A plausible hypothesis for the degradation was the possibility of citronellyl acetate decomposition in the acidic vehicle. A series of formulations were prepared incorporating the selected vehicle with 12.5% citronellyl acetate and varying amounts of acetic acid to test the hypothesis. The formulations prepared included 0, 0.1, 1, 2, 3, or 5% acetic acid, and one formulation replaced acetic acid with enough hydrochloric acid (HCl) to bring the final pH of the product to 3.5. The products were packaged into amber, 4 oz. polyethylene terephthalate (PET) bottles with 120 mL of product per bottle. Each bottle was sealed with a PP cap. Samples were again stored at 25°C, 40°C, and 60°C. The 60°C storage and assessment was used only for research purposes, while the RT and AST storage and pulls again compiled with current GMP and ICH guidelines. A sample of each was assayed for citronellyl acetate, according to the HPLC method previously described, just prior to storage for an initial reference point. The bottles stored at 60°C were sampled and assayed after 2 weeks of storage. The bottles stored at RT and AST were sampled and assayed after 1 and 2 months of storage. Table 21 summarizes the HPLC assay results for the stability study.

The stability study showed the direct correlation of citronellyl acetate decomposition with increasing acid concentrations. The incorporation of HCl resulted in drastically more citronellyl acetate decomposition, which was expected since it is a stronger acid compared to acetic acid, a weak acid. Based on physical inspection of the samples, the product was compatible with the PET packaging. The only formulation that proved to possess acceptable stability characteristics was the one without any acetic acid.

A simple solution was conceived to overcome the stability obstacles. Since the citronellyl acetate was stable in the product with absence of acetic acid, the acetic acid and water were isolated from the formulation and packaged in a separate container to form an "add-mix system." One container of the system, termed "Active Ingredient Component"

Table 21. Results of HPLC assay for citronellyl acetate for stability studies.

% Acetic acid	Storage Conditions / Storage Time (Results in %LC citronellyl acetate)					
	Initial	60°C / 2 wk	25°C / 1 mo	25°C / 2 mo	40°C / 1 mo	40°C / 2 mo
0.0	98.0	85.8	94.5	98.3	94.0	95.2
0.1	97.4	82.3	93.9	96.2	91.2	91.7
1.0	92.4	53.4	*	*	*	*
2.0	93.6	25.0	*	*	*	*
3.0	*	45.8	*	*	*	*
5.0	94.0	29.8	88.6	88.7	77.3	71.2
HCl	90.6	6.2	*	*	*	*
*samples not tested						

(AIC), contained all other ingredients except acetic acid and water and comprised 88 1/3% of the total formulation. The second container in the system, termed "Diluent Component" (DC), contained the entire amount of acetic acid and water required to complete the formulation (other 11 2/3%). For use, the contents of the DC were added to those in the AIC and mixed well. This "add-mix system" theoretically avoided the instability issue due to the separation of citronellyl acetate and acetic acid. Because water was removed from the AIC, its concentration was fixed for all additional formulations, and Steol CS-230 became the diluent. Table 22 outlines the add-mix system's composition before and after mixing with respective fill volumes and container sizes.

Another dose response study was designed by formulating the add-mix system with various levels of citronellyl acetate between 0.0 to 8.0%, and Ian Burgess at Medical Entomology Centre tested the formulations for *in vitro* pediculicidal activity using the modified method previously described. The resulting data was statistically analyzed using the student version of JMP IN Version 3.2.1 in attempt to identify the lowest concentration of citronellyl acetate required in the preferred vehicle for 50% and 100% *in vitro* efficacy, to fit a linear model to the data, and to verify the acceptability of the fitted model. In addition, ANOVA analysis was performed with Microsoft Excel 2000 on each formulation to identify whether it possessed statistically significant efficacy over that of the water control. Table 23 summarizes the strengths tested and raw data from the study.

Table 24 displays the results generated by the ANOVA analysis (with $\alpha = 0.025$) testing the hypothesis that the mean activity of the sample is equal to that of the water control. Therefore, p-values greater than α (0.025) indicate no statistically significant activity while those less than α possess statistically significant activities. Figure 12 shows the graphical representation of the fitted linear model generated by JMP. Table 25 includes summary of fit and results from the lack of fit (LOF) test performed to assess the applicability of the linear model. Figures 13 and 14 display the results of JMP data generated from a "screening fit" for identification of LD_{50} and LD_{100} concentrations.

Table 22. Example of add-mix system composition with 12.5% citronellyl acetate.

Ingredient	AIC % (w/w)	DC % (w/w)	Mixed % (w/w)
Citronellyl Acetate	14.15	0.0	12.5
IPA	22.64	0.0	20.0
Steol CS-230	62.05	0.0	55.0 (diluent)
Methylparaben	0.2	0.2	0.2
Propylparaben	0.1	0.1	0.1
Glacial Acetic Acid	0.0	42.86	5.0
Purified Water	0.0	56.84	5.55
Fill Volume in Package size	106 mL in 4 oz bottle	14 mL in a 0.5 oz bottle	120 mL in 4 oz, AIC bottle

Table 23. Summary of the dose response study with "add-mix system."

Citronellyl Acetate %	Replicate	Total lice	Killed	Moribund	% Mortality	Corrected % Ped. Act.
0.1	1	20	0	0	0.0	-5.3
0.1	2	19	2	1	15.8	6.4
0.1	3	20	1	0	5.0	0.0
0.5	1	22	4	0	18.2	13.9
0.5	2	20	1	1	10.0	0.0
0.5	3	20	2	1	15.0	10.5
1.0	1	20	2	0	10.0	5.3
1.0	2	20	3	1	20.0	11.1
1.0	3	21	1	0	4.8	-0.3
1.75	1	20	0	1	5.0	0.0
1.75	2	20	0	0	0.0	-11.1
1.75	3	20	0	0	0.0	-5.3
2.5	1	20	8	2	50.0	47.4
2.5	2	19	2	4	31.6	24.0
2.5	3	20	7	3	50.0	47.4
5.0	1	20	9	3	60.0	57.9
5.0	2	20	11	6	85.0	83.3
5.0	3	20	10	3	65.0	63.2

Table 23. Continued.

Citronellyl Acetate %	Replicate	Total lice	Killed	Moribund	% Mortality	Corrected % Ped. Act.
8.0	1	20	18	2	100.0	100.0
8.0	2	19	10	9	100.0	100.0
8.0	3	21	13	6	90.5	90.0
0.75	1	21	3	3	28.6	24.8
0.75	2	20	3	5	40.0	33.3
0.75	3	21	5	8	61.9	59.9
0.0	1	20	5	2	35.0	31.6
0.0	2	20	2	5	35.0	27.8
0.0	3	20	0	3	15.0	10.5
Water control	1	20	1	0	5.0	0.0
Water control	2	20	2	0	10.0	0.0
Water control	3	20	0	1	5.0	0.0

Table 24. Results of ANOVA analysis ($\alpha = 0.025$).

Strength	p-value
Vehicle	0.03442
0.1	0.96014
0.5%	0.05648
0.75%	0.02062
1.0%	0.3606
1.75%	0.10119
2.5%	0.00427
5.0%	0.00126
8.0%	1.49×10^{-5}

Table 25. Linear model fitted, summary of fit, and results of LOF test.

Description	Result
Linear model fitted	Cor % Ped Act = 6.24578 + 11.1848 % Act
Summary of fit	
RSquare	0.717838
RSquare Adj	0.706552
Root Mean Square Error	18.41196
Mean of Response	30.6037
Observations (or Sum Wgts)	27
LOF test	p-value = Prob>F = 0.0002

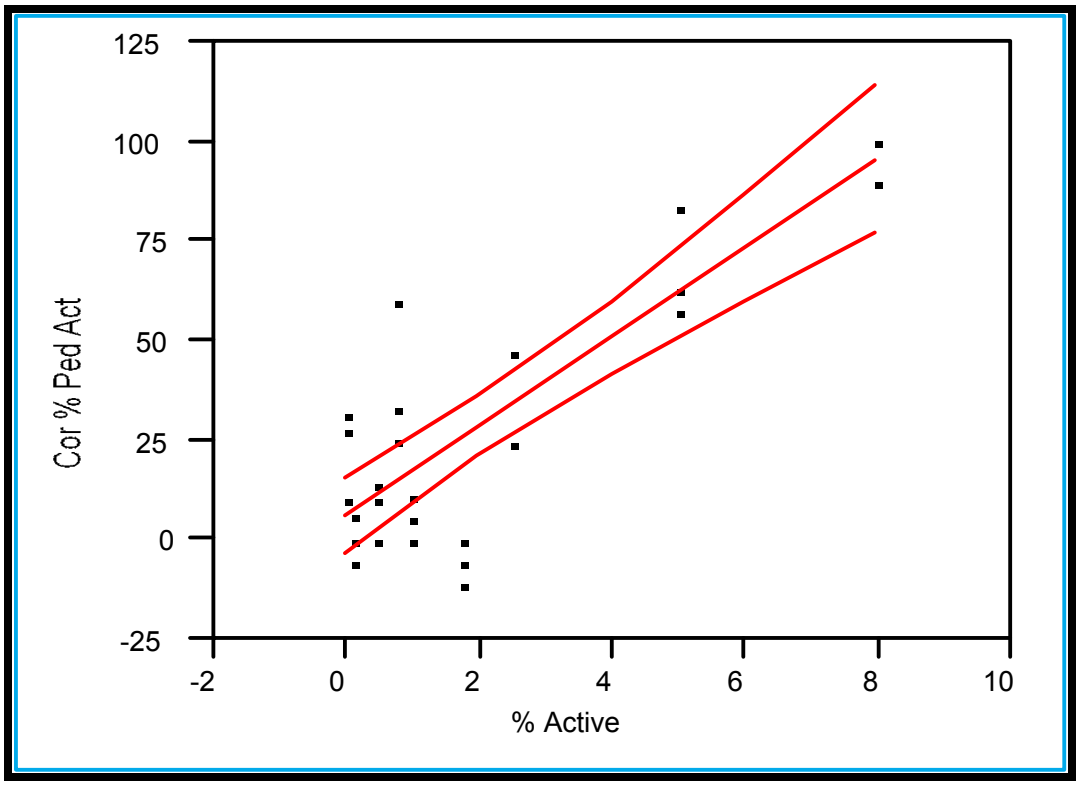


Figure 12. Graphical representation of linear model fit generated by JMP.

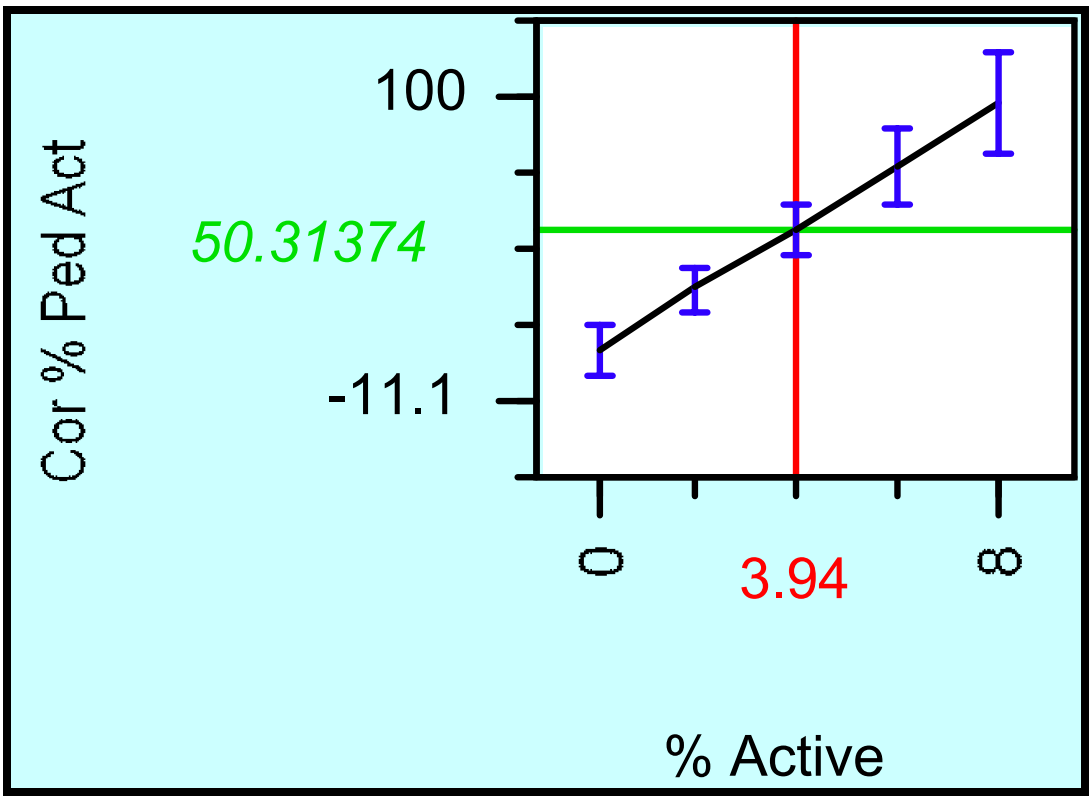


Figure 13. Results of JMP screening fit identifying citronellyl acetate's LD_{50} to lab lice.

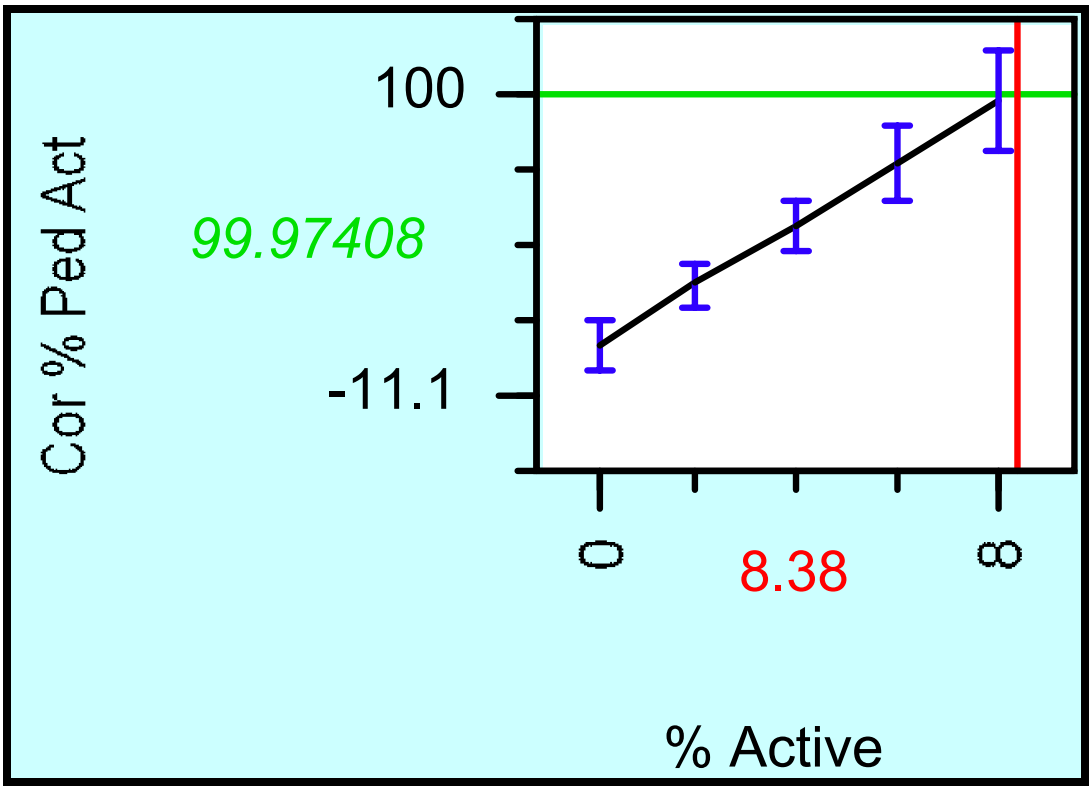


Figure 14. Results of JMP screening fit identifying citronellyl acetate's LD_{100} to lab lice.

Based on the ANOVA analysis, citronellyl acetate concentrations of 0.75, 2.5, 5.0, and 8.0% demonstrated statistically significant efficacy. However, the minimum concentration required for 100% *in vitro* pediculicidal efficacy was calculated as 8.4%, based on the JMP screening model. Surprisingly, the resulting curve from the dose escalation study did not fit a linear model well based on the JMP LOF test.

The results of the dose response study identified products with citronellyl acetate concentrations less than 2.5% to possess marginal or no activity. Therefore, the 0.1, 0.75, 1.75, and 2.5% products from the dose response studies were used to assess the effect of increasing the exposure time. Medical Entomology Centre performed the pediculicidal assessments using the modified method with 20 and 30-minute exposure times. The data was analyzed with JMP to fit a linear model in attempt to identify any possible trends. Tables 26 through 28 summarize the data, and Figure 15 shows the four linear regression plots generated by JMP.

Two strengths, 0.1% and 1.75%, showed an increase of efficacy with good linear fit while the 0.75% and 2.5% strengths showed a decrease in efficacy but did not possess good linear fit. Based on the contradictory results, exposure time did not significantly affect efficacy, which was surprising since a substantial increase was expected.

SUMMARY:

The experiment documented the development and identification of a citronellyl acetate formulation possessing 100% pediculicidal and ovicidal efficacy *in vitro*. Incorporation of an alcohol and increasing the amounts of acetic acid and sodium laureth sulfate in the vehicle greatly enhanced citronellyl acetate's penetration into and lethal qualities to both live lice and their eggs. However, stability assessment uncovered the paradox that increasing the amount of acetic acid also causes excessive decomposition of citronellyl acetate. The assessments also indicated the product was incompatible with HDPE.

Table 26. 10-minute exposure time data.

Citronellyl Acetate %	Replicate	Total lice	Killed	Moribund	% Mortality	Corrected % Ped. Act.
0.1	1	20	0	0	0.0	-5.3
0.1	2	19	2	1	15.8	6.4
0.1	3	20	1	0	5.0	0.0
0.75	1	21	3	3	28.6	24.8
0.75	2	20	3	5	40.0	33.3
0.75	3	21	5	8	61.9	59.9
1.75	1	20	0	1	5.0	0.0
1.75	2	20	0	0	0.0	-11.1
1.75	3	20	0	0	0.0	-5.3
2.5	1	20	8	2	50.0	47.4
2.5	2	19	2	4	31.6	24.0
2.5	3	20	7	3	50.0	47.4
control	1	20	1	0	5.0	0.0
control	2	20	2	0	10.0	0.0
control	3	20	0	1	5.0	0.0

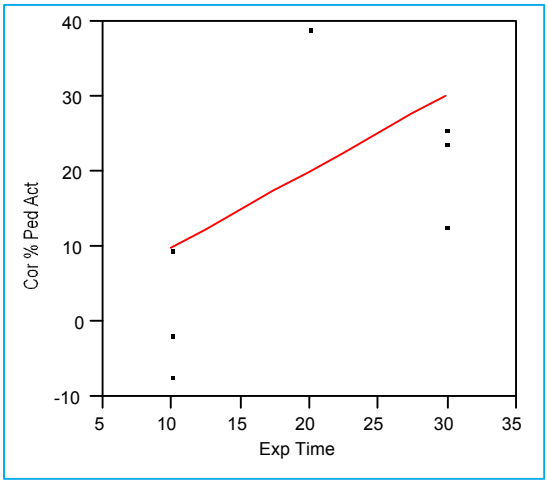
Table 27. 20-minute exposure time data.

Citronellyl Acetate %	Replicate	Total lice	Killed	Moribund	% Mortality	Corrected % Ped. Act.
0.1	1	20	2	6	40.0	37.4
0.1	2	20	2	6	40.0	40.0
0.1	3	20	2	6	40.0	40.0
0.75	1	20	1	2	15.0	11.3
0.75	2	23	3	2	21.7	21.7
0.75	3	20	3	0	15.0	15.0
1.75	1	23	5	4	39.1	36.5
1.75	2	20	4	9	65.0	65.0
1.75	3	18	4	5	50.0	50.0
2.5	1	20	2	2	20.0	16.5
2.5	2	21	0	5	23.8	23.8
2.5	3	20	2	3	25.0	25.0
control	1	24	1	0	4.2	0.0
control	2	18	0	0	0.0	0.0
control	3	18	0	0	0.0	0.0

Table 28. 30-minute exposure time data.

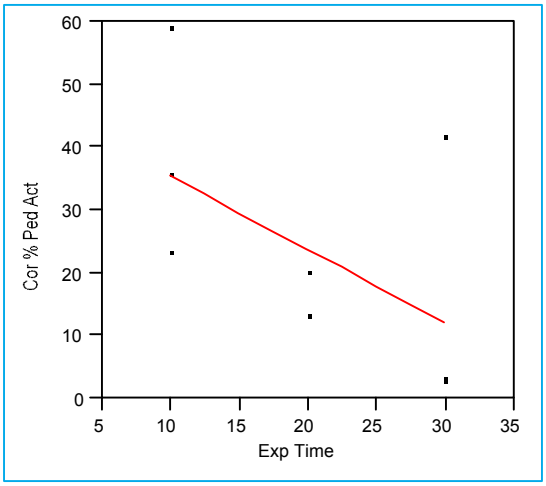
Citronellyl Acetate %	Replicate	Total lice	Killed	Moribund	% Mortality	Corrected % Ped. Act.
0.1	1	21	1	2	14.3	14.3
0.1	2	26	3	4	26.9	23.1
0.1	3	20	2	3	25.0	25.0
0.75	21	0	1	4.8	4.8	21
0.75	20	1	0	5.0	0.0	20
0.75	21	4	5	42.9	42.9	21
1.75	19	0	2	10.5	10.5	19
1.75	19	1	2	15.8	11.4	19
1.75	22	2	6	36.4	36.4	22
2.5	24	4	4	33.3	33.3	24
2.5	20	1	0	5.0	0.0	20
2.5	22	2	6	36.4	36.4	22
control	20	0	0	0.0	0.0	20
control	20	1	0	5.0	0.0	20
control	20	0	0	0.0	0.0	20

0.1%



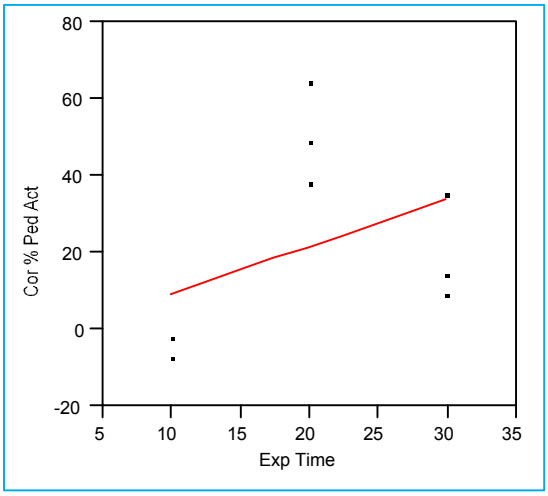
LOF test Prob>F =0.0007

0.75%



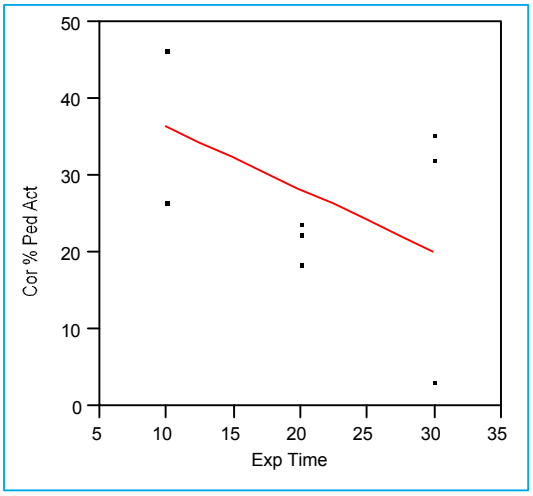
LOF test Prob>F =0.3515

1.75%



LOF test Prob>F =0.0015

2.5%



LOF test Prob>F =0.1705

Figure 15. JMP Linear Regression Plots for Exposure Time Effect.

The alcohol selected was IPA incorporated at 20% based on handling and cost issues. Acetic acid concentration was adjusted to 5% based on an attempt to optimize stability and efficacy profiles. An add-mix system was developed to isolate the acetic acid and water from the remainder of the formulation to protect against decomposition of citronellyl acetate. PET packaging replaced the HDPE for the AIC of the add-mix system and proved to be compatible with the formulation.

A dose response study using the add-mix system identified the minimum concentrations of citronellyl acetate required for statistically significant *in vitro* efficacy and the projected concentration required to kill 50% and 100% of the laboratory lice. Results indicated that a minimum of 0.75% is required to exhibit any activity, a minimum of 3.94% is required for 50% kill, and a minimum of 8.4% is required for complete kill. However, the study failed to demonstrate a linear relationship between citronellyl acetate concentration and efficacy. In addition, studies with 20 and 30-minute exposure times failed to demonstrate a statistically significant increase in efficacy with the increase in exposure.

The data generated from the screening and vehicle development studies were compiled into two patent applications. One demonstrated the 100% pediculicidal and ovicidal qualities of the formulation with 8.0% or greater active ingredient concentration. The other demonstrated the statistically significant pediculicidal and ovicidal qualities of the formulation when incorporating 0.75%-8.0% active ingredient concentration. Ultimately the first patent application was approved and issued as US Patent # 5,902,595 entitled "Pest Removing Composition" on May 11, 1999, and this was the first patent to reveal the insecticidal qualities of GRAS status monoterpenes. The second application was filed both for foreign and domestic approval and is pending under PCT Application US00/21417.

While the initial challenge of identifying a new, potentially efficacious treatment for head lice appears to have been met, future studies were required to assess the potential safety of its use. Since the majority of the head lice patient population is pediatric based, it

was necessary to perform extensive pre-clinical toxicity assessments in attempt to elucidate any potential safety issues prior to actual use with children. Additional vehicle research was required to optimize the stability, safety, and efficacy profiles of treatment with citronellyl acetate.

POTENTIAL HUMAN TOXICITY ASSESSMENT OF CITRONELLYL ACETATE IN AN "ADD-MIX" DELIVERY VEHICLE

BACKGROUND:

Previous research identified the potential efficacy of citronellyl acetate in an "add-mix" delivery system for treatment of human head lice infestations. Because the majority of head lice infestations are found in children, understanding the potential toxicity issues inherent with new treatments is very important, especially when considering that new treatments for head lice almost always involve the use of a pesticide.

Figure 16 shows the reported structure of citronellyl acetate. Citronellyl acetate has been in public use since the 1920's mainly as a fragrance in the cosmetic industry in amounts less than 20,000 pounds per year. It was granted GRAS status in 1965 and is approved by the US Food and Drug Administration (FDA) for food use.[132] The Council of Europe (1970) listed citronellyl acetate, giving an ADI of 0.25 mg/kg. The Food Chemicals Codex (1972) has a monograph on citronellyl acetate. According to the "Fragrance Raw Materials Monographs," citronellyl acetate is approved for use to a maximum of 0.4% in perfume. In addition, carcinogenicity studies have been performed indicating that citronellyl acetate is not carcinogenic even with systemic exposure to 29% citronellyl acetate solution five times a week for 103 total weeks.[133]

Citronellyl acetate is readily available as a food grade material with purity ranging from about 90-95%. Through some research, Elan Chemical Co. in Newark, New Jersey, was discovered to be the largest manufacturer of citronellyl acetate in the U.S. Elan produces citronellyl acetate from esterification of food grade citronellol with acetic anhydride in a one step synthesis. Through a fractional distillation step, they are able to

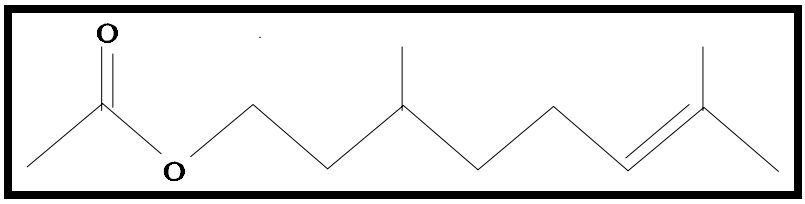


Figure 16. Reported structure of citronellyl acetate.

achieve the food grade purity of 90-95%. While this purity may be acceptable for a food additive, active pharmaceutical ingredients (APIs) typically require a purity of 98% or greater. Therefore, Elan added an additional fractional distillation step to the process, and the resulting product possessed purity slightly greater than 98%, which was acceptable for initial assessment as an API.

While the historically safe use of citronellyl acetate both topically and systemically in humans is well documented, various basic pre-clinical toxicology assessments were performed on the add-mix formulation prior to its use on humans. The product used for the assessments incorporated the higher purity citronellyl acetate from Elan at the highest concentration used for the *in vitro* efficacy studies, 12.5%. Table 29 summarizes the "add-mix" formulation used for the pre-clinical assessments.

Due to the GRAS status of citronellyl acetate and historically safe use both topically and systemically in humans, an abbreviated toxicology program was designed and performed. Because the results would be filed with the FDA, pre-clinical toxicology supplies were manufactured in compliance with cGMPs and assessments complied with current Good Laboratory Practice (cGLP) guidelines. Pre-clinical toxicology assessments consisted of *in vitro* assessment of dermal absorption, acute oral toxicity to mice, acute oral toxicity to rats, primary skin irritation in rabbits, acute dermal toxicity to rabbits, and acute ocular irritation to rabbits. Due to cost and time restraints, the dermal absorption study was performed at the University of Belfast in Northern Ireland. All other studies were conducted at Springborn Laboratories in Spencerville, Ohio, for cGLP compliance.

IN VITRO ASSESSMENT OF DERMAL ABSORPTION - MATERIALS :

The test articles for all studies consisted of the add-mix system incorporating 12.5% citronellyl acetate. The contents of the diluent component were added to the active ingredient component and mixed well just prior to application.

For the assessment of dermal absorption, human skin samples, specifically from legs, were used. The samples were obtained from surplus supplies due to routine surgical

Table 29. Summary of add-mix formulation with citronellyl acetate.

Ingredient	AIC % (w/w)	DC % (w/w)	Mixed % (w/w)
Citronellyl Acetate	14.15	0.0	12.5
IPA	22.64	0.0	20.0
Steol CS-230	62.05	0.0	55.0 (diluent)
Methylparaben	0.2	0.2	0.2
Propylparaben	0.1	0.1	0.1
Glacial Acetic Acid	0.0	42.86	5.0
Purified Water	0.0	56.84	5.55

procedures at Belfast City Hospital in Northern Ireland. Modified Franz diffusion cells consisted of FDC-400, flat flange, 15 mm orifice diameter mounted on a FDCD-3 diffusion cell drive console capable of providing synchronous stirring at 600 rpm (Crown Glass Co., Somerville, N.J., USA.). A circulating water bath was used to maintain constant temperature at 37°C (TE-8J circulating water bath from Techne) through the diffusion cell water jackets. Standard stainless steel filter supports (Millipore UK) were used for mounting the skin samples. Additionally, reagent grade phosphate-buffered saline (PBS, pH 7) was used as the receiving fluid, and amaranth solution was used for leakage testing.

IN VITRO ASSESSMENT OF DERMAL ABSORPTION – METHODS:

For *in vitro* assessment of potential dermal absorption, only human leg skin samples perceived to be healthy on visual inspection were used for testing. Subcutaneous fat was carefully removed by dissection and the skin was cut into pieces of a suitable size for mounting in the diffusion cell. Prepared skin samples were stored frozen at -18°C until required. Skin samples were allowed to defrost fully overnight at ambient temperature before use.

The diffusion apparatus consisted of three water-jacketed diffusion cells maintained at 37°C. The cells, of the modified Franz type, were mounted in series on a drive console providing synchronized stirring of all three cells. Specifically, modified Franz diffusion cells, FDC-400, flat flange, 15 mm orifice diameter, were mounted in triplicate on an FDCD-3 diffusion cell drive console providing synchronous stirring at 600 r.p.m. Constant temperature was maintained via water circulation at 37°C through the diffusion cell water jackets. The receiving chamber of each cell was filled with 12 ml of phosphate-buffered saline (PBS, pH 7). Skin samples were supported on a stainless steel filter support and secured with a flat flange, open orifice glass top and a stainless steel clamp. Skin samples mounted in the diffusion apparatus were equilibrated for 1 hour at 37°C before addition of the test solution. The exposed skin area per cell was calculated to be 176.7 mm^2.

Leakage testing was used to confirm that no leakage of a test solution could occur around the clamped skin sample from the donor through to the receiver compartment. Amaranth solution (1.0 mL) was pipetted into the open cylindrical top of the clamped diffusion cell such that it was in direct contact with the stratum corneum side of a clamped skin sample. After one hour, the cell was opened and the reservoir solution (PBS, ph 7) tested for dye content. The experiment was performed in triplicate, and for passing, no dye can be detected in the receiving compartment.

Three samples of the test article (each comprising one bottle of active with one bottle of diluent) were designated as TEST 1, TEST 2 and TEST 3. Before beginning the test, the total contents of the diluent bottle were added to the total contents of the active bottle, and the activated shampoo was shaken thoroughly for 1 minute. Immediately following activation, 1.0 mL of the test article was added into the open cylindrical top of the Franz Cell such that it was in direct contact with the stratum corneum side of a clamped skin sample. In all cases, the contact time between shampoo (or blank) solution and skin was 10 minutes. Six samples were run in this manner for each of three bottles of the test article, yielding 18 samples in total. Also, 6 blank samples were obtained by substituting the test article with an identical volume of pH 7.2 PBS. In all cases, a fresh skin sample was used for each individual experiment.

Following conclusion of the 10-minute contact time, the test article solution (or PBS blank) was removed from the skin surface by suction. The cell was carefully opened to avoid possible contamination of the receiver compartment, and 5.0 mL of the receiving fluid was pipetted into a glass sample vial containing 10 mg of a proprietary preservative system (Nipastat Sodium, a mixture of methyl and propyl parabens as their water-soluble sodium salts). These sample vials were used for determination of citronellyl acetate concentration by HPLC.

The HPLC assay method used was almost identical to that described previously for stability assessment. In short, an HPLC system capable of UV detection at 200 nm was

used with a Whatman Partisphere RTF C_{18}, 5µm, 15cm x 4.6mm column and guard column. The method incorporated an injection volume of 10 µL, flow rate of 1.5 mL/min., and mobile phase of 45% acetonitrile dissolved in purified water (55%) with 0.01% sodium phosphate monobasic. Run time was shortened to 14 minutes with ambient column temperature, elution time of citronellyl acetate was 7.9 minutes, and the solution used for dissolving the sample and standards was 75% acetonitrile solution in water.

IN VITRO ASSESSMENT OF DERMAL ABSORPTION – RESULTS AND DISCUSSION:

In vitro assessment of possible dermal penetration of citronellyl acetate was assessed using the procedure previously described. The add-mix system with 12.5% citronellyl acetate (formulation previously described in Table 29) served as the test article to assess possible penetration resulting from the "worst case scenario" of exposure (highest strength for 10 minutes). Three identical samples of the test articles were supplied and tested. For the purposes of this analysis, the concentration of citronellyl acetate was ultimately calculated as the difference between the measurements for the samples and the corresponding blank. Tables 30 and 31 summarize the resulting citronellyl acetate concentrations.

The largest single concentration difference between an individual sample and blank corresponded to a citronellyl acetate concentration of 1.94 ppm. Based on this sample alone, the maximum theoretical amount of citronellyl acetate absorption following application of 60 mL of product with a 10-minute exposure is about 1 mg. However, the measurements for PBS blanks ranged from 5.40-8.17 ppm and for samples from 5.44-8.10 ppm. In each series, the mean concentration in the PBS blanks was greater than the mean concentration for the samples. Additionally, the individual replicate and overall mean sample concentrations were less than the blank's. The results indicate no substantial percutaneous absorption of citronellyl acetate following topical application of the product.

In vitro dermal absorption studies using excised skin can provide a reasonable indication of penetration characteristics since absorption through the skin (percutaneous

Table 30. Summary of citronellyl acetate concentrations in individual receiving fluid samples.

Test Article #1		Test Article #2		Test Article #3	
Sample #	Conc. (ppm)	Sample #	Conc. (ppm)	Sample #	Conc. (ppm)
1.1 (blank)	7.88	2.1 (blank)	7.09	3.1 (blank)	5.40
1.1A	6.66	2.1A	7.26	3.1A	7.24
1.2A	8.31	2.2A	8.10	3.2A	7.36
1.3A	5.67	2.3A	7.26	3.3A	5.44
1.2 (blank)	7.82	2.2 (blank)	7.89	3.2 (blank)	8.17
1.1B	7.14	2.1B	6.34	3.1B	*****
1.2B	7.61	2.2B	7.61	3.2B	7.19
1.3B	7.49	2.3B	7.79	3.3B	5.72

Table 31. Summary of mean citronellyl acetate concentrations in samples and blanks.

Test Article #1		Test Article #2		Test Article #3	
Description	Conc. (ppm)	Sample #	Conc. (ppm)	Sample #	Conc. (ppm)
Mean Sample	7.85	Mean Sample	7.39	Mean Sample	6.59
Mean Blank	7.15	Mean Blank	7.49	Mean Blank	6.79
Difference	-0.70	Difference	-0.10	Difference	-0.20
Total Mean Sample	7.07	Total Mean Blank	7.38	Total Difference	-0.31

absorption) is a passive process.[138] The penetrant diffuses through the skin barrier, the stratum corneum, along a concentration gradient. Several comparative studies on percutaneous absorption obtained by both *in vivo* and *in vitro* procedures suggest that reliable measurements are possible from *in vitro* data. Therefore, no substantial systemic exposure to citronellyl acetate is expected upon topical application to humans.

ACUTE ORAL TOXICITY ASSESSMENT IN MICE – MATERIALS:

For acute oral toxicity assessment in mice, young adult CD-1® mice Crl:CD-1® (ICR) BR were obtained from Charles River Laboratories, Inc., Portage, Michigan. The animals were housed individually in suspended stainless steel cages. Food consisted of PMI Certified Rodent Chow #5002 obtained from Purina Mills, Inc. Drinking water consisted of municipal tap water treated by reverse osmosis and was delivered by an automatic watering system. Test articles were delivered using a ball tipped stainless steel gavage needle attached to a syringe.

ACUTE ORAL TOXICITY ASSESSMENT IN MICE – METHODS:

The assessment of acute oral toxicity to mice was designed as a limit test with a single dose of 5000 mg test article per 1 kg body weight. All aspects of the testing were performed in compliance with cGLPs. All housing and care were based on the standards recommended by the Guide for the Care and Use of Laboratory Animals.[134] Environmental conditions were maintained at 65-71°F and 39-53% relative humidity, light timers were set to maintain a 12-hour light/12-hour dark cycle, and room ventilation was set to produce 10-15 air changes an hour. Room temperature and humidity were recorded a minimum of once daily. Food was provided ad libitum throughout the study except during the designated fasting periods. Supplier's Certificates of Analysis were obtained documenting the nutritional composition and any possible environmental contaminants in the feed. Drinking water was supplied ad libitum by an automatic watering system.

On day 0, 5 male and 5 female CD-1® mice were selected randomly, weighed, and fasted for 4 to 5 hours prior to dosing. The test article was mixed and shaken for 30

seconds. The undiluted, mixed test article (100% concentration) was administered orally as a single dose of 5000 mg/kg. Since the dose was delivered with a syringe equipped with a ball tipped stainless steel gavage needle, the equivalent volumetric dose was 5.15 mL/kg. Each dose was calculated from each animal's fasted body weight on day 0, and after dosing, the animals were returned to ad libitum feeding.

Animals were observed for clinical abnormalities twice, post-dose, on day 0 and once daily thereafter (days 1-14). A general health and mortality check was performed twice daily (morning and afternoon) each day of the study. Individual animal body weights were obtained prior to fasting and dosing on day 0 and on days 7 and 14. All animals were euthanized by carbon dioxide inhalation at termination of the study (day 14) and necropsied. Body cavities (cranial, thoracic, abdominal, and pelvic) were opened and examined, but no tissues were retained.

Data generated from the limit test were analyzed, and an LD_{50} value was estimated. Figure 17 shows the estimation formulas. Body weight means and standard deviations were calculated separately for males and females.

ACUTE ORAL TOXICITY ASSESSMENT IN MICE – RESULTS AND DISCUSSION:

A toxicity study was performed to assess the short-term toxicity of the test product in CD-1® mice when administered as a single dose by gavage. The study was designed as a limit test intended to provide insight into the potential hazards of product ingestion. The study was conducted in compliance with cGLPs according to the method previously described with a dose of 5000 mg/kg.

No mortality occurred during the study in any of the 10 mice. Clinical observations recorded included decreased activity, slow breathing, salivation, dark material around mouth, dark material around nose, wobbly gait, and urine staining (slight and moderate). Table 32 summarizes the occurrences of each observation. Body weight data is summarized in Tables 33 and 34. Weight loss was noted in one female during the day

<50% Mortality: LD_{50} estimated as greater than administered dose

=50% Mortality: LD_{50} estimated as equal to the administered dose

>50% Mortality: LD_{50} estimated as less than administered dose

Figure 17. Estimation of LD_{50} from limit test.

Table 32. Clinical observations and occurrence during acute oral toxicity testing in mice.

Observation	# animals affected
Decreased activity	5
Slow breathing	9
Salivation	7
Dark material around mouth	7
Dark material around nose	1
Wobbly gait	1
Urine stain – slight	1
Urine stain – moderate	1

Table 33. Body weight data for males during acute oral toxicity testing in mice.

		Weight (g) on Day			
Animal #	Sex	0 (pre-fast)	0 (post-fast)	7	14
30494	M	26.4	25.1	28.8	32.4
30495	M	26.4	24.3	30.9	33.4
30498	M	27.0	25.2	27.9	31.0
30500	M	25.1	23.1	27.9	29.8
30503	M	28.9	26.9	30.2	33.0
Mean		26.8	24.9	29.1	31.9
Std dev.		1.38	1.39	1.36	1.49

Table 34. Body weight data for females during acute oral toxicity testing in mice.

		Weight (g) on Day			
Animal #	Sex	0 (pre-fast)	0 (post-fast)	7	14
30513	F	28.0	26.0	29.1	32.0
30516	F	27.2	25.1	25.2	27.6
30517	F	29.5	27.7	31.4	33.0
30523	F	25.7	24.2	26.7	28.4
30526	F	29.1	27.5	30.6	26.4
Mean		27.9	26.1	28.6	29.5
Std dev.		1.53	1.51	2.61	2.87

7-14 interval, but weight gain was noted for all other animals during the test period. No gross internal findings were observed at necropsy on day 14. Under the conditions of the test and using the estimation formula shown in Figure 17, the acute oral LD_{50} of the product was estimated to be greater than 5000 mg/kg in the mouse.

ACUTE ORAL TOXICITY ASSESSMENT IN RATS – METHODS:

The assessment of acute oral toxicity to rats was also designed as a limit test but incorporated two different single doses, 2000 mg and 5000 mg test article per 1 kg body weight. All aspects of the testing were performed in compliance with cGLPs. All housing and care were based on the standards recommended by the Guide for the Care and Use of Laboratory Animals.[134] Environmental conditions were maintained at 70-73°F and 38-58% relative humidity, light timers were set to maintain a 12-hour light/12-hour dark cycle, and room ventilation was set to produce 10-15 air changes an hour. Room temperature and humidity were recorded a minimum of once daily, and food was provided ad libitum throughout the study except during the designated fasting periods. Supplier's Certificates of Analysis were obtained documenting the nutritional composition and any possible environmental contaminants in the feed. Drinking water was supplied ad libitum by an automatic watering system.

On the day prior to dosing, 10 male and 10 female Sprague-Dawley rats were randomly selected, weighed, and fasted overnight. On day 0, the animals were separated into two groups of 5 males and 5 females each. The test article was mixed and shaken for 30 seconds, and the undiluted, mixed test article (100% concentration) was administered orally as a single dose of 2000 mg/kg to one group and 5000 mg/kg to the other. Since the dose was delivered with a syringe equipped with a ball tipped stainless steel gavage needle, the equivalent volumetric doses were 2.06 mL/kg and 5.15 mL/kg, respectively. Each dose was calculated based on each animal's fasted body weight on day 0, and after dosing, the animals were returned to ad libitum feeding.

Animals were observed for clinical abnormalities 3 times, post-dose, on day 0 and once daily thereafter (days 1-14). A general health and mortality check was performed twice daily (morning and afternoon) each day of the study. Individual animal body weights were obtained prior to fasting and dosing on day 0 and for all surviving animals on days 7 and 14. Animals found dead after day 0 were also weighed. All animals were euthanized by carbon dioxide inhalation at termination of the study on day 14 and necropsied. Body cavities (cranial, thoracic, abdominal, and pelvic) were opened and examined, but no tissues were retained.

Data generated from the limit test were analyzed and an LD_{50} value was estimated as shown previously in Figure 17. Body weight means and standard deviations were calculated separately for males and females.

ACUTE ORAL TOXICITY ASSESSMENT IN RATS – RESULTS AND DISCUSSION:

For comparison to mice, a toxicity study was also performed to assess the short-term toxicity of the test product in Sprague-Dawley rats when administered as a single dose by gavage. The study was designed as a limit test intended to provide insight into the potential hazard from ingestion of the product. The study was conducted in compliance with cGLPs according to the method previously described with two separate groups of 10 rats receiving either a single 2000 mg/kg dose or 5000 mg/kg dose.

No mortality occurred in any rats that received the 2000 mg/kg dose. For the 5000 mg/kg dose, all mortality occurred by the third day of the study. Two rats died on the day of dosing (day 0), four died on day 1, and 1 died on day 3. A summary of clinical observations recorded for both groups is displayed in Tables 35 and 36. The most notable observations included decreased activity, breathing abnormalities, dark material around facial area, wobbly gait, urine staining, ocular discharge, and dilated pupil(s). Body weights are summarized in Tables 37 and 38, and weight gain was noted for all survivors.

For animals that died prior to day 14, gross necropsy findings observed included abnormal content and reddened mucosa in the digestive tract and reddened thymus. No

Table 35. Clinical observations and occurrence during acute oral toxicity testing in rats with the 2000 mg/kg dose.

Observation	# animals affected
Rales	1
Salivation	6
Dark material around mouth	3
Dark material around eye	1
Urine stain – slight	2

Table 36. Clinical observations and occurrence during acute oral toxicity testing in rats with the 5000 mg/kg dose.

Observation	# animals affected (per each)
Decreased activity, wobbly gait	10
Piloerection, dark material around eye	8
Salivation	7
Urine stain – slight	6
Ocular discharge, labored breathing	5
Eyelids partially closed, dilated pupils	4
Hunched posture	3
Dark material around mouth, rales, scabs (nose area), eye lesion due to orbital bleeding, cool to touch, few feces, decreased food consumption	1

Table 37. Body weight data for males during acute oral toxicity testing in rats.

2000 mg/kg dose		Weight (g) on Day			
Animal #	Sex	-1 (pre-fast)	0 (post-fast)	7	14
10450	M	280	250	321	346
10452	M	276	255	321	356
10457	M	273	245	313	353
10463	M	279	252	311	352
10465	M	281	254	314	348
Mean		278	251	316	351
Std dev.		3.3	4.0	4.7	4.0
5000 mg/kg dose		Weight (g) on Day			
Animal #	Sex	-1 (pre-fast)	0 (post-fast)	7	14
10227	M	241	209	250	305
10233	M	232	207	Dead day 1 (wt = 194)	Dead
10239	M	236	204	Dead day 1 (wt = 198)	Dead
10246	M	244	211	259	319
10250	M	250	219	278	343
Mean		241	210	262	322
Std dev.		7.0	5.7	14.3	19.2

Table 38. Body weight data for females during acute oral toxicity testing in rats.

2000 mg/kg dose		Weight (g) on Day			
Animal #	Sex	-1 (pre-fast)	0 (post-fast)	7	14
10481	F	213	195	222	237
10480	F	219	200	224	232
10486	F	216	196	230	234
10490	F	213	196	226	238
10496	F	218	197	226	232
Mean		216	197	226	235
Std dev.		2.8	1.9	3.0	2.8
5000 mg/kg dose		Weight (g) on Day			
Animal #	Sex	-1 (pre-fast)	0 (post-fast)	7	14
10227	F	206	183	Dead day 0	Dead
10233	F	210	183	Dead day 0	Dead
10239	F	204	183	Dead day 1 (wt = 173)	Dead
10246	F	210	188	Dead day 1 (wt = 178)	Dead
10250	F	202	177	Dead day 3 (wt = 166)	Dead
Mean		206	183	N/A	N/A
Std dev.		3.6	3.9	N/A	N/A

significant findings were observed at necropsy of the animals that completed the study on day 14. Under the conditions of the test and using the estimation formula (Figure 17), the acute oral LD_{50} was estimated to be between 2000 mg/kg and 5000 mg/kg in the rat.

ACUTE DERMAL TOXICITY ASSESSMENT TO RABBITS – MATERIALS:

For assessments of acute dermal toxicity to rabbits, New Zealand White rabbits of both sexes weighing about 2.0 to 3.5 kg were obtained (Myrtle's Rabbitry, Thompson Station, Tennessee, or other USDA approved supplier). All other supplies were identical to those used for the oral toxicology assessments except that PMI Certified Rabbit Chow #5322 (Purina) was used for food. Additional supplies consisted of 4-ply porous gauze dressing backed with a plastic strap, elastic wrap, adhesive tape, and animal hair clippers.

ACUTE DERMAL TOXICITY ASSESSMENT TO RABBITS – METHODS:

The assessment of acute dermal toxicity in rabbits was also designed as a limit test but only incorporated a single dose of 2000 mg per 1 kg body weight. All aspects of the testing were performed in compliance with cGLPs. All housing and care were based on the standards recommended by the Guide for the Care and Use of Laboratory Animals.[134] Environmental conditions were maintained at 64-72°F and 40-56% relative humidity, light timers were set to maintain a 12-hour light/12-hour dark cycle, and room ventilation was set to produce 10-15 air changes an hour. Room temperature and humidity were recorded a minimum of once daily. Food was provided ad libitum throughout the study except during the designated fasting periods. Supplier's Certificates of Analysis were obtained documenting the nutritional composition and any possible environmental contaminants in the feed. Drinking water was supplied ad libitum by an automatic watering system.

On the day prior to dosing, 5 male and 5 female New Zealand White rabbits were selected randomly. Using an animal clipper, the fur of each rabbit was removed from the dorsal trunk area to include the scapula to the wing of the ileum and halfway down the flank on each side of the animal. Care was taken to avoid abrasion of the skin during the clipping process. The clipped area equated to approximately 10% of the total animal's

body surface area (BSA). On the following day (day 0), the animals were weighed prior to dosing. The test article was mixed and shaken for 30 seconds, and the undiluted, mixed test article (100% concentration) was administered topically as a single dose of 2000 mg/kg to the 10% BSA exposed from the clipping process. Since the dose was delivered with a syringe, the equivalent volumetric dose was 2.06 mL/kg. The test article was spread evenly over the test area and held in contact with the skin using an appropriately sized 4-ply porous gauze dressing backed with a plastic strap which was placed over the gauze dressing (occlusive binding). Prevention of removal and ingestion of the test article was achieved by placing an elastic wrap over the trunk and test area. The elastic wrap was further secured with adhesive tape around the trunk at the cranial and caudal ends. Each dose was calculated based on each animal's fasted body weight on day 0. After an approximate 24-hour exposure period, the gauze dressing, plastic, and elastic wrap were removed and the corners of the test site were delineated using a marker. Residual amounts of the test article were removed using gauze moistened with deionized water, and the area was dried with clean, dry gauze.

Animals were observed for erythema and edema following patch removal on day 1, once daily thereafter (days 2-14), and responses were scored according to the Macroscopic Dermal Grading System based on Draize.[135] Tables 39-41 summarize the Macroscopic Dermal Grading System for erythema and edema observations, notable dermal lesions, and additional findings.

Additionally, animals were observed for clinical abnormalities twice post-dose, on day 0, and once daily thereafter (days 1-14). A general health and mortality check was performed twice daily (morning and afternoon) each day of the study. Individual animal body weights were obtained prior to dosing on day 0 and on days 7 and 14. All animals were euthanized by intravenous injection of sodium phenobarbital at termination of the study on day 14 and necropsied. Body cavities (cranial, thoracic, abdominal, and pelvic) were opened and examined, but no tissues were retained.

Table 39. Macroscopic dermal grading system for erythema and edema observations.

ERYTHEMA AND EDEMA OBSERVATIONS		
OBSERVATION	DEFINITION	CODE
Erythema - Grade 0	No Erythema	0
Erythema - Grade 1	Very slight erythema (barely perceptible)	1
Erythema - Grade 2	Well-defined Erythema	2
Erythema - Grade 3	Moderate to severe erythema	3
Erythema - Grade 4	Severe erythema (beet redness)	4
Maximized Grade 4	Notable dermal lesions (see below)	M – 4 (see below)
Edema - Grade 0	No edema	0
Edema - Grade 1	Very slight edema (barely perceptible)	1
Edema - Grade 2	Slight edema (edges of area well defined by definite raising)	2
Edema - Grade 3	Moderate edema (raised approximately 1 millimeter)	3
Edema - Grade 4	Severe edema (raised more than 1 millimeter and extends beyond the area of exposure)	4

(NOTE to Table 39: Each animal was assigned an erythema and edema score. The most severely affected area within the test site was graded. If eschar, blanching, ulceration and/or necrosis greater than grade 1 was observed, then the "Maximized Grade 4" was assigned to the test site in place of the erythema score and the type of notable dermal lesion(s) (e.g., eschar - grade 2, blanching - grade 3, etc.) was noted. The presence of any other dermal changes (e.g., desquamation, fissuring, eschar exfoliation, etc.) was also recorded.)

Table 40. Macroscopic dermal grading system for notable dermal lesions.

NOTABLE DERMAL LESIONS		
OBSERVATION	CODE	DEFINITION
Eschar - Grade 1	ES-1	Focal and/or pinpoint areas up to 10% of test site.
Eschar - Grade 2	ES-2	> 10% < 25% of test site.
Eschar - Grade 3	ES-3	> 25% < 50% of test site.
Eschar - Grade 4	ES-4	> 50% of test site.
Blanching - Grade 1	BLA-1	Focal and/or pinpoint areas up to 10% of test site.
Blanching - Grade 2	BLA-2	> 10% < 25% of test site.
Blanching - Grade 3	BLA-3	> 25% < 50% of test site.
Blanching - Grade 4	BLA-4	> 50% of test site.
Ulceration - Grade 1	U-1	Focal and/or pinpoint areas up to 10% of test site.
Ulceration - Grade 2	U-2	> 10% < 25% of test site.
Ulceration - Grade 3	U-3	> 25% < 50% of test site.
Ulceration - Grade 4	U-4	> 50% of test site.
Necrosis - Grade 1	NEC-1	Focal and/or pinpoint areas up to 10% of the test site. (Note color of necrosis)
Necrosis - Grade 2	NEC-2	> 10% < 25% of test site (Note color of necrosis)
Necrosis - Grade 3	NEC-3	> 25% < 50% of test site (Note color of necrosis)
Necrosis - Grade 4	NEC-4	> 50% of test site (Note color of necrosis)

Table 41. Macroscopic dermal grading system for additional dermal findings.

ADDITIONAL DERMAL FINDINGS		
OBSERVATION	DEFINITION	CODE
Desquamation	Characterized by scaling or flaking of dermal tissue with or without denuded areas.	DES
Fissuring	Characterized by cracking of the skin with or without moist exudate. Fissuring should be checked prior to removing the animal from the cage and manipulating the test site.	FIS
Eschar Exfoliation	The process by which areas of eschar flake off the test site.	EXF
Test Site Staining	Skin located at test site appears to be discolored, possibly due to test article (note color of staining).	TSS (color)
Erythema Extends Beyond the Test Site	The erythema extends beyond the test site. Note: A study director should be contacted for erythema extending beyond the test site.	ERB
Superficial Lightening	Characterized by pale area(s) (almost a burn-like appearance) in the test site. However, erythema may still be observed through the pale area. Note: This observation may affect the overall erythema score of the test site. This observation may progress to other observations resulting in notable dermal lesions, but SL itself will not be considered a notable dermal lesion that will result in a dermal score to be maximized since it does not result in any in-depth injury (see below for coding).	--

Table 41. Continued.

ADDITIONAL DERMAL FINDINGS		
OBSERVATION	DEFINITION	CODE
Superficial Lightening - Grade 1	Focal and/or pinpoint areas up to 10% of the test site	SL-1
Superficial Lightening - Grade 2	> 10% < 25% of test site	SL-2
Superficial Lightening - Grade 3	> 25% < 50% of test site	SL-3
Superficial Lightening - Grade 4	> 50% of test site	SL-4
Dermal Irritation - Outside of the Test Site	Noticeable irritation outside of test site probably due to the binding tape material. This notation will only be made for reactions greater than what are normally observed from tape removal that does not interfere with the scoring of the test site.	IT

Data generated from the limit test were analyzed and an LD_{50} value estimated as shown previously in Figure 17. Body weight means and standard deviations were calculated separately for males and females.

No mortality occurred during the test. Clinical abnormalities observed included decreased activity, and dermal irritation was noted at the application site and scored. Tables 42 and 43 summarize the individual observations and scoring, and Tables 44 and 45 summarize body weight data. Body weight gain was observed for all animals. Under the conditions of the test and using the estimation formula (Figure 17), the acute dermal LD_{50} of the test article was estimated to be greater than 2000 mg/kg in the rabbit.

PRIMARY SKIN IRRITATION TO RABBITS – MATERIALS:

For assessments of primary skin irritation in rabbits, New Zealand White rabbits of both sexes weighing about 2.0 to 3.5 kg were obtained (Myrtle's Rabbitry, Thompson Station, Tennessee, or other USDA approved supplier). All other supplies were identical to those used for the dermal toxicology assessments.

PRIMARY SKIN IRRITATION TO RABBITS – METHODS:

The assessment of primary skin irritation in rabbits was also performed in compliance with cGLPs. All housing and care were based on the standards recommended by the Guide for the Care and Use of Laboratory Animals.[134] Environmental conditions were maintained at 67-75°F and 39-65% relative humidity, light timers were set to maintain a 12- hour light/12-hour dark cycle, and room ventilation was set to produce 10-15 air changes an hour. Room temperature and humidity were recorded a minimum of once daily. Food was provided ad libitum throughout the study except during the designated fasting periods. Supplier's Certificates of Analysis were obtained documenting the nutritional composition and any possible environmental contaminants in the feed. Drinking water was supplied ad libitum by an automatic watering system.

On the day prior to dosing, 6 male New Zealand White rabbits were selected randomly. Using an animal clipper, the fur of each rabbit was removed from the dorsal

Table 42. Clinical observations for first 5 rabbits during dermal toxicity study.

Observation w/ Grade	Animal # / Days Observation was Present				
	55293	55296	55297	55299	55301
Decrsd. Act.	0-1	0-1	0-1	0	0
Erythema – 2	12-13	11-14	10-11	11-14	14
Erythema – 1	14		12-14		
Superficial Lightening - 2	1	1			1
Superficial Lightening – 1		2	1-2	1	2
Maximized – 4	1-11	1-10	1-9	1-10	1-13
Blanching - 2	1	1	1-3	1-2	1-2
Blanching – 1	2-4	2-3	4	3-4	3-5
Eschar - 4	4-7	3-6	5-6	4-7	4-7
Eschar – 3	3, 8-9	2,7-8	4,7-8	8-9	3,8-11
Eschar – 2	2,10-11	9-10	3,9	3,10	2,12-13
Eschar – 1	12-13	1,11-13	1-2,10-14	1-2,11-12	1,14
Eschar exfoliation	5-11	5-13	5-9	6-12	5-14
Necrosis – 4, greenish brn					1
Necrosis – 3, greenish brn	1	1	1-2	1-3	2-3
Necrosis – 2, greenish brn	2-3		3-4		
Necrosis – 1, greenish brn	4	2-3			4
Desquamation	6-14	4-14	5-14	4-14	5-14
Edema – 4	1-2	1	1	1	1
Edema – 3	3-4	2-5	2-7	2-5	2-6
Edema – 2	5-9	6-9	8-9	6-9	7-11
Edema – 1	10-11	10-14	10-13	10-14	12-14

Table 43. Clinical observations for last 5 rabbits during dermal toxicity study.

Observation w/ Grade	Animal # / Days Observation was Present				
	55332	55333	55334	55335	55336
Decrsd. Act.	0-1	0	0	0-1	0
Dermal Irrit.– outside site	1				
Erythema – 2	11-14		12-14		13-14
Superficial Lightening - 3			1		
Superficial Lightening - 2	1	1			1
Superficial Lightening – 1	2	2	2	1	
Maximized – 4	1-10	1-14	1-11	1-14	1-12
Blanching – 2	1-3	1-2	1-3	2-3	1-3
Blanching – 1	4	3	4-5	1,4	
Eschar – 4	4-7	4-7	5-7	4-7	4-6
Eschar – 3	8	3,8-13	8-10	8	7-9
Eschar – 2	3,9-10	14	4,11	3,9-14	3,10-12
Eschar – 1	2,11-13	1-2	12-14	1-2	1-2,13-14
Eschar exfoliation	5-13	6-14	6-13	6-14	5-12
Necrosis – 4, greenish brn				1-2	1-2

Table 44. Body weight data for males during acute dermal toxicity testing in rabbits.

		Weight (g) on Day		
Animal #	Sex	0	7	14
55293	M	2603	2718	2964
55296	M	2498	2632	2890
55297	M	2559	2641	2912
55299	M	2685	2822	3038
55301	M	3593	2784	3033
Mean		2558	2719	2967
Std dev.		68.2	84.4	67.7

Table 45. Body weight data for females during acute dermal toxicity testing in rabbits.

		Weight (g) on Day		
Animal #	Sex	0	7	14
55332	F	2670	2870	3124
55333	F	2660	2891	3157
55334	F	2733	2914	3242
55335	F	2578	2862	3229
55336	F	2459	2493	2762
Mean		2620	2806	3103
Std dev.		105.5	176.1	196.7

trunk area of each animal. Care was taken to avoid abrasion of the skin during the clipping process. On the following day (day 0), the animals were weighed prior to dosing, and the test article was mixed and shaken for 30 seconds. The undiluted, mixed test article (100% concentration) was applied to a 1-inch square gauze patch, (0.5 mL of the test article per patch). The patch with the test article was held in contact with the skin at the cut edges with non-irritating tape. Prevention of removal and ingestion of the test article was achieved by placing an elastic wrap over the trunk and test area (semi-occlusive binding). The elastic wrap was further secured with adhesive tape around the trunk at the cranial and caudal ends. After a 4-hour exposure period, the gauze dressing, plastic, and elastic wrap were removed and the corners of the test site were delineated using a marker. Residual amounts of the test article were removed using gauze moistened with deionized water, and the area was dried with clean, dry gauze.

Animals were examined for signs of erythema and edema at approximately 1, 24, 48, and 72 hours and up to 21 days after patch removal. Responses were scored according to the Macroscopic Dermal Grading System based on Draize previously summarized in Tables 39-41.[135] Additionally, any unusual observations and/or mortality were recorded. General health and mortality checks were performed twice daily (morning and afternoon) each day of the study. Individual animal body weights were obtained prior to dosing on day 0. All animals were euthanized by intravenous injection of sodium phenobarbital following the final scoring, but gross necropsy examinations were not required.

The Primary Irritation Index (PII) of the test article was calculated by adding 1, 24, 48, and 72 hour erythema and edema scores for all animals and dividing that total by the number of test sites times 4. The calculated PII was classified according to the Dermal Evaluation Criteria summarized in Table 46.[136]

PRIMARY SKIN IRRITATION TO RABBITS – RESULTS AND DISCUSSION

Exposure to the test article produced well-defined erythema on 1 of 6 test sites. Notable lesions, including blanching and necrosis, were observed on 5 of 6 test sites, and

Table 46. Dermal Evaluation Criteria.

Primary Irritation Index (P.I.I.)	Irritation Rating
0.00	Nonirritant
0.01 - 1.99	Slight Irritant
2.00 - 5.00	Moderate Irritant
5.01 - 8.00	Severe Irritant

very slight to moderate edema was seen on all 6 test sites at the 1-hour scoring interval. The dermal irritation progressed to eschar on all test sites by day 7. This dermal irritation resolved completely in all animals by study day 21. Additional findings included eschar, exfoliation, superficial lightening, and desquamation noted on 4, 6, and 6 of the total 6 test sites, respectively. Table 47 summarizes the individual dermal irritation scores for each rabbit at each scoring interval with comments based on the definitions previously shown in Tables 39-41.

The Primary Irritation Index was calculated using the formula previously described in the methods. The calculated PII was 6.75, which classifies the test article as a "severe irritant" to rabbit skin based on the Dermal Evaluation Criteria exhibited in Table 46.

PRIMARY EYE IRRITATION IN RABBITS – MATERIALS:

For assessments of primary eye irritation in rabbits, New Zealand White rabbits weighing about 2.0 to 3.5 kg were obtained (Myrtle's Rabbitry, Thompson Station, Tennessee, or other USDA approved supplier). All other supplies were identical to those used for the dermal toxicology and primary skin irritation studies previously described.

PRIMARY EYE IRRITATION IN RABBITS – METHODS:

The assessment of primary eye irritation in rabbits was designed to incorporate both rinse and no rinse procedures. Both procedures were performed with New Zealand White rabbits, but the rinse group consisted of 3 rabbits while the no rinse group consisted of 6 rabbits. All aspects of the testing were performed in compliance with cGLPs. All housing and care were based on the standards recommended by the Guide for the Care and Use of Laboratory Animals.[134] Environmental conditions were maintained at 61-73°F and 40-70% relative humidity, light timers were set to maintain a 12-hour light/12-hour dark cycle, and room ventilation was set to produce 10-15 air changes an hour. Room temperature and humidity were recorded a minimum of once daily. Food was provided ad libitum throughout the study except during the designated fasting periods. Supplier's Certificates of Analysis were obtained documenting the nutritional composition

Table 47. Individual dermal irritation scores at each scoring interval.

Animal # Sex / Wt	Scoring Interval	Erythema Score	Edema Score	Comments
55256 Male 2.713 kg	1 hr	M-4	3	BLA-2, SL-2
	24 hr	M-4	3	BLA-1, ES-1, NEC-2(B)
	48 hr	M-4	3	BLA-1, ES-1, NEC-3(B)
	72 hr	M-4	3	BLA-1, ES-1, NEC-1(B)
	7 days	M-4	2	ES-4
	10 days	M-4	2	ES-2, DES
	14 days	1	0	DES
	21 days	0	0	
55259 Male 3.028 kg	1 hr	M-4	3	SL-2, NEC-2(B)
	24 hr	M-4	3	BLA-1, ES-1, NEC-2(B)
	48 hr	M-4	2	BLA-2, ES-1, NEC-2(B)
	72 hr	M-4	3	BLA-2, ES-2, NEC-1(B)
	7 days	M-4	2	ES-2, EXF, DES
	10 days	2	1	DES
	14 days	0	0	DES

Table 47. Continued.

Animal # Sex / Wt	Scoring Interval	Erythema Score	Edema Score	Comments
55263 Male 2.995 kg	1 hr	2	1	BLA-1, SL-2,
	24 hr	3	2	BLA-1, SL-3
	48 hr	M-4	2	BLA-2, SL-3
	72 hr	M-4	3	BLA-2, SL-1
	7 days	M-4	1	ES-2, EXF, DES
	10 days	2	0	ES-1, DES
	14 days	1	0	DES
	21 days	0	0	DES
55268 Male 2.624 kg	1 hr	M-4	3	BLA-2, SL-1, NEC-1(B)
	24 hr	M-4	3	BLA-2, SL-1, NEC-1(B)
	48 hr	M-4	3	BLA-3, SL-1, NEC-1(B)
	72 hr	M-4	3	BLA-2, ES-1, NEC-1(B)
	7 days	M-4	1	ES-4, EXF, DES
	10 days	12	0	ES-1, DES
	14 days	0	0	DES
55270 Male 2.742 kg	1 hr	M-4	3	BLA-2, SL-2, NEC-1(B)
	24 hr	M-4	4	BLA-3, SL-1, NEC-1(B)
	48 hr	M-4	4	BLA-3, SL-1, NEC-2(B)
	72 hr	M-4	3	BLA-3, ES-2, NEC-1(B)
	7 days	M-4	1	ES-2, EXF, DES
	10 days	2	1	ES-1, DES
	14 days	0	0	DES

Table 47. Continued.

Animal # Sex / Wt	Scoring Interval	Erythema Score	Edema Score	Comments
55275 Male 2.793 kg	1 hr	M-4	3	BLA-2, SL-1, NEC-2(B)
	24 hr	M-4	3	BLA-2, ES-1, NEC-2(B)
	48 hr	M-4	3	BLA-2, ES-1, NEC-3(B)
	72 hr	M-4	3	BLA-2, ES-3, NEC-2(B)
	7 days	2	1	ES-1, DES
	10 days	2	21	ES-1, DES
	14 days	0	0	DES

and any possible environmental contaminants in the feed. Drinking water was supplied ad libitum by an automatic watering system.

On the day prior to dosing (day 0), rabbits were selected randomly. Both eyes of each animal selected for use were examined macroscopically for ocular irritation with the aid of an auxiliary light source. In addition, the corneal surface was examined using fluorescein sodium dye. One drop of a fluorescein/physiological saline mixture was gently dropped onto the superior sclera of the each eye. After about a 15-minute exposure, the eyes were rinsed with a physiological saline solution. The corneal surface was examined for dye retention under a long-wave UV light source. Animals exhibiting preexisting ocular irritation, corneal injury, or dye retention were eliminated from the study.

After a minimum of 1 hour after successful completion of the preliminary examination, 0.1 mL of the mixed test article was instilled into the conjunctival sac of the right eye of each of the total 9 rabbits after gently pulling the lower lid away from the eye. After instillation, the eyelids were gently held together for about 1 second in order to limit loss of the article. The contralateral eye of each individual rabbit remained untreated to serve a control. For the rinse group of 3 rabbits, the test and control eyes were rinsed with physiological saline about 30 seconds after instillation to remove the test article. The remaining 6 rabbits were not rinsed and served as the no rinse group.

The eyes of each animal were macroscopically examined with the aid of an auxiliary light source for signs of irritation at 1, 24, 48, and 72 hours and up to 30 days after dosing of the test article depending on response. Responses were scored according to the Ocular Grading System based on Draize.[13,5] Tables 48-54 summarize the Ocular Grading System for corneal, iris, and conjunctival irritation, and Figures 18-20 show the calculations for corneal, iris, and conjunctival scoring.

A bimicroscopic slit-lamp was utilized to further examine and clarify ocular lesions as necessary. After the macroscopic examinations at the 24-hour scoring interval, the fluorescein examination procedure was repeated on both test and control eyes of each

Table 48. Ocular grading system for cornea scoring.

(O) CORNEAL OPACITY--DEGREE OF DENSITY (AREA MOST DENSE TAKEN FOR READING)	
OBSERVATION	CODE
No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible	1
Easily discernible translucent area, details of iris slightly obscured	2
Nacreous (opalescent) area, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through opacity	4
No ulceration or opacity	0
(A) AREA OF CORNEA INVOLVED (TOTAL AREA EXHIBITING ANY OPACITY, REGARDLESS OF DEGREE)	
One quarter (or less) but not zero	1
Greater than one quarter, but less than half	2
Greater than half, but less than three quarters	3
Greater than three quarters, up to whole area	4

Cornea Score = O x A x 5 Total Maximum = 80

Figure 18. Calculation of cornea score.

Table 49. Ocular grading system for iris scoring.

(I) IRITIS	
OBSERVATION	CODE
Normal	0
Markedly deepened rugae (folds above normal), congestion, swelling, moderate circumcorneal hyperemia or injection, any or all of these or combination of, iris is still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any or all of these)	2

Iris Score = I x 5 Total Maximum = 10

Figure 19. Calculation of iritis score.

Table 50. Ocular grading system for conjunctival scoring.

(R) CONJUNCTIVAL REDNESS (REFERS TO PALPEBRAL AND BULBAR CONJUNCTIVAE EXCLUDING CORNEA AND IRIS)	
OBSERVATION	CODE
Blood vessels normal	0
Some blood vessels definitely hyperemic (injected) above normal (slight erythema)	1
Diffuse, crimson color, individual vessels not easily discernible (moderate erythema)	2
Diffuse beefy red (marked erythema)	3

Table 50. Continued.

(S) CONJUNCTIVAL SWELLING (LIDS AND/OR NICTITATING MEMBRANE)	
No swelling	0
Any swelling above normal (includes nictitating membrane, slightly swollen)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids more than half closed	4
(D) CONJUNCTIVAL DISCHARGE	
No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of the lids, hairs, & considerable area around eye	3

Conjunctival Score = (R+S+D) x 2 Total Maximum = 20

Figure 20. Calculation of conjunctival score.

Table 51. Ocular grading system for corneal neovascularization.

CORNEAL NEOVASCULARIZATION		
OBSERVATION	CODE	DEFINITION
Neovascularization - Very Slight	VAS-1	Total area of vascularized corneal tissue is < 10% of corneal surface
Neovascularization - Mild	VAS-2	Total area of vascularized corneal tissue is > 10% but < 25% of corneal surface
Neovascularization - Moderate	VAS-3	Total area of vascularized corneal tissue is > 25% but < 50% of corneal surface
Neovascularization - Severe	VAS-4	Total area of vascularized corneal tissue is > 50% of corneal surface

Table 52. Ocular grading system for secondary findings.

SECONDARY OCULAR FINDINGS		
OBSERVATION	CODE	DEFINITION
Sloughing of the corneal epithelium	SCE	Corneal epithelial tissue is observed to be peeling off the corneal surface.
Corneal bulging	CB	The entire corneal surface appears to be protruding outward further than normal.
Slight dulling of normal luster of the cornea	SDL	The normal shiny surface of the cornea has a slightly dulled appearance.
Raised area on the corneal surface	RAC	A defined area on the corneal surface that is raised above the rest of the cornea. This area is generally associated with neovascularization and has an off-white to yellow color.
Corneal edema	CE	The cornea has a swollen appearance.
Test article present in eye	TAE	Apparent residual test article is observed on the eye or in the conjunctival sac/inner canthus.
Observation confirmed by slit lamp	OCS	A slit lamp examination was performed to confirm the initial observation.
Corneal mineralization	CM	Small white or off-white crystals that are observed in the corneal tissue.

Table 53. Ocular grading system for fluorescein examination.

FLUORESCEIN EXAMINATION OF CORNEA	
OBSERVATION	CODE
Fluorescein Dye Retention	
Fluorescein dye retention associated with the area of corneal opacity	FAO
Fluorescein dye retention is not associated with any other finding	FNF
Negative Results	
No fluorescein retention is observed	(-)
Secondary Ocular Findings	
Superficial mechanical abrasion to the cornea observed during the fluorescein examination period	MI
Fine stippling on the cornea observed during the fluorescein examination procedure	ST

Table 54. Ocular grading system for post-dose clinical observations.

POST-DOSE CLINICAL OBSERVATIONS	
OBSERVATION	CODE
Animal vocalized following dosing	VOC
Animal excessively pawed test eye following dosing	PAW
Animal exhibited excessive hyperactivity following dosing	HYP
Animal exhibited excessive head tilt following dosing	HT
Animal exhibited excessive squinting of test eye following dosing	SQ

animal, and any residual test article remaining in the eye was rinsed out at this point with physiological saline for the no rinse group. Animals noting findings from the 24-hour fluorescein examination were subsequently examined at each additional time interval until a negative response was obtained and/or all corneal opacity had cleared.

The study was conducted for the longer of the 72-hour scoring interval if no irritation existed or every 7 days for affected animals to a 30-day maximum length. Additionally, any unusual observations and mortality were recorded, and general health and mortality checks were performed twice daily (morning and afternoon) each day of the study. All animals found dead or euthanized for cause (by intravenous injection of sodium phenobarbital) were necropsied with examination of body cavities (cranial, thoracic, abdominal, and pelvic) that were opened (no tissues were retained). Each surviving animal was eventually euthanized by intravenous injection of sodium phenobarbital following the final scoring period, but gross necropsy examinations were not required for these.

For each group, the ocular irritation score was calculated for each parameter (corneal, iritis, conjunctival) as shown in Figures 18-20, and the totals were added for each animal at each interval. The group mean irritation score was then calculated for each scoring interval based on the number on animals initially dosed in each group. In the event of a death, the total number of animals in the group was reduced by the total number of deaths. This was repeated for each subsequent scoring interval for the purposes of calculating mean ocular irritation scoring for each interval. The calculated group mean ocular irritation scores for each interval were used to ultimately classify the test article according to the Ocular Evaluation Criteria shown in Table 55.[137]

PRIMARY EYE IRRITATION IN RABBITS - RESULTS AND DISCUSSION:

For the no rinse group, exposure to the test article produced corneal opacity in all test eyes by the 24-hour scoring interval (confirmed by dye retention). The corneal opacity resolved in 2 of the 6 test eyes by study termination. Iritis was observed in all test eyes at the 1-hour interval and resolved completely in all eyes by study day 30. Conjunctivitis was

Table 55. Ocular Evaluation Criteria.

Maximum mean Score (days 0-3)	Maximum mean score	Persistence of Individual Scores	Descriptive Rating and Class
0.00 - 0.49	24 hours = 0		Non-Irritating 1
	24 hours > 0		Practically Non-Irritating 2
0.50 - 2.49	24 hours = 0		Non-Irritating 1
	24 hours > 0		Practically Non-Irritating 2
2.50 - 14.99	48 hours = 0		Slight Irritant 3
	48 hours > 0		Mild Irritant 4
15.00 - 24.99	72 hours = 0		Mild Irritant 4
	72 hours > 0		Moderate Irritant 5
25.00 - 49.99	7 day ≤ 20	> half of day 7 scores ≤ 10	Moderate Irritant 5
		> half of day 7 scores > 10, but no score > 20	Moderate Irritant 5
		> half of day 7 scores > 10, and any score > 20	Severe Irritant 6
	7 day > 20		Severe Irritant 6

Table 55. Continued.

Maximum mean Score (days 0-3)	Maximum mean score	Persistence of Individual Scores	Descriptive Rating and Class
50.00 – 79.99	7 day ≤ 40	> half of day 7 scores ≤ 30	Severe Irritant 6
		> half of day 7 scores > 30, but no score > 60	Severe Irritant 6
		> half of day 7 scores > 30, and any score > 60	Very Severe Irritant 7
	7 day > 40		Very Severe Irritant 7
80.00 - 99.99	7 day ≤ 80	> half of day 7 scores ≤ 60	Very Severe Irritant 7
		> half of day 7 scores > 60, but no score > 100	Very Severe Irritant 7
		> half of day 7 scores > 60, and any score > 100	Extremely Severe Irritant 8
	7 day > 80		Extremely Severe Irritant 8
100.00 - 110.00	7 day ≤ 80		Very Severe Irritant 7
	7 day > 80		Extremely Severe Irritant 8

noted in all test eyes at the 1-hour interval, but it resoled completely in 4 of the 6 eyes by study termination. Additional ocular findings included sloughing of the corneal epithelium (1 eye), corneal neovascularization (5 eyes), raised area on the corneal surface (1 eye), and slight dulling of the normal luster of the cornea (4 eyes). Table 56 summarizes the individual ocular irritation scores, Table 57 summarizes the additional findings from examination for the no rinse group with comments based on the definitions previously shown in Tables 51-54, and Table 58 summarizes the mean ocular scores for each interval for the no rinse group. One animal was found dead on day 17, and gross internal necropsy observations included abnormal content in the abdominal and thoracic cavities, dark red mucosa in the trachea, and foci on the liver.

For the rinsed group, exposure to the test article produced corneal opacity in all test eyes by the 24-hour scoring interval (confirmed by dye retention). The corneal opacity resolved in 2 of the 3 test eyes by study termination. Iritis was observed in all test eyes at the 1-hour interval and resolved completely in all eyes by study day 10. Conjunctivitis was noted in all test eyes at the 1-hour interval, but it resolved completely in 2 of the 3 eyes by study termination. Additional ocular findings included sloughing of the corneal epithelium (1 eye), corneal neovascularization (1 eyes), and slight dulling of the normal luster of the cornea (2 eyes). Table 59 summarizes the individual ocular irritation scores, and Table 60 summarizes the additional findings from examination with comments based on the definitions previously shown in Tables 51-54. Table 61 summarizes the mean ocular scores for each interval for the rinsed group.

Using the Ocular Evaluation Criteria exhibited in Table 55, the test article was found to be “corrosive” and a “very severe irritant” to the ocular tissue of the rabbit under the conditions of the test. Applying the same criteria for the rinsed group, the test article was found to be a “severe irritant” to the ocular tissues of the rabbit under the test conditions.

Table 56. Individual ocular irritation scores for the no rinse group.

Animal # Sex / Wt.	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
55160 Female 3.517 kg	1 hr	1	3	15	1	5	2	2	2	12	32
	24 hrs	2	4	40	1	5	2	2	3	14	59
	48 hrs	3	4	60	1	5	3	2	3	16	81
	72 hrs	3	4	60	1	5	3	2	3	16	81
	7 days	3	3	45	1	5	2	2	2	12	62
	10 days	3	3	45	1	5	2	2	2	12	62
	14 days	3	3	45	1	5	3	2	2	14	64
	21 days	2	1	10	1	5	2	1	0	6	21
	30 days	2	1	10	0	0	1	1	0	4	14
55345 Female 2.946 kg	1 hr	0	0	0	1	5	3	3	3	18	23
	24 hrs	2	4	40	1	5	3	2	2	14	59
	48 hrs	2	4	40	1	5	3	2	2	14	59
	72 hrs	3	3	45	1	5	3	2	2	14	64
	7 days	3	3	45	1	5	3	2	1	12	62
	10 days	3	3	45	0	0	2	1	1	8	53
	14 days	1	1	5	0	0	1	1	1	6	11

Table 56. Continued.

Animal # Sex / Wt.	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
55346 Female 2.965 kg	1 hr	0	0	0	1	5	2	2	3	14	19
	24 hrs	2	4	40	1	5	3	2	2	14	59
	48 hrs	2	4	40	1	5	3	2	2	14	59
	72 hrs	3	4	60	1	5	3	2	2	14	79
	7 days	3	1	15	1	5	2	2	0	8	28
	10 days	4	1	20	0	0	1	1	0	4	24
	14 days	1	1	5	0	0	1	1	0	4	9
	21 days	1	1	5	0	0	0	1	0	2	7
	28 days	1	1	5	0	0	0	0	0	0	5
55339 Female 3.014 kg	1 hr	1	4	20	1	5	3	2	3	16	41
	24 hrs	2	4	40	1	5	3	2	2	14	59
	48 hrs	2	4	40	1	5	3	2	2	14	59
	72 hrs	3	4	60	1	5	3	2	2	14	79
	7 days	3	2	30	0	0	2	1	0	6	36
	10 days	1	1	5	0	0	1	0	0	2	7
	14 days	0	0	0	0	0	1	0	0	2	2
	21 days	0	0	0	0	0	0	0	0	0	0

Table 56. Continued.

Animal # Sex / Wt.	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
55351 Female 2.769kg	1 hr	0	0	0	1	5	2	3	3	16	21
	24 hrs	2	4	40	1	5	2	2	2	12	57
	48 hrs	2	4	40	1	5	3	2	2	14	59
	72 hrs	3	4	60	1	5	3	2	2	14	79
	7 days	3	3	45	1	5	2	2	1	10	60
	10 days	3	1	15	0	0	2	2	2	12	27
	14 days	3	1	15	0	0	2	2	1	10	25
	21 days	1	1	5	0	0	0	2	0	4	9
	28 days	1	1	5	0	0	0	0	0	0	5
55352 Female 2.834 kg	1 hr	0	0	0	1	5	2	2	2	12	17
	24 hrs	1	3	15	1	5	2	2	2	12	32
	48 hrs	2	2	20	1	5	2	1	2	10	35
	72 hrs	3	2	30	0	0	2	1	0	6	36
	7 days	1	1	5	0	0	1	1	0	4	9
	10 days	0	0	0	0	0	0	0	0	0	0

Table 57. Summary of examination findings for the no rinse group.

Interval	Animal # Comments – Fluorescein Exam Findings (Secondary Ocular Findings)					
	55160	*55345*	*55346*	*55339*	*55351*	*55352*
1 hr	(SCE)	(SDL)	(SDL)		(SDL)	(SDL)
24 hrs	FAO	FAO	FAO	FAO	FAO	FAO
48 hrs	FAO	FAO	FAO	FAO	FAO	FAO
72 hrs	FAO	FAO	FAO	FAO	FAO	FAO
7 days	FAO (VAS-1)	FAO (VAS-1)	FAO	FAO (VAS-1)	FAO (VAS-1)	(VAS-1)
10 days	FAO (VAS-1, RAC)	FAO (VAS-1)	FAO	FAO (VAS-1)	FAO (VAS-1)	
14 days	FAO (VAS-2)	(VAS-1)	FAO		FAO (VAS-1)	
21 days	FAO (VAS-1)		FAO		FAO	
28 days			FAO			
30 days	FAO (VAS-1)					

Table 58. Mean ocular scores for no rinse group.

Interval	Mean Ocular Score
1 hr	25.50
24 hrs	54.17
48 hrs	58.67
72 hrs	69.67
7 days	42.83
10 days	28.83
14 days	18.50
21 days	7.40
28 days	2.50

Table 59. Individual ocular irritation scores for the rinsed group.

Animal # Sex / Wt.	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
55353 Female 2.951 kg	1 hr	1	4	20	1	5	2	2	2	12	37
	24 hrs	2	4	40	1	5	3	2	2	14	59
	48 hrs	2	4	40	1	5	3	2	2	14	59
	72 hrs	3	4	60	1	5	3	2	1	12	77
	7 days	0	0	0	1	5	1	1	0	4	9
	10 days	0	0	0	0	0	0	1	0	2	2
	14 days	0	0	0	0	0	0	1	0	2	2
	21 days	0	0	0	0	0	0	0	0	0	0

Table 59. Continued.

Animal # Sex / Wt.	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
55358 Female 2.789 kg	1 hr	0	0	0	1	5	2	3	3	16	21
	24 hrs	2	4	40	1	5	2	2	2	12	57
	48 hrs	2	4	40	1	5	3	2	1	12	57
	72 hrs	3	4	60	1	5	3	2	1	12	77
	7 days	3	2	30	1	5	2	2	1	10	45
	10 days	3	2	30	0	0	2	2	1	10	40
	14 days	3	2	30	0	0	2	2	1	10	40
	21 days	2	1	10	0	0	1	2	0	6	16
	28 days	1	1	5	0	0	0	1	0	2	7
55359 Female 2.870 kg	1 hr	0	0	0	1	5	2	2	3	14	19
	24 hrs	2	3	30	1	5	2	2	2	12	47
	48 hrs	3	3	45	1	5	3	2	2	14	64
	72 hrs	3	3	45	0	0	2	2	1	10	55
	7 days	0	0	0	0	0	1	1	0	4	4
	10 days	0	0	0	0	0	1	1	0	4	4
	14 days	0	0	0	0	0	2	0	0	2	2
	21 days	0	0	0	0	0	0	0	0	0	0

Table 60. Summary of examination findings for the rinsed group.

Interval	Animal # & Comments – Fluorescein Exam Findings (Secondary Ocular Findings)		
	55353	*55358*	*55359*
1 hr	(SCE)	(SDL)	(SDL)
24 hrs	FAO	FAO	FAO
48 hrs	FAO	FAO	FAO (OD-Y)
72 hrs	FAO	FAO	FAO
7 days		FAO (VAS-1)	
10 days		FAO (VAS-2)	
14 days		FAO (VAS-2)	
21 days		FAO (VAS-1)	
28 days		(VAS-1)	

Table 61. Mean ocular scores for rinse group.

Interval	Mean Ocular Score	Interval	Mean Ocular Score
1 hr	25.67	10 days	15.33
24 hrs	54.33	14 days	14.67
48 hrs	60.00	21 days	5.33
72 hrs	69.67	28 days	2.33
7 days	19.33		

OVERALL SUMMARY OF PRE-CLINICAL TOXICITY EXPERIMENTATION:

Several pre-clinical toxicity assessments were performed in compliance with cGLPs on the product comprised of 12.5% citronellyl acetate in the add-mix system. Interpretation of the *in vitro* assessment of possible dermal penetration predicts almost no chance of systemic exposure to citronellyl acetate following topical treatment to human skin. The product possesses very low oral toxicity profiles with an LD_{50} of greater than 5000 mg/kg to mice, and an LD_{50} greater than 2000 mg/kg but less than 5000 mg/kg in rats. The product also possesses a very low dermal toxicity profile with an LD_{50} greater than 2000 mg/kg in rabbits. The product was found to be severely irritating to rabbit skin when exposed for 4 hours, and also to be severely irritating to rabbit eyes with a 30 second exposure and then rinsing.

Based on the results of the toxicity testing, the main issues of concern for future human testing is the potential of skin and eye irritation. However, the potential of skin irritation is greatly reduced since the product will be used with only a 10-minute exposure period as opposed to the 4-hour exposure for the pre-clinical toxicity assessment. Great care should be taken to rinse the product completely from the hair after the 10-minute exposure period has elapsed. Precautions should be taken in future human studies to prevent eye contact with the product, and it may be advisable to allow the children to cover their eyes with a towel during treatment and rinsing. Overall, the product's safety profile was excellent, and it poses little risk of serious adverse effects upon initial human exposure.

ULTIMATE VEHICLE OPTIMIZATION FOR DELIVERY OF CITRONELLYL ACETATE IN TREATMENT OF HUMAN HEAD LICE INFESTATIONS

BACKGROUND:

Previous experiments have shown the feasibility of treating human head lice with citronellyl acetate incorporated into a suitable pharmaceutical delivery system. Based on the successful completion of *in vitro* safety and efficacy assessments, human clinical trial protocols were prepared, and the data was compiled into an Investigational New Drug

(IND) Application filed with the FDA. The IND was accepted, a designation number assigned (IND 54-249), and permission was granted to proceed with human clinical assessments. The studies were performed by International Dermatology Research, Inc. (IDR) and were sponsored by Effcon Laboratories, Inc.

The formulation previously identified to possess preferred *in vitro* efficacy and reasonable safety profiles was selected for use in the human studies. The clinical supplies were identical to those formulations previously studied (add-mix system) and were produced with the same method of manufacturing. The process was scaled to 400 L, and each batch was produced and analyzed in compliance with cGMPs. Supplies consisted of the add-mix system packaged with a nit comb. Table 62 summarizes the formulations.

In total, three human clinical studies were performed with the objectives of assessing safety and identifying the minimum citronellyl acetate concentration required for a 95% or greater clinical cure rate. The studies included a Phase I design to initially assess the efficacy and safety of the product in a limited number of patients (XP001), an "*in vivo*" ovicidal activity assessment (mimicking the *in vitro* assessment) using eggs collected from human subjects (XP003), and a Phase II, dose response design (XP002). All aspects of the studies were performed in compliance with current Good Clinical Practices (cGCPs), cGMPs, and cGLPs, as applicable.

An open label, uncontrolled pilot clinical study (XP001) was performed to initially evaluate the safety and efficacy of the formulation for eradication of human head lice when applied by health care professionals. Patients were diagnosed with a head lice infestation, and the first 20 qualified patients were treated on Day 1 with the 7.5% citronellyl acetate formulation. After wetting the hair, a towel was held over the patient's eyes, and the test product was applied. After the product was allowed to sit on the hair for 10 minutes, it was thoroughly rinsed out. Each patient then underwent a detailed grooming process to remove all eggs using a nit removal comb. Patients returned on Day 8 for clinical assessment and received a second treatment of the 7.5% product under the same

Table 62. Summary of human clinical supplies.

Ingredient	Composition After Mixing % (w/w)
Citronellyl Acetate	0, 2.5, 5.0, 7.5, 10.0 or 12.5
IPA	20.0
Steol CS-230	55.0
Methylparaben	0.2
Propylparaben	0.1
Glacial Acetic Acid	5.0
Purified Water	diluent

procedures (eggs were not collected). A final clinical assessment was completed 14 days later (about Day 22). The procedures above were repeated for the second set of 20 qualified patients treated with the 12.5% strength product.

For pediculicidal assessments, lice were collected before and after treatment by straining the rinse water. The lice were placed in a petri dish, examined for viability, and incubated for 24 +/- 2 hours under appropriate conditions. For ovicidal assessments, ten viable eggs were collected before and after treatment by removing (cutting) the hair with the attached egg. The hairs (with attached egg) were placed in a petri dish and incubated for 14 days at appropriate conditions.

The primary efficacy outcome criterion used was clinical outcome evaluated 14 days after the final application. Outcome was defined as "cured", "substantial improvement", "re-infestation", or "failure" based on the number of lice and eggs found in the hair. Secondary efficacy criteria included viability assessment of lice and eggs collected before and after treatment. Safety evaluation was based on the type and incidence of adverse events reported by the patients or observed by the investigator and staff. Any itching and erythema of the scalp were recorded before and after treatment. Tables 63-65 display the statistical summaries of pediculicidal activity, ovicidal activity, and clinical outcome for the study.

Itching was worse on Day 8 than Day 1 for one subject. Erythema was absent on Day 1 and was mild on Day 8, 15, or 22 for five patients treated with the 7.5% product. For one patient treated with 12.5% strength, mild erythema was observed on Day 1 before and after treatment, and moderate erythema was seen on Day 8 before and after treatment. All instances of itching and erythema appeared to be related to the underlying lice infestations. One patient experienced mild general malaise on Day 1 that lasted for 1 day and was not considered to be related to the study medication. One patient experienced a moderate headache on Day 8 for 4 hours, but this also was not considered to be related to the study medication.

Table 63. Statistical summary of pediculicidal activity for XP001.

Treatment	Total # Lice	# Dead	# Moribund	# Alive	% Activity
Visit 1 - 7.5%	450	450	0	0	100%
Visit 2 - 7.5%	203	203	0	0	100%
Visit 1 - 12.5%	466	466	0	0	100%
Visit 2 - 12.5%	369	369	0	0	100%

Table 64. Statistical summary of ovicidal activity for XP001.

Treatment	Total # Eggs	# Hatched	# Unhatched	% Activity
7.5% Pre-trmt	200	17	183	8.5%
7.5% Post-trmt	200	33	167	16.5%
12.5% Pre-trmt	200	107	93	53.5%
12.5% Pre-trmt	200	128	72	64.0%

Table 65. Statistical summary of clinical outcome for XP001.

Outcome	Test Product (% citronellyl acetate) # of Subjects (%)	
	7.5%	12.5%
Cured	7 (17.5%)	13 (32.5%)
Subst. Improvement	12 (30.0%)	7 (17.5%)
Total Cured / Subst. Improved	19 (95%)	20 (100%)
Failure	1 (2.5%)	0 (0%)
Total Patients	20	20

In conclusion, both the 7.5% and 12.5% citronellyl acetate formulations effectively reduced or eliminated head lice infestations. However, neither of the strengths resulted in a 95% or greater cure rate. Both concentrations were 100% pediculicidal, but neither demonstrated significant ovicidal activity under the conditions of the study. The product was well tolerated with few adverse events resulting from treatment.

Due to the surprising lack of *in vivo* ovicidal activity, the ASTM *in vitro* ovicidal assessment method was duplicated using human head lice ova collected from human subjects to evaluate the discrepancies between the *in vitro* and *in vivo* results (XP003). All aspects of the study were designed identically to the previous testing with the ASTM method in attempt to discern any difference in activity between eggs collected from humans versus those from the laboratory lice. For the comparison purpose of the study, the eggs were immersed in the test product for the entire 10-minute exposure period.

Eggs were collected from patients diagnosed with head lice infestations. Viable nits (20-70) were obtained from each patient by cutting the hair with the nit attached. The hairs were placed in a petri dish and examined under a microscope for viability. Any nits that appeared "not viable" were discarded. Blocks of 10 nits were allocated to replicates such that each petri dish contained 30 nits (10 nits from 3 different subjects) with no patient contributing more than 10 nits to any single replicate. Tap water and the vehicle with no active were used as controls, and five other concentrations of citronellyl acetate were tested. The five test products incorporated final citronellyl acetate concentrations of 2.5%, 7.5%, 12.5%, 7.5% diluted 50/50 with tap water, and 12.5% diluted 50/50 with tap water. Table 66 summarizes the resulting ovicidal activities of the formulations.

Products containing 2.5% or greater citronellyl acetate possessed significantly more ovicidal qualities than the control and placebo when using human head lice ova. Also, the difference in ovicidal activity between human head lice ova versus laboratory lice ova was not significant under the conditions used for the test. The study documents the lack of

Table 66. Summary of ovicidal activities for XP003.

Product (% citronellyl acetate concentration) / % Ovicidal Activity						
Control	Placebo	2.5%	7.5%	7.5% dil.	12.5%	12.5% dil.
2.0%	11.6%	97.3%	100%	99.9%	100%	99.4%

ovicidal activity present in the initial human clinical trial (XP001) was not due to a difference in ova treated for the *in vitro* and *in vivo* methods.

The sponsor decided to continue with the next clinical assessment with a few changes to the protocol. The test product was applied to dry hair, and combing was limited to a maximum of 20 minutes. Patients did not return until Day 15 for clinical assessment and possible re-treatment. Clinical assessment was completed 14 days after the last application of test product. For pediculicidal analysis, an additional viability assessment was made at 1-hour after incubation. Also, the study was conducted in two stages. In Stage 1, placebo and the 2.5%, 5.0%, and 7.5% strengths were tested. Treatment for Stage 2 was based on the results of Stage 1. If the products used in the first stage yielded 95% or greater cure rates, additional patients were treated with the same concentrations. If not, patients were treated with 10.0% and 12.5% strengths.

The study (XP002) was designed as a double blind, placebo controlled, dose escalation study. It was performed to evaluate the efficacy and safety of the formulation incorporating increasing strengths of citronellyl acetate in the eradication of human head lice when treated by health care professionals. The objectives of the study were to determine the minimum concentration of citronellyl acetate required to provide a cure rate of 95% or greater and was 100% pediculicidal and ovicidal after a single treatment.

After the completion of Stage 1 produced no products with 95% or greater cure rates, the 10.0% and 12.5% were selected for testing in Stage 2 using the same procedure as Stage 1. The protocol planned for enrollment of up to 150 patients, but evaluation of 80-120 patients was expected to be sufficient to meet the study objectives. All 40 patients enrolled in Stage 1 completed, and all 30 patients enrolled in Stage 2 were used to assess safety while 29 for efficacy (1 subject was eliminated due to a protocol violation). The study was discontinued after the completion of the first 30 subjects in Stage 2 due to a lack of 95% or greater cure rates.

As with XP001, the primary efficacy outcome criterion was clinical outcome evaluated 14 days after the final application. Outcome was classified as "cured", "re-infestation", or "failure" based on the presence of lice and eggs found in the hair. Secondary efficacy criteria included severity of infestation and viability assessment of lice and nits collected before and after treatment. Safety evaluation was performed as in XP001. Tables 67 and 68 summarize the resulting data.

No strength proved to possess significant ovicidal activity. The percent of eggs that failed to hatch during the 14-day incubation period ranged from 1-11% for eggs collected before treatment and 10-32% after treatment. There were no substantial differences among treatment groups.

No deaths, serious adverse events, or discontinuations due to adverse events occurred during the study. One or more adverse events, all of which were limited to the scalp, were reported for 29 patients. Application site reaction was reported for 25 patients, and pruritus was reported for 4 patients. All adverse events were of mild to moderate severity and resolved without intervention by the investigator. These adverse events were classified as "probably related" to the study medication.

In conclusion, all treatments resulted in cure rates better than 50% and effectively reduced or eliminated head lice infestations. While more irritation was observed in the study, the product was still well tolerated. A slight dose response was demonstrated in the study, but a citronellyl acetate concentration resulting in 95% or greater clinical cure or 100% pediculicidal and ovicidal activity was not identified under the conditions of the study.

The failure of the initial human clinical studies to identify a product that produced either 95% or greater cure rates or 100% pediculicidal and ovicidal activity presented a major obstacle to proceeding to a Phase III human clinical study and gaining ultimate regulatory approval. Compounding the issue were the other undesirable attributes of the product. The add-mix product would lend itself to poor patient compliance and greater

Table 67. Statistical summary of clinical outcome for XP002.

Outcome	Product (% citronellyl acetate concentration) # Patients (%)					
	Placebo	2.5%	5.0%	7.5%	10.0%	12.5%
Cured	13 (65%)	6 (60%)	5 (56%)	7 (70%)	7 (70%)	8 (80%)
Failure	7 (35%)	4 (40%)	4 (44%)	3 (30%)	3 (30%)	2 (20%)
Re-infested	0	0	0	0	0	0
Total Patients	20	10	10	10	10	10

Table 68. Summary of pediculicidal activity assessments for XP002.

Assessment Time	Strength / % Activity						
	Stage 1				Stage 2		
	Placebo	2.5%	5.0%	7.5%	Placebo	10.0%	12.5%
1 hr	55.4%	74.4%	78.7%	86.1%	66.0%	96.5%	94.6%
24 hrs	97.4%	99.1%	99.6%	100%	100%	100%	99.6%

risk of misuse. The toxicity tests uncovered the formulation was a severe skin and eye irritant, side effects that would eventually surface with wide scale human use. This, coupled with the fact that the formulation contained 5% acetic acid and possessed water-like viscosity, could lead to an unacceptable safety risk to children. Therefore, a strategic decision was made to initiate further vehicle optimization in attempt to maximize efficacy while minimizing the risk of potential side effects to humans.

The human clinical studies demonstrated the lack of acceptable correlation between the *in vitro* and *in vivo* ovicidal activity assessment methods. For more realistic assessment of potential *in vivo* activity for the new formulations, modified *in vitro* ovicidal test methods were developed and implemented for all future assessments in attempt to improve *in vitro/in vivo* correlation and the ability to predict future *in vivo* performance.

Research was initiated in attempt to develop a product that would be less irritating, could be packaged in a single container, and would possess 100% pediculicidal and ovicidal qualities when applied to human heads. First attempts to overcome some of these issues were previously discussed and documented with the preparation of "anhydrous" and o/w emulsion formulations (also documented in US Patent No. 5,902,595). These initial formulations were developed as creme rinses incorporating conditioners in lieu of the surfactant, sodium laureth sulfate. While the emulsion approach greatly increased stability, *in vitro* testing uncovered that while some were pediculicidal, none were ovicidal. Due to the lack of *in vitro* ovicidal efficacy, additional emulsified formulations were developed in attempt to "re-incorporate" the sodium laureth sulfate and acetic acid into a single component.

In addition, efforts were focused on increasing viscosity. Hypothetically, increasing the viscosity of the formulations would increase stability and efficacy. Increasing the viscosity of the formulation would impede molecular motion and slow down particle collisions possibly affecting the degradation kinetics and slowing the decomposition of the active ingredient. More importantly, increasing viscosity would increase the contact time

and decrease evaporation of the active ingredient while on the pest. This would result in the pest being exposed to more active ingredient for longer durations of time, potentially increasing efficacy.

A vast amount of literature was collected from excipient vendors on the current and common techniques and excipients used for the preparation of thickened pharmaceutical o/w emulsions. Typical thickeners such as Carbopol™ (carbomer), PVP (polyvinylpyrrolidone), Pemulen® (Acrylates/C10-30 Alkyl Acrylate Crosspoly-mer), and Klucel HF® (hyroxypropyl cellulose, HPC) were identified for use in pharmaceutical and cosmetic emulsions, creams, ointments, and gels.

Additionally, a patent search uncovered a proprietary gel delivery vehicle claimed to increase penetration of drugs while minimizing irritation.[139] The patented vehicle incorporated relatively high concentrations of alcohol (50% or greater) in a propylene carbonate base thickened with HPC. Also, the patent documented the inclusion of butylated hydroxytoluene (BHT) as an antioxidant used to minimize oxidative drug decomposition.

Some of the techniques and excipients were incorporated in attempt to optimize the overall drug product formulation with citronellyl acetate. The techniques and excipients uncovered by the research were implemented and utilized to prepare numerous new vehicles for delivery of citronellyl acetate. Also, many variations of the techniques and different combinations of excipients were attempted and evaluated.

NEW METHOD FOR *IN VITRO* OVICIDAL ACTIVITY ASSESSMENT TO LICE OVA - MATERIALS:

Materials for the ovicidal assessments were identical to those previously described for the method modified to incorporate the human hair tresses. The only additional supplies used were human hair wigs and plastic wig stands resistant to the formulation ingredients (not Styrofoam), and a stereomicroscope was used for examination. The human hair wigs were obtained from a private wig maker in Cambridge, England.

NEW METHOD FOR *IN VITRO* OVICIDAL ACTIVITY ASSESSMENT TO LICE OVA - METHODS:

The newly modified method was nearly identical to the previous modified method discussed. All tests were performed at Medical Entomology Centre in Cambridge, England. Louse eggs were obtained by providing actively laying adults with untreated human hair of European origin bound into tresses of approximately 200-500 hairs. The tress served as an egg laying substrate on which the lice laid eggs over a 48-hour period. The lice were removed from the tresses, and some tresses were divided into smaller ones bearing a suitable number of eggs. The tresses were allocated on a random basis to marked 90 mm plastic Petri dishes.

A wig made from human hair of European origin was obtained and fitted to a malleable block stand. The hair tresses bearing louse eggs were tagged with colored, plastic coated, paperclips, and woven into the wig in different sections. The formulation under investigation was mixed thoroughly and applied to its respective section of the wig just as if it was being applied to hair on a human head. The product was massaged into the wig in the immediate vicinity with a "shampooing motion." After application, the product was allowed to remain on the wig for the remainder of the identified exposure time after which the wig was rinsed thoroughly using 250 mL aliquots of warm (34°C) tap water. The aliquots of water were poured through the hair as many times as was required to remove the detectable physical traces of each treatment.

After rinsing, the tresses of hair were removed and blotted dry using medical wipe tissues. They were returned to their marked Petri dishes and incubated until all the nymphs in the control group had completed hatching (approximately 12 days). The portion of hairs bound by the tape in each tress was removed prior to assessment since the tape may have interfered with exposure to the test preparations.

In addition to the new application technique, two additional evaluation techniques were also used. These new techniques were added in an effort to determine the level of

each product's penetration through the various protective structures of the ova. First, the number of half-hatched embryos was tabulated and recorded. Second, the eggs that possessed no eyespot formation were added, and this total was divided by the total number of eggs treated in the replicate and multiplied by 100 to calculate the "percent undeveloped."

For assessment of the validity of the new method, *in vitro* assessments were performed using the new method and the same target drug product previously identified and assessed with the other *in vitro* and *in vivo* methods. Specifically, the test articles included the add-mix formulation with 10.0%, and 12.5% citronellyl acetate, and these products were prepared identically to previous samples.

NEW METHOD FOR *IN VITRO* OVICIDAL ACTIVITY ASSESSMENT TO LICE OVA – RESULTS AND CONCLUSIONS:

The modified application method of the new test was considered more typical of the normal treatment regime than the immersion dip test. The new method should eliminate sources of error inherent with the previous, vastly different application techniques. Intuitively, this application method correlates much better with the *in vivo* application than the submersion techniques previously used.

Theoretically, the additional evaluations would provide improved prediction of *in vivo* success. Documentation of level of hatching is basically a "pass/fail" assessment of penetration. For example, if the product penetrates through the porous operculum but not through the membrane protecting the embryo, the product will be trapped just outside of the membrane (common with Rid™ and Nix™). In this case, the embryo will continue to develop and begin to hatch. Once the nymph has broken the membrane, it is exposed to the active trapped around it. If high enough concentrations of the active are present, the nymph will be killed while hatching. This scenario was documented as a "half-hatched" egg. Assessment and location of eyespot formation provides a more detailed evaluation into penetration characteristics and possible mode of action. Development and location of

eyespots signify a developing nervous system and the extent to which it has developed. At the time of treatment (within 2 days after being laid), the embryos inside the egg do not possess a developed nervous system or eyespots. Therefore, a lack of eyespot formation after treatment indicates the product's ability to penetrate inside the egg and kill the embryo at the stage of treatment. Also, a lack of eyespot formation indicates the product does not act exclusively on the CNS since a CNS has not even developed. However, eyespot formation after treatment indicates the product either acts via the CNS or did not penetrate into the egg. The location of the eyespot can even predict the level of penetration or action.

The new ovicidal activity assessment method was used to assess the add-mix system with 10.0 and 12.5% citronellyl acetate. By using these strengths and a 10-minute exposure period, the data could be compared directly with both the previous *in vitro* and *in vivo* results. Table 69 displays the test results.

Implementation of the new method resulted in the 10.0% and 12.5% formulations possessing 96.2% and 98.1% ovicidal activity, respectively. While these overall results did not correlate well with the 10-32% experienced *in vivo*, assessment of undeveloped eggs indicated that only 53.4% and 72.0%, respectively, of the eggs remained undeveloped. Obviously, the new method shows that while the overall effect may be positive (ie: almost 100% ovicidal activity) the underlying ability of the product to penetrate may not be in proportion or correlate. The "% Undeveloped" results can begin to explain the lack of ovicidal activity in less controlled *in vivo* studies, as this study was carried out in a controlled laboratory setting and still resulted in some embryo development. Even though the study did not prove to correlate directly with *in vivo* results, it did provide much more insight into the level of the product's penetration into the egg, which can aid in prediction of *in vivo* activity. Since the new method and evaluation techniques proved to provide much more useful data, they were used as the preferred tools for ovicidal assessments of new formulations.

Table 69. Results of *in vitro* ovicidal testing with new method.

Treatment	Number of eggs			Undev'd	% Mortality	% Undev'd
	Total	Hatched	Half-hatched			
10.0%	494	19	11	264	96.2	53.4
12.5%	207	4	2	149	98.1	72.0
Control	164	142	4	13	13.4	7.9

FINAL VEHICLE OPTIMIZATION – MATERIALS:

Various different formulations were prepared for assessment. Table 70 outlines all materials used for the preparation of supplies. All formulations were prepared using an electric stirrer equipped with a 3-blade impellor.

FINAL VEHICLE OPTIMIZATION – METHODS:

Numerous additional formulations were prepared including o/w emulsions and clear gels. A thickened version of the Active Ingredient Component in the existing add-mix formula was also prepared. The methods used to prepare the new formulations are outlined in Tables 71-76. These methods may have or may not have incorporated a thickening step. If incorporated, two different techniques were used to thicken the formulations. The first thickening technique was performed merely through the simple addition of the appropriate concentrations of HPC or Pemulen® (higher concentrations than typically used). The second technique incorporated a neutralization step of a cationic polymer such as Pemulen® (at typical concentrations) with a base. In all cases, the thickening step was performed last. Additional clear gel formulations were made with the identical method shown in Table 76 with substitution of various excipients, substitution of Pemulen® for Klucel® HF, and the addition of a neutralization step as a final step.

FINAL VEHICLE OPTIMIZATION – RESULTS AND DISCUSSION:

In total, over 60 different formulations were prepared in attempt to finalize the optimization of delivery vehicle for citronellyl acetate. Initially numerous o/w emulsion formulations were prepared in attempt to "protect" the citronellyl acetate from acid degradation since the citronellyl acetate would reside in the oil phase while the acid in the water phase. The formulations were prepared using one of the methods previously described. Table 77 summarizes the ranges of excipient levels of the initial formulations developed.

The formulations were assessed for physical and chemical stability, and some were tested for *in vitro* efficacy. While many of the formulations possessed dramatically

Table 70. Materials for vehicle optimization.

Ingredient	Supplier	Grade / Trade name
Citronellyl Acetate	Delmar Chemicals	Pharmaceutical
IPA	Aaper Alcohol	USP
Acetic Acid	JT Baker	USP
Purified water	In-house	USP
Pemulen® TR-1	BF Goodrich	USP
Mineral oil	Light, Penta	USP
Methylparaben	Ashland Chemical	NF
Propylparaben	Ashland Chemical	NF
Ethanol (95%)	Aaper Alcohol	USP
BHT	Spectrum Chemicals	Food
Propylene Glycol	VW&R	USP
Glycerin	VW&R	USP
Klucel HF® (HPC)	Aqualon	NF
Sodium Hydroxide	JT Baker	USP/NF
Propylene Carbonate	Arconate® HP, Lyondell Chemical Co.	HP
PVP	Luviskol® K, BASF	Cosmetic
Triethanolamine (TEA)	Fisher	Reagent Grade
Sodium laureth sulfate (25%)	Steol CS-230	USP

Table 71. Preparation of thickened existing Active Ingredient Component formula without neutralizer.

Step #	Description
1	Add alcohol to an adequately sized mixing vessel (#1) and begin mixing
2	While mixing, add methylparaben and propylparaben and mix until completely dissolved
3	While mixing, add citronellyl acetate, then Steol CS-230, then purified water mixing for 2 minutes after each addition
4	Heat the solution from step #3 to 40-45°C
5	Add Pemulen to the hot solution from step #4 and mix well while maintaining the temperature at 40-45°C until completely hydrated
6	Cool to room temperature (about 25°C)

Table 72. First preparation of o/w emulsion formulations without neutralizer.

Step #	Description
1	Add citronellyl acetate and mineral oil to an adequately sized mixing vessel (#1), begin mixing, and heat to 60-65°C
2	While mixing and maintaining the temperature at 60-65°C, add Pemulen and mix until well dispersed
3	In a separate, adequately sized vessel (#2), mix water and alcohol and heat to 50-55°C
4	While mixing and maintaining the temperature at 50-55°C, disperse the PVP in the solution from step #3 (in vessel #2) and continue mixing until the PVP is well hydrated
5	Add glacial acetic acid to the hot solution from step #4 (vessel #2) and mix well while maintaining the temperature at 50-55°C
6	While mixing and maintaining the temperature at 50-55°C, add the solution in vessel #1 to that in vessel #2 and mix until completely uniform
7	Cool to room temperature (about 25°C)

Table 73. Second preparation of o/w emulsion formulations without neutralizer.

Step #	Description
1	Add water, alcohol, and acetic acid to an adequately sized mixing vessel (#1), begin mixing, and heat to 40-50°C
2	While mixing and maintaining the temperature at 60-65°C, add methylparaben and propylparaben and mix until completely dissolved
3	While mixing and maintaining the temperature at 40-50°C, disperse the PVP in the solution from step #2 (in vessel #1) and continue mixing until the PVP is well hydrated
4	Add citronellyl acetate and mineral oil to a separate, adequately sized mixing vessel (#2), begin mixing, and heat to 60-65°C
5	Add Pemulen to the hot solution from step #4 (vessel #2) and mix well while maintaining the temperature at 40-50°C until completely hydrated
6	While mixing and maintaining the temperature at 50-55°C, add the solution in vessel #1 to that in vessel #2 and mix until completely uniform
7	Cool to room temperature (about 25°C)

Table 74. Third preparation of o/w emulsion formulations without neutralizer.

Step #	Description
1	Add water, alcohol, and acetic acid to an adequately sized mixing vessel (#1) and mix for 2 minutes
2	While mixing, add methylparaben and propylparaben, begin heating the solution to 40-50°C, and mix until completely dissolved
3	While mixing and maintaining the temperature at 40-50°C, disperse the PVP in the solution from step #2 and continue mixing until the PVP is well hydrated
4	In a separate, adequately sized mixing vessel (#2), add citronellyl acetate and mineral oil, begin mixing, and heat to 35-45°C
5	While mixing and maintaining temperature at 40-50°C, add the contents of vessel #2 to that in vessel #1
6	Add Pemulen to the hot solution from step #5 and mix well while maintaining the temperature at 40-50°C until completely hydrated
7	Cool to room temperature (about 25°C)

Table 75. Preparation of o/w emulsion formulations with neutralizer.

Step #	Description
1	Add citronellyl acetate and mineral oil to an adequately sized mixing vessel (#1), begin mixing, and heat to 60-65°C
2	While mixing and maintaining the temperature at 60-65°C, add Pemulen and mix until well dispersed
3	In a separate, adequately sized vessel (#2), mix water and alcohol, and heat to 50-55°C
4	While mixing and maintaining the temperature at 50-55°C, disperse the PVP in the solution from step #3 (in vessel #2), and continue mixing until the PVP is well hydrated
5	Add neutralizer (TEA or NaOH) to the hot solution from step #4 (vessel #2) and mix well
6	While mixing and maintaining the temperature at 50-55°C, add the solution in vessel #1 to that in vessel #2, and mix until completely uniform
7	Cool to room temperature (about 25°C)

Table 76. Preparation of clear gel formula as described in US Patent No. 5,902,595 with citronellyl acetate.

Step #	Description
1	Add alcohol to an adequately sized mixing vessel (#1) and begin mixing
2	While mixing, add BHT and mix until completely dissolved
3	While mixing, add propylene carbonate, then propylene glycol, and then glycerin mixing for 2 minutes after each addition
4	While mixing, slowly add Klucel (or other type of thickener) and mix until completely hydrated and a uniform gel is achieved

Table 77. Summary of excipient ranges for initial o/w emulsions formulations.

Ingredient	% (w/w)
Citronellyl acetate	8.0, 10.0, 12.5
Methylparaben	0.0, 0.2
Propylparaben	0.0, 0.1
PVP	0.0, 1.0
Pemulen®	0.25-2.5
Glacial Acetic Acid	0.0%, 5.0%
IPA	20.00
Sodium Laureth Sulfate (25%)	0.0, 50.0
TEA	0.0, 0.4
Purified Water	diluent

improved stability profiles, none exhibited even the slightest trace of *in vitro* ovicidal activity. Because the numerous o/w emulsion formulation attempts proved futile, focus was shifted to the development of a thickened solution formulation that could be packaged into a single container. These types of products are usually clear gel formulations in which the excipients and drug are dissolved in an aqueous or alcoholic base and then thickened with a suitable natural or synthetic polymer. Initial formulations were prepared using the proprietary vehicle and method described in US Patent No. 5,902,595. Five formulations were prepared to assess the effects of the type of alcohol, assess the necessity of propylene carbonate, and assess the effects of thickening the formulation on *in vitro* pediculicidal activity (5 sec. dip method). The formulations were prepared as previously described in the methods section, and Table 78 summarizes them and their pediculicidal activities.

All formulations were clear gels, and those that were thickened possessed a viscosity of about 4000-6000 cps. Assessments revealed the presence or use of propylene carbonate did not affect efficacy, and it was eliminated from future formulations. However, a twofold increase in effectiveness was observed for incorporation of IPA versus ethanol in otherwise identical, non-thickened formulations. Also, at least a twofold increase in effectiveness was seen with thickening in otherwise thinner, identical formulations.

Based on the pediculicidal activity data, 2 of the formulations were used for *in vitro* ovicidal assessment incorporating the new "wig" method and a 10-minute exposure period. These 2 formulations were numbers 3 and 5 from Table 78. Table 79 summarizes the results. Both the thin IPA formulation and the thickened ethanol formulation were ovicidal and resulted in basically no development of the nymph inside the egg after exposure.

The *in vitro* data resulted in the selection of IPA as the alcohol and the use of a thickener in all subsequent formulations. The first additional formulations were hybrids of formulations 3 and 5 displayed in Table 78. Both new formulations incorporated IPA as the alcohol and Klucel® HF for thickening. A series of formulations were made consisting of the vehicle and various amounts of citronellyl acetate. Additional formulations were

Table 78. Thickened AIC formulation.

Ingredient (Note control = 3.2% activity)	Formulation # % (w/w)				
	1	2	3	4	5
Citronellyl acetate	8.0	8.0	8.0	8.0	8.0
Ethanol (95%)	51.95	50.95	0	51.95	50.95
IPA	0	0	51.95	0	0
Klucel® HF	0	1.0	0	0	1.0
BHT	0.05	0.05	0.05	0.05	0.05
Propylene carbonate	20.0	20.0	0	0	0
Propylene glycol	10.0	10.0	20.0	20.0	20.0
Glycerin	10.0	10.0	20.0	20.0	20.0
In vitro Ped. Act	7.9%	93.2%	80.3%	41.7%	88.9%

Table 79. Results of ovicidal assessment using the new "wig" method and 2 formulations.

Formulation #	% Ovicidal Act.	% Undeveloped
3	99.2	98.1
5	100	99.5

were prepared substituting Pemulen® TR-1 in lieu of Klucel® HF to assess the effect of type of thickener used. These formulations were tested for *in vitro* efficacy, and Table 80 summarizes the formulations and their *in vitro* pediculicidal activities.

Again all formulations resulted in clear gels, but the Klucel® HF formulations possessed viscosities of about 4000-6000 cps while the Pemulen® TR-1 formulations possessed viscosities of about 1500-3000 cps. Additionally, the Pemulen® TR-1 produced formulations with preferred aesthetic qualities such as less "tackiness" and improved "smoothness," and they less required much less time to prepare.

The data indicated that neither the type of thickener nor minor fluctuations in viscosity drastically affect the efficacy. The lowest strength tested that produced 100% pediculicidal activity incorporated 8% citronellyl acetate within the clear gel vehicle. While this previous vehicle development research revealed the potential success of a clear gel vehicle for the delivery of citronellyl acetate, all of the formulations incorporated a significant amount of alcohol. The potential irritating effects and flammable nature of a product incorporating of high concentrations of alcohol raised concerns. For example, an unsuspecting parent may use a hairdryer that produces a spark thus igniting the product on the child's head, or a smoking parent, unaware of the risk, could light a cigarette during the waiting time thus producing the same results. Therefore, additional vehicle optimization efforts were focused on the minimization of IPA in the vehicle.

A series of formulations were prepared with various concentrations of IPA and were tested for *in vitro* pediculicidal and ovicidal efficacy (wig method). All of the formulations incorporated 8.0% citronellyl acetate and were identical with the exception of alcohol concentration. Due to the minimization efforts, the role of diluent shifted from the alcohol to propylene glycol. Table 81 summarizes the formulations and results.

As evident from the study, activity was not related to the IPA concentration. Therefore, a series of formulations were prepared with and 10% IPA for an *in vitro* dose response study.

Table 80. Summary of Klucel® HF thickened gels with varying citronellyl acetate concentrations and their corresponding *in vitro* pediculicidal activities.

Ingredient	% (w/w)						
IPA	58.45	57.95	56.45	53.95	53.45	48.95	49.95
BHT	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Propylene glycol	20.00	20.00	20.00	20.00	19.70	20.00	20.00
Glycerin	20.00	20.00	20.00	20.00	19.80	20.00	20.00
Klucel HF® (HPC)	1.00	1.00	1.00	1.00	0.00	0.00	0.00
Pemulen® TR1	0.00	0.00	0.00	0.00	2.00	3.00	2.00
Citronellyl acetate	0.50	1.00	2.50	5.00	5.00	8.00	8.00
% Ped. Act.	67.2	42.9	93.1	79.7	74.6	100.0	100.0

Table 81. Clear gel formulations with various levels of IPA and their *in vitro* activities.

Ingredient	% (w/w)				
IPA	10.00	20.00	30.00	40.00	Control
BHT	0.05	0.05	0.05	0.05	-
Propylene glycol	59.95	49.95	39.95	29.95	-
Glycerin	20.00	20.00	20.00	20.00	-
Pemulen® TR1	2.00	2.00	2.00	2.00	-
Citronellyl acetate	8.00	8.00	8.00	8.00	-
% Ped. Act.	100.0	100.0	100.0	100.0	12.0
% Ovicidal Act.	100.0	100.0	100.0	99.5	10.2
% Undeveloped Eggs	99.5	99.8	98.0	98.0	5.3

IN VITRO PEDICULICIDAL AND OVICIDAL DOSE RESPONSE STUDY WITH THE CLEAR GEL VEHICLE – MATERIALS:

Materials for the study were identical to those previously for sample preparations and also those used for the modified *in vitro* methods.

IN VITRO PEDICULICIDAL AND OVICIDAL DOSE RESPONSE STUDY WITH THE CLEAR GEL VEHICLE – METHODS:

The dose response study samples were prepared as previously described in Table 76. *In vitro* assessments were tested using the 5-second immersion method for pediculicidal tests and the wig method for ovicidal tests. However, the number of replicates was increased for each test, and the tests were spread over a minimum number of days. For the pediculicidal assessments, 5 replicates of 20 lice each were tested over a minimum of 3 separate days. For ovicidal assessments, 3 replicates of a minimum of 50 eggs each were also tested over 3 separate days. An additional calculation was performed for the pediculicidal data. The percent of lice classified as "Killed" (ie: not classified as "Moribund" or "Alive") by each treatment was calculated.

Statistical analyses were performed with appropriate computer software. Calculations of means, ANOVA analyses, and generation of basic graphs were performed with Microsoft Excel 2000. Additional statistical software, student version of JMP IN version 3.2.1 from the SAS Institute, was used to statistically identify LD_{50}'s and LD_{100}'s , to perform linear regression modeling of data, and to check the fit of the linear model.

IN VITRO PEDICULICIDAL AND OVICIDAL DOSE RESPONSE STUDY WITH THE CLEAR GEL VEHICLE – RESULTS AND DISCUSSION:

The dose response study was designed by formulating the new clear gel vehicle with 10% IPA and various levels of citronellyl acetate between 0.0 to 10.0%. Ian Burgess at Medical Entomology Centre tested the formulations for *in vitro* pediculicidal activity using the modified methods previously described. In addition to the inclusion of a vehicle and water control, an "active control" was incorporated. This active control consisted of Nix™

that was repackaged into generic packaging identical to that of the other samples and was numbered with an unrelated lot number for blinding purposes. Table 82 outlines the clear gel formulations that were prepared for the dose response study and the resulting data from the assessments. Table 83 summaries the results for the water and active controls.

All formulations possessed viscosities between 1500-3000 cps. The formulations with 8.0% and 10.0% citronellyl acetate resulted in some haziness present in the gel. All other formulations were clear gels. Table 84 displays the results generated by the ANOVA analysis (with $\alpha = 0.025$) used to determine activity statistically different to that of the water control (p-values > α indicate no significant activity). Figures 21 and 22 show the fitted linear model graphs generated by JMP for pediculicidal and ovicidal activities, respectively. Tables 85 and 86 include summary of fit and results from each lack of fit (LOF) test performed to assess the applicability of the linear models. Figures 23 and 24 display the "Quantile Density Plots" generated from JMP. Figures 25-29 display JMP data generated from a "screening fit" performed to identify the LD_{50} and LD_{100} to lab lice and their eggs and the predicted concentration required for 100% "undeveloped eggs".

IN VITRO PEDICULICIDAL AND OVICIDAL DOSE RESPONSE STUDY WITH THE CLEAR GEL VEHICLE – SUMMARY:

A minimum of 5.0% citronellyl acetate was required to exhibit statistically significant pediculicidal activity (p-value = 1.5×10^{-6}), and a minimum of 2.5% was required to exhibit statistically significant ovicidal activity (p-value = 0.004057). The results of the study indicated that pediculicidal activity was linearly related to dose (LOF test p-value = 0.2151 vs. $\alpha = 0.025$), but ovicidal activity did not fit a linear model well (LOF test p-value = 0.0025 vs. $\alpha = 0.025$). The minimum concentration of citronellyl acetate predicted to elicit 100% pediculicidal activity was 9.0%, and the minimum concentration predicted to elicit 100% ovicidal activity was 8.3%. However, a minimum of 11.1% citronellyl acetate was predicted to yield 100% undeveloped eggs. As expected, Nix resulted in basically 0% undeveloped embryos since it is not able to cross the egg membrane and acts on the CNS,

Table 82. Summary of dose response study supplies and their corresponding activities.

Ingredient	% (w/w)						
IPA	10.00	10.00	10.00	10.00	10.00	10.00	10.00
BHT	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Propylene glycol	67.95	66.95	65.45	62.95	61.45	59.95	57.95
Glycerin	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Pemulen® TR1	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Citronellyl acetate	0.00	1.00	2.50	5.00	6.50	8.00	10.00
% Killed	14.9	5.1	28.7	25.0	81.2	97.9	99.0
% Corrected Ped. Act.	21.3	6.7	54.2	59.7	89.4	100	100
% Undev'd Eggs	16.4	21.3	12.0	98.3	74.2	85.9	65.5
Corrected % Ovicidal Act.	1.1	37.7	37.8	100	84.2	98.5	97.9

Table 83. Activities of active and water controls.

	Nix™	Water
% Killed	80.6	6.9
% Corrected Ped. Act.	100.0	0.0
% Undev'd Eggs	3.0	6.4
Corrected % Ovicidal Act.	100.0	0.0

Table 84. ANOVA analysis of pediculicidal and ovicidal activities ($\alpha = 0.025$).

Strength	Data Analyzed		
	% Ped. Act. p-value	% Ovicidal Act. p-value	% Undev'd Eggs p-value
Vehicle	0.324999	0.992901	0.228258
1.0%	0.759547	0.045519	0.038553
2.5%	0.03279	0.004057	0.259956
5.0%	1.5×10^{-6}	4.8×10^{-5}	1.2×10^{-5}
6.5%	4.2×10^{-6}	0.001045	0.001009
8.0%	4.2×10^{-6}	4.8×10^{-5}	0.000412
10.0%	2.1×10^{-10}	6.1×10^{-5}	0.000118

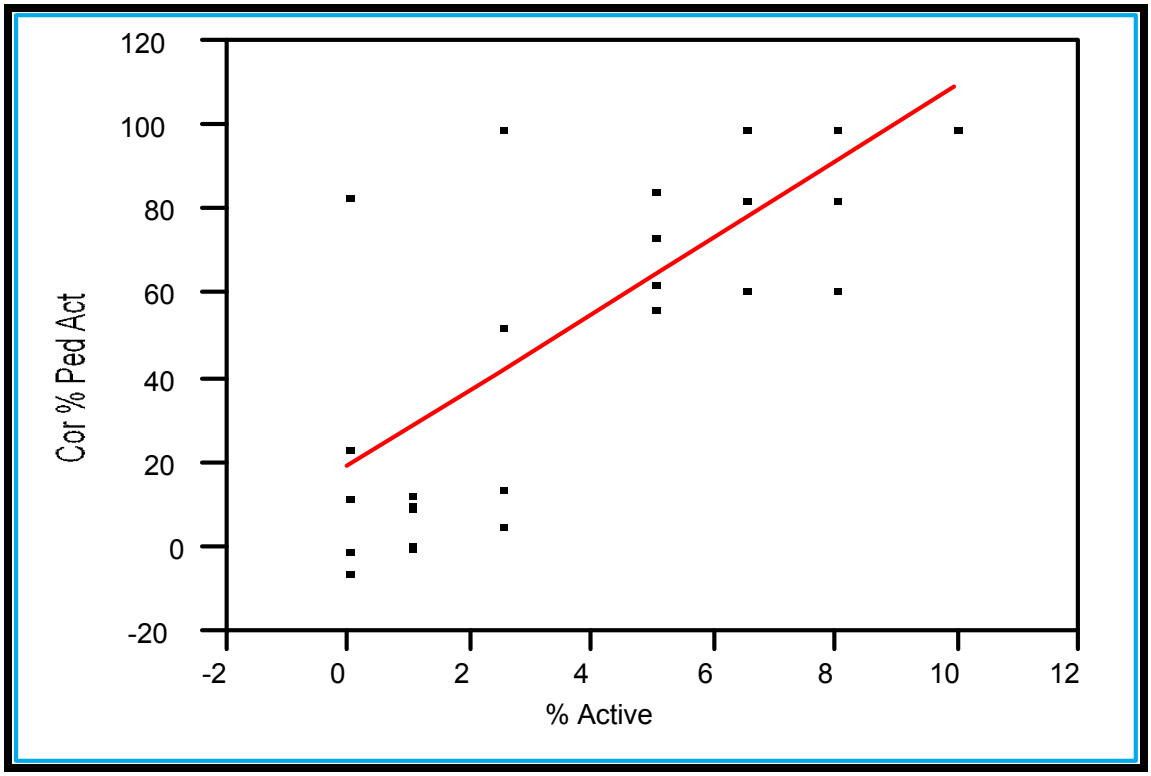


Figure 21. Linear fitted model for pediculicidal activity.

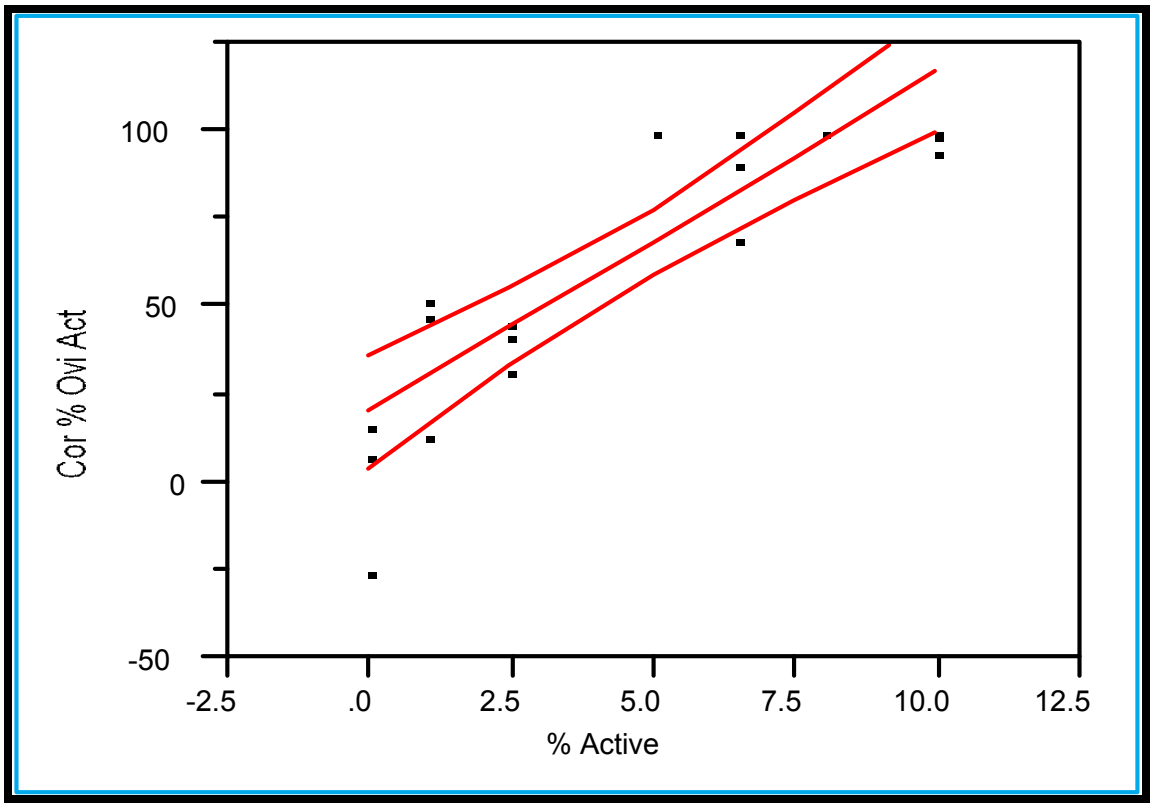


Figure 22. Linear fitted model for ovicidal activity.

Table 85. Linear model fitted, summary of fit, and results of LOF test for pediculicidal activity.

Description	Result
Linear model fitted	Cor % Ped Act = 19.296 + 8.9954 % Act
Summary of fit	
RSquare	0.618706
RSquare Adj	0.607151
Root Mean Square Error	25.03162
Mean of Response	61.70286
Observations (or Sum Wgts)	35
LOF test	p-value = Prob>F = 0.2151

Table 86. Linear model fitted, summary of fit, and results of LOF test for ovicidal activity.

Description	Result
Linear model fitted	Cor % Ovi Act = 20.1047 + 9.68446 % Act
Summary of fit	
RSquare	0.543953
RSquare Adj	0.51995
Root Mean Square Error	25.87095
Mean of Response	50.10048
Observations (or Sum Wgts)	21
LOF test	p-value = Prob>F = 0.0025

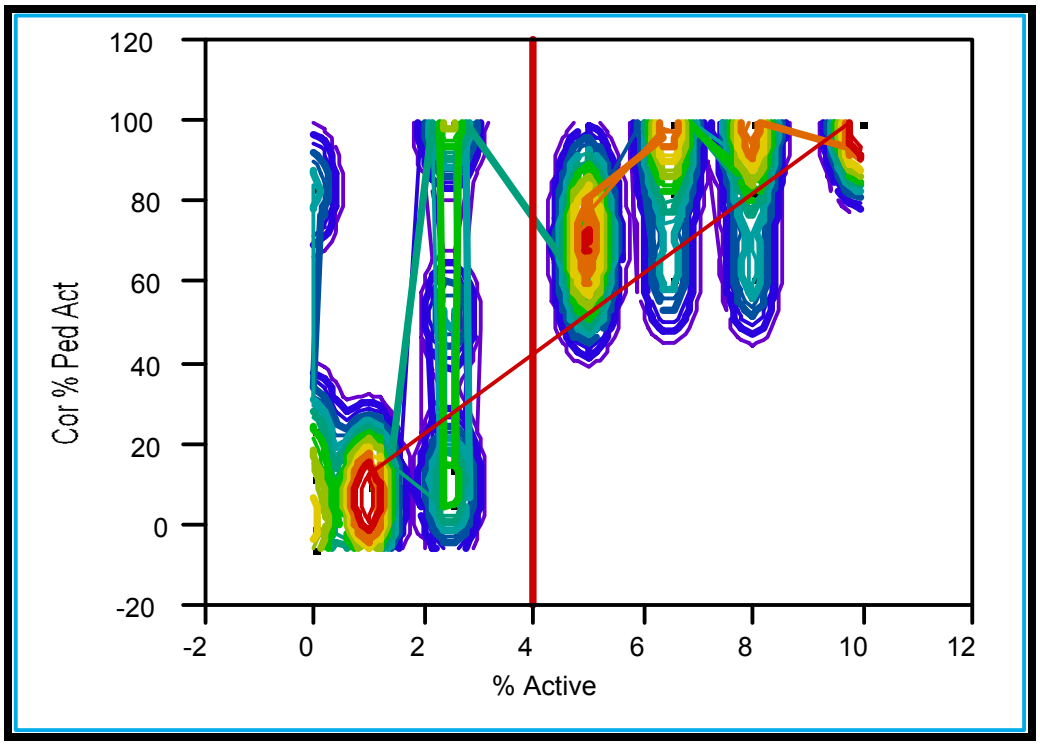


Figure 23. Quantile density plot for pediculicidal activity (JMP).

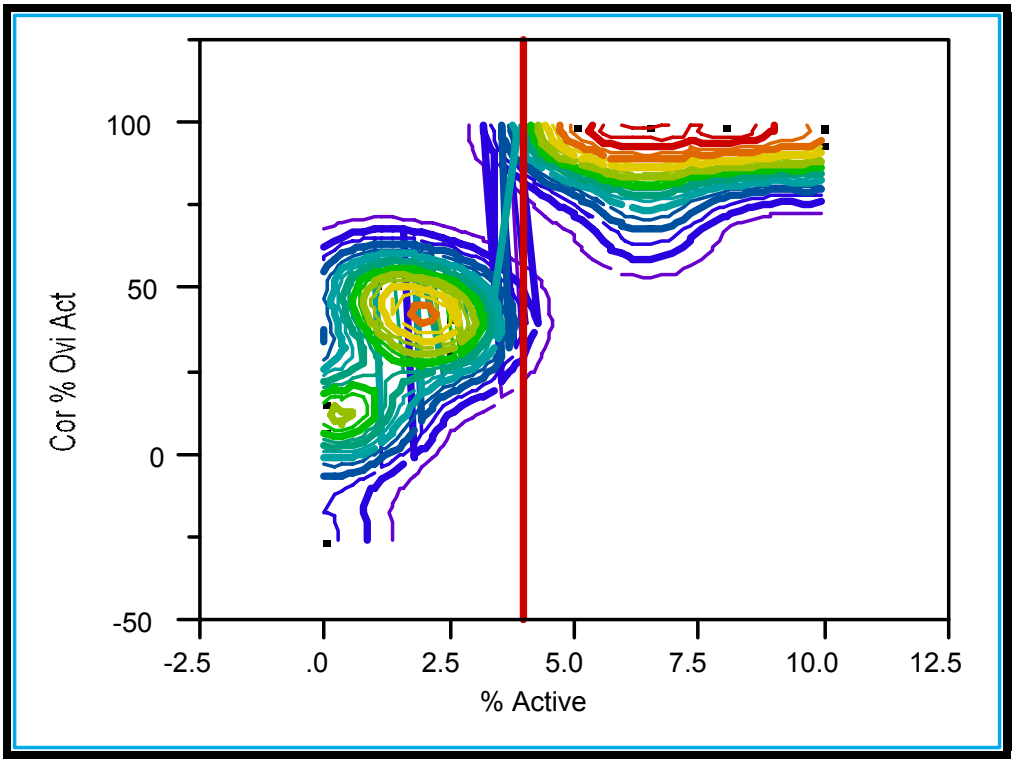


Figure 24. Quantile density plot for ovicidal activity (JMP).

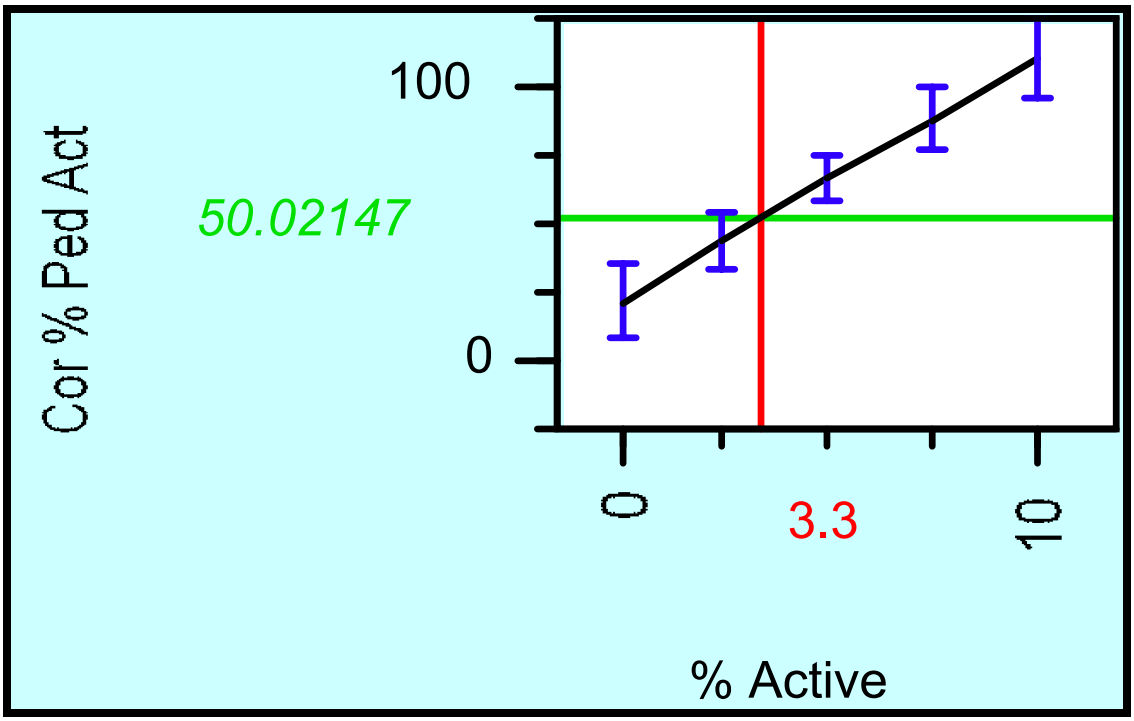


Figure 25. JMP screening fit for identification of LD_{50} for pediculicidal activity.

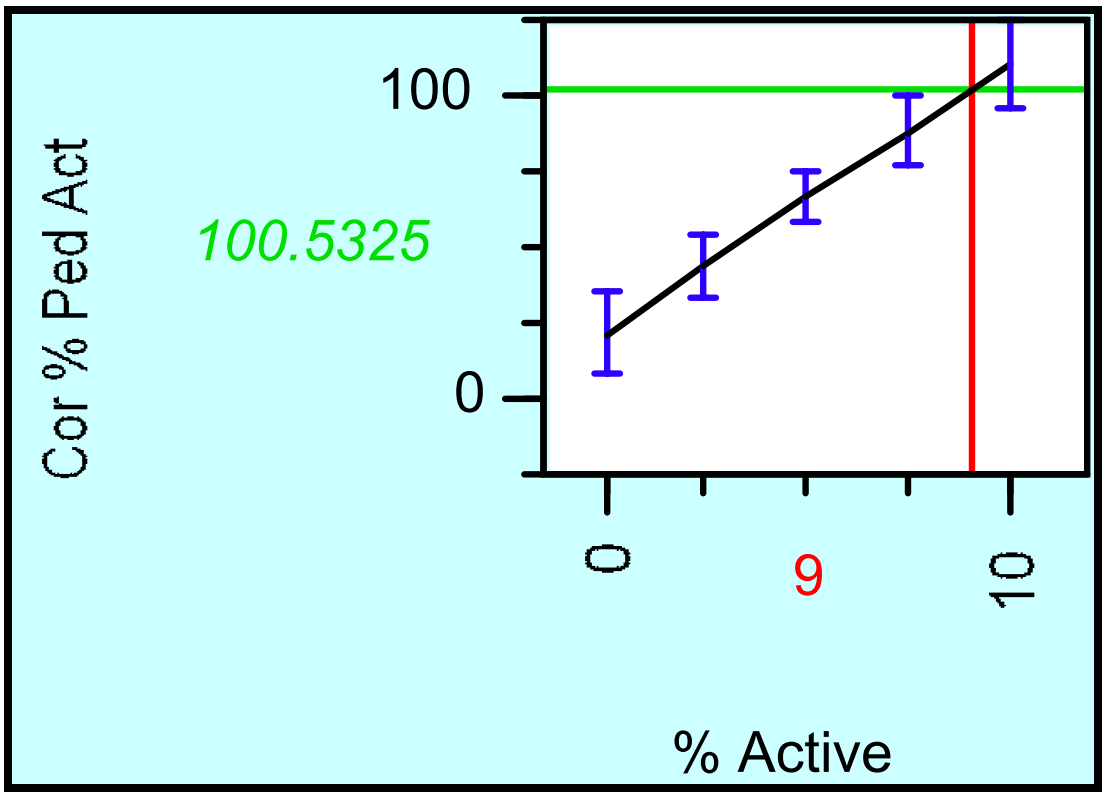


Figure 26. JMP screening fit for identification of LD_{100} for pediculicidal activity.

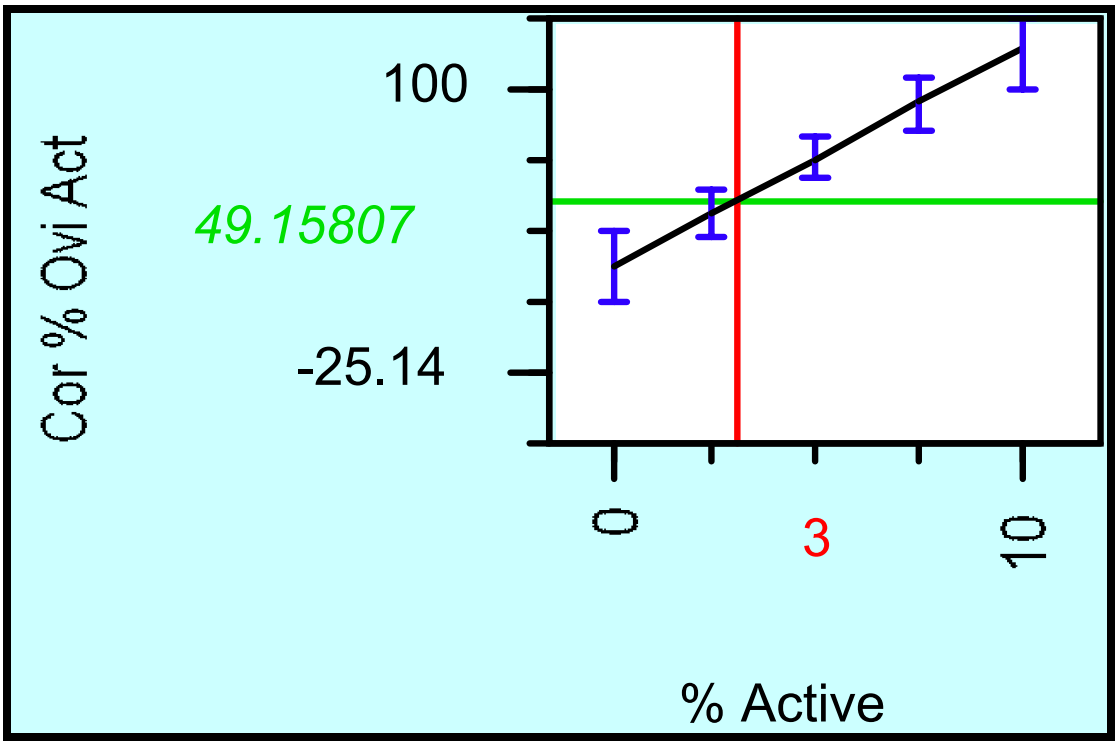


Figure 27. JMP screening fit for identification of LD_{50} for ovicidal activity.

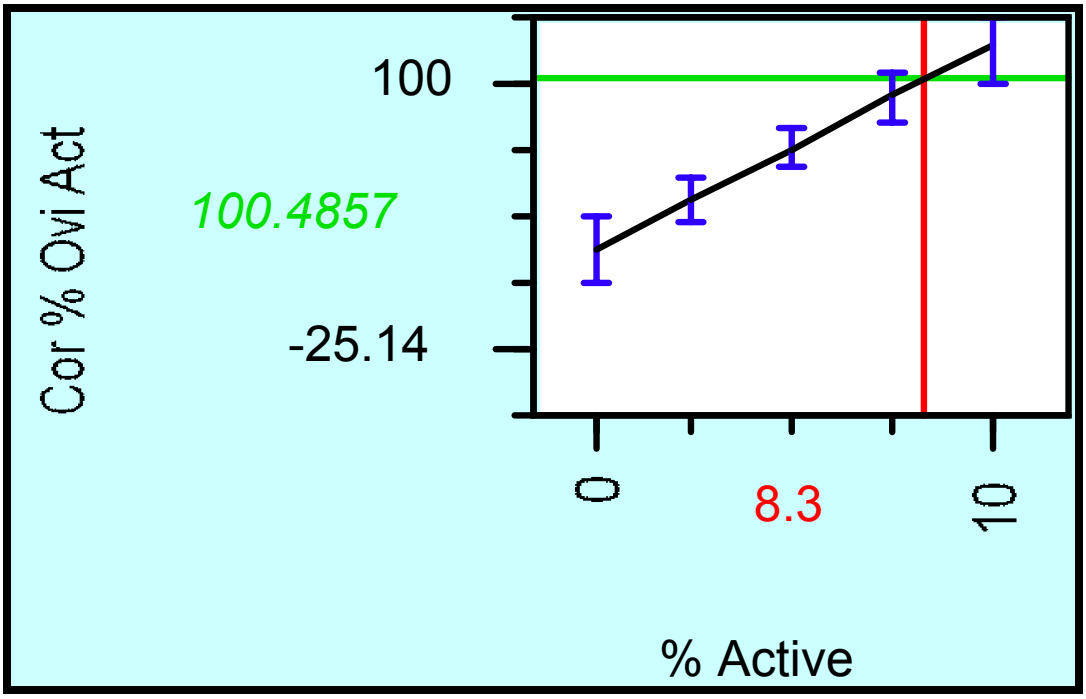


Figure 28. JMP screening fit for identification of LD_{100} for ovicidal activity.

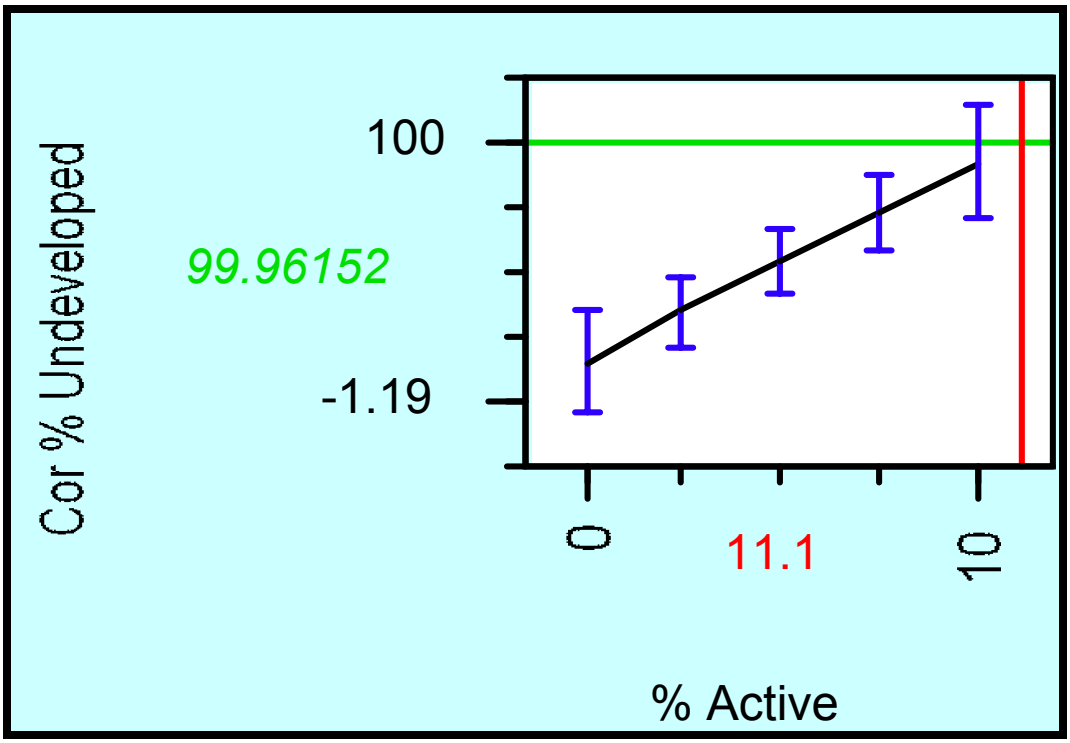


Figure 29. JMP screening fit for identification of concentration required for 100% "undeveloped eggs."

but citronellyl acetate concentrations of 5.0% or greater resulted in a statistically significant percent of eggs that remained undeveloped (p-value = 1.2×10^{-5}).

ULTIMATE VEHICLE OPTIMIZATION, *IN VITRO* CONFIRMATION, AND TOXICITY ASSESSMENT – MATERIALS:

The materials used for the ultimate optimization of the clear gel formulations were identical to those previously described for the clear gel vehicle development. Additionally all materials required for the toxicity assessments (dermal and eye irritation) were identical to those previously described.

ULTIMATE VEHICLE OPTIMIZATION, *IN VITRO* CONFIRMATION, AND TOXICITY ASSESSMENT – METHODS:

Both neutralized and non-neutralized formulations were prepared similarly to those previously described. Tables 87 and 88 summarize the specific methods of preparation. Viscosity measurements were obtained using a digital Brookfield viscometer. The assessment incorporated a number 27 spindle and was made while maintaining temperature of 25°C. First, the viscometer was calibrated prior to use with two previously qualified standard solutions. Then, the product was added to the sample container, and the instrument was allowed to run until equilibrium was achieved for the output.

An additional new *in vitro* pediculicidal activity assessment was implemented for ultimate confirmation of potential efficacy and assessment of the optimized products. The new method mimicked the ASTM method except that human head lice were collected and used for the evaluation. Specifically, human head lice were collected from patients receiving routine treatment and grooming at Lice Source Services, Inc. (Plantation, Florida). For the assessment, lice were collected from patients that had recently experienced multiple product failures. This assured a sampling of lice suspected to possess resistance to current treatments. The lice were collected on nylon gauze patches inside of a petri dish. The lice were then used for experimentation within 30 minutes after collection. The lice were separated into groups of 10 and then treated.

Table 87. Preparation of clear gel formulation without neutralization.

Step #	Description
1	Add alcohol to an adequately sized mixing vessel and begin mixing
2	While mixing add BHT and mix until completely dissolved
3	While mixing, add citronellyl acetate and mix for 2 minutes
4	While mixing, add propylene glycol and glycerin (or other solvents in lieu of) and mix for 2 minutes
5	While mixing, slowly add Pemulen to the vortex and mix until well hydrated and uniformly thickened

Table 88. Preparation of clear gel formulation with neutralization.

Step #	Description
1	Add alcohol to an adequately sized mixing vessel and begin mixing
2	While mixing add BHT and mix until completely dissolved
3	While mixing, add citronellyl acetate and mix for 2 minutes
4	While mixing, add propylene glycol and glycerin (or other solvents in lieu of) and mix for 2 minutes
5	While mixing, slowly add Pemulen to the vortex and mix until well hydrated
6	Add neutralizer (TEA or NaOH) to the solution from step #5 and mix until uniformly thickened

Two application techniques were incorporated. For the first two formulations, the lice were either submerged in the test product for the entire exposure time period. For the third formulation, the lice were submerged for 1 minute and removed but not rinsed for the remainder of the exposure period (10 minutes for all assessments). The lice were rinsed thoroughly with tap water, blotted dry, and placed on a new, dry piece of nylon gauze. The lice were observed for signs of life under a stereomicroscope at various time intervals up to 3 hours after exposure. Lice were classified as "appeared dead" if no physical movement existed or "alive" if the lice were crawling or otherwise physically moving. Percentages were calculated for each classification at each time interval. The data was charted using Microsoft Excel 2000 for comparison of each observation interval for each formulation tested.

Primary skin and eye irritation tests were performed identically to those previously described for the add-mix system. The testing complied with cGLPs.

ULTIMATE VEHICLE OPTIMIZATION, *IN VITRO* CONFIRMATION, AND TOXICITY ASSESSMENT – RESULTS AND DISCUSSION:

As previously stated, formulations with higher amounts of citronellyl acetate resulted in some haziness. A quick and simple experiment was designed to uncover the source of the haziness. Citronellyl acetate was added directly to the proportional amounts of the glycerin and propylene glycol. When the citronellyl acetate was added to the glycerin with little presence of alcohol, the same haziness appeared, but did not it the propylene glycol. Therefore, glycerin was removed from the formulation and replaced with additional propylene glycol since it was not completely compatible with the active. Also based on these brief studies, IPA concentration was determined to be critical for adequate solvation of citronellyl acetate. The concentration of IPA can be varied between 10% and 20% in future formulations to produce a clear gel formulation depending on the amount of citronellyl acetate incorporated.

Initial attempts to incorporate a neutralization step were made using TEA. However, when TEA was added to the propylene glycol based formulations, it became emulsified in the solution (turned a milky white color) without thickening it. Sodium hydroxide was substituted for the TEA and resulted in a thickened clear gel formulation with less than half of the amount of Pemulen® required. Additionally, some formulations were prepared by substitution of the propylene glycol base with Steol CS-230 ("surfactant" based formula). When attempts were made to thicken these surfactant based formulations with Klucel® or by using a neutralization step with Pemulen®, no thickening occurred with the use of TEA or sodium hydroxide, regardless of the amount added.

Based on the initial formulation attempts, all additional "non-surfactant" based formulations thickened by a neutralization step incorporated sodium hydroxide as the neutralizer. All surfactant based formulations were thickened with higher concentrations of Pemulen® eliminating a neutralization step.

Three optimized clear gel formulations were prepared in attempt to finalize the vehicle development research. All three formulations incorporated either 10.0% or 12.5% citronellyl acetate and either 15% or 20% IPA. Two formulations used propylene glycol as the base, and both were thickened utilizing less Pemulen® and a neutralization step with sodium hydroxide. The third formulation substituted Steol CS-230 for the entire amounts of propylene glycol and glycerin and was thickened through higher levels of Pemulen®. This third formulation also did not incorporate the BHT due to immediate availability issues. Table 89 summarizes the three formulations prepared for *in vitro* confirmation testing and their viscosities.

In vitro confirmation testing was performed on the three formulations using the new method incorporating human head lice with suspected knockdown gene (*kdr*-gene) resistance previously described. The numbers of lice that appeared dead and alive were tabulated for each observation interval, and the corresponding percentages were calculated. Figures 30-32 display the comparative results of the testing for each formulation. The

Table 89. Three potentially optimized formulations for *in vitro* confirmation testing.

Ingredient	Formulation # % (w/w)		
	1	2	3
IPA	15.00	15.00	20.00
BHT	0.05	0.05	0.00
Propylene glycol	75.06	72.56	0.00
Steol CS-230	0.00	0.00	65.50
Pemulen® TR1	0.85	0.85	2.00
Citronellyl acetate	10.00	12.50	12.50
Viscosity (cps)	4300	5750	1450

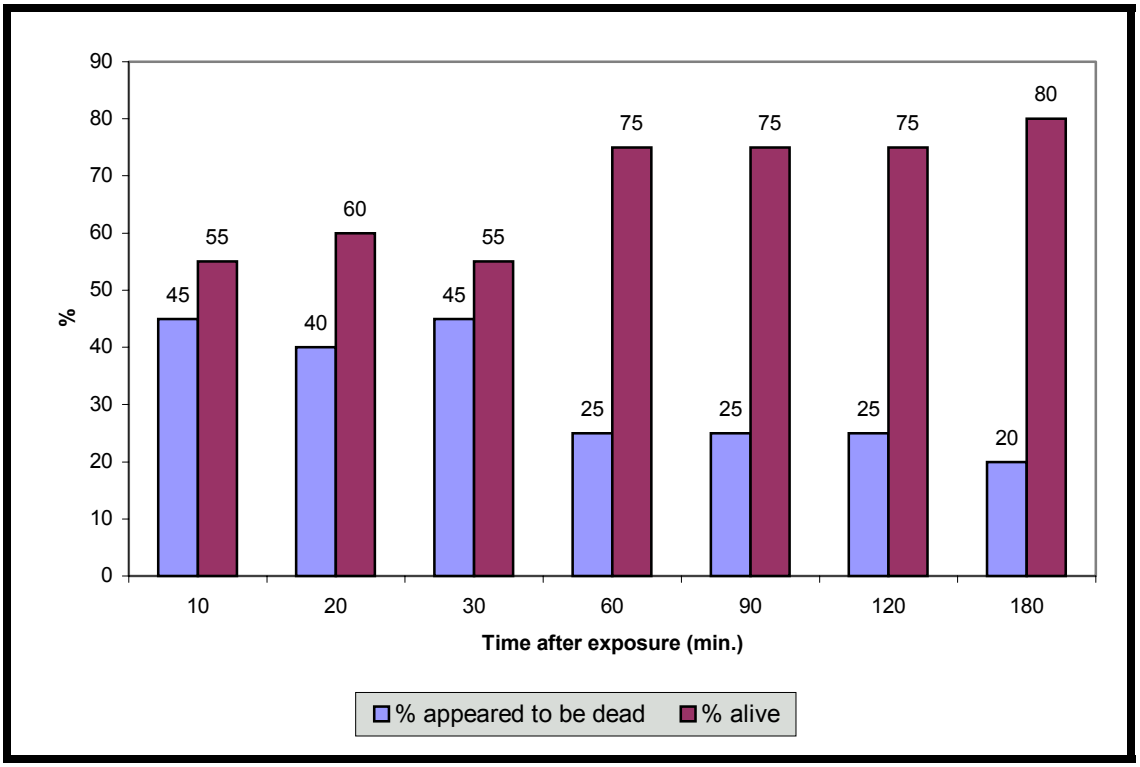


Figure 30. Results of confirmation testing for potentially optimized formulation #1.

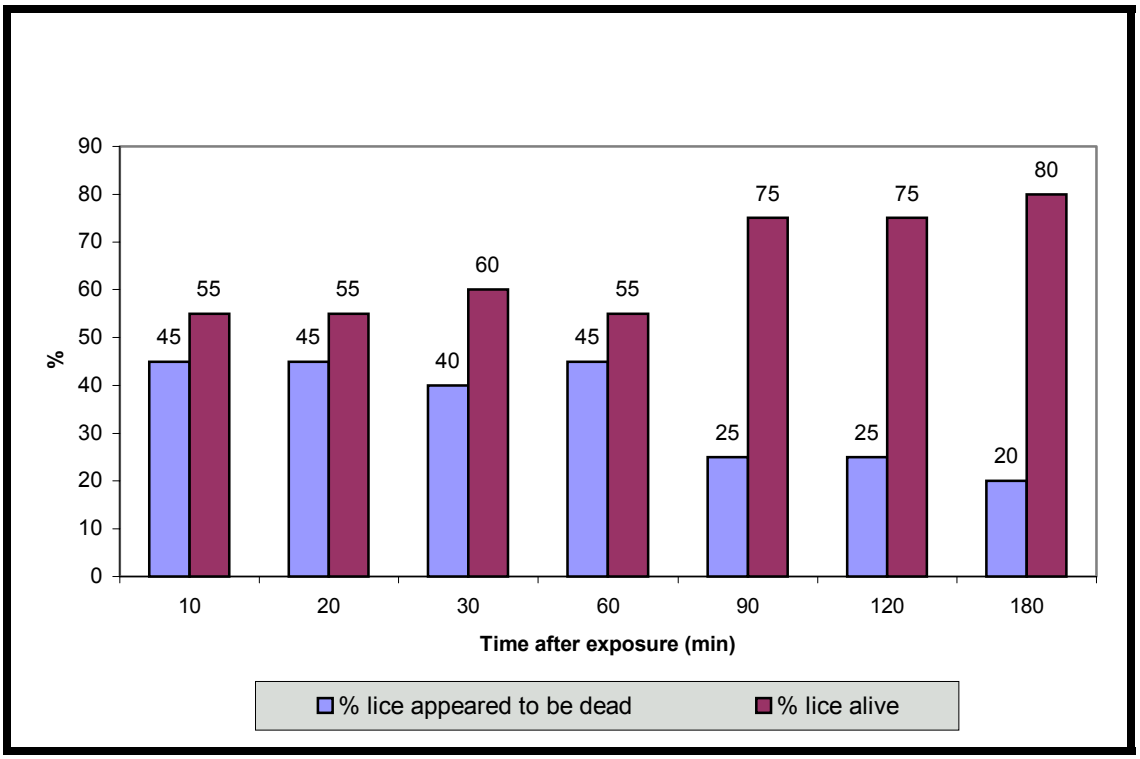


Figure 31. Results of confirmation testing for potentially optimized formulation #2.

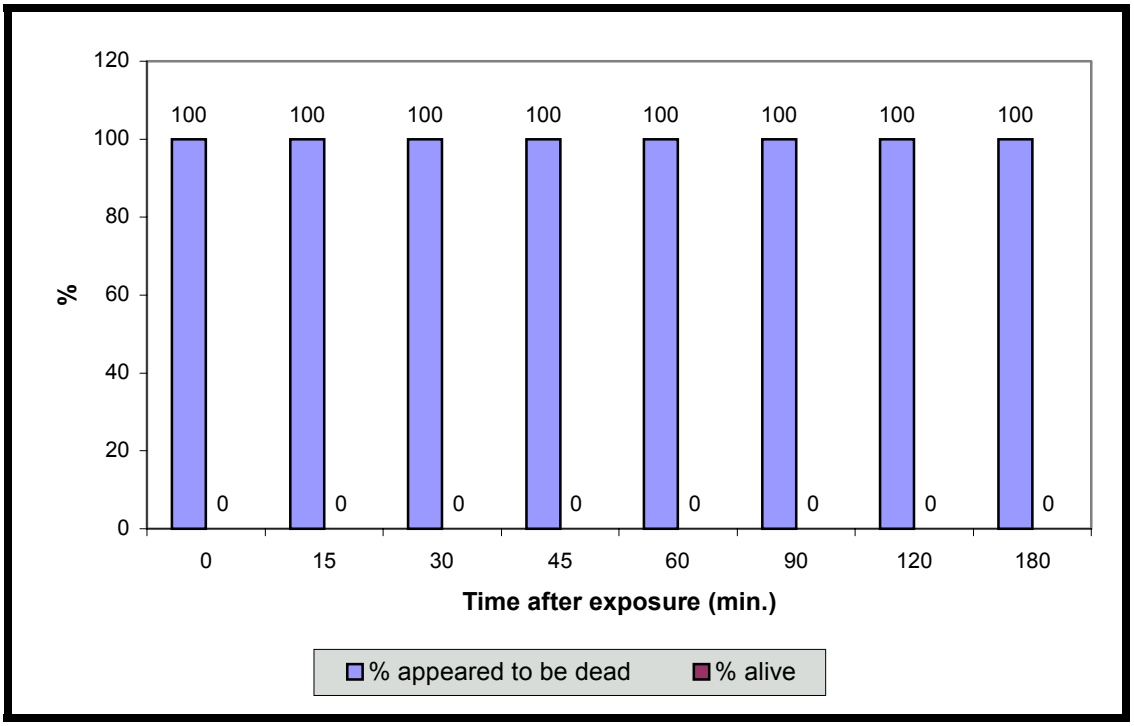


Figure 32. Results of confirmation testing for potentially optimized formulation #3.

results of formulations 1 and 2 clearly show the "knockdown" resistance attributes shown by these lice suspected of possessing the *kdr*-gene. Initial observation times indicate a high level of apparent kill, but as time elapsed the lice recovered and lived. Therefore, while these treatments may show efficacy to non-resistant lice, they most likely will not display any efficacy to the resistant ones. However, formulation 3 (with Steol CS-230) apparently overcame the *kdr*-gene resistance mechanism and resulted in 100% kill. This indicates that the product will most likely be effective against resistant lice in the field. Obviously, the study results identified the surfactant based clear gel product as the ultimate product optimized for efficacy.

The optimized surfactant based formulation was duplicated and used for comparative pre-clinical toxicology assessments. These assessments were performed and results classified identically to those for the add-mix system for a direct comparison of toxicity. The only variation in the methods was that body weights were not recorded for these assessments. The tests were limited to primary skin and eye irritation assessment for comparison of the optimized gel formulation's irritation characteristics to those found to be the most severe for the previous add-mix system. The primary skin and primary eye irritation testing was performed in compliance with cGLPs.

For the skin irritation testing, an additional exposure time was assessed for the new formulation. Therefore, the study assessed irritation associated with both a 1 and 4-hour exposure to the test product. Tables 90 and 91 summarize the results. The Primary Irritation Index was calculated for the optimized product with both 1 and 4-hour exposures. The resulting PIIs were 3.58 for the 1-hour exposure and 4.54 for the 4-hour exposure, both of which classify as a "moderate irritant." The optimized gel formulation resulted in drastically improved dermal irritation profiles as compared to the add-mix system that resulted in a PII of 6.75 indicating that the product was severely irritating.

Primary eye irritation testing with the optimized product included both a rinsed and "not rinsed" group. The results and observations of the assessment are summarized in

Table 90. Results of skin irritation testing with a 4-hour exposure to the optimized gel formulation.

Animal # Sex	Scoring Interval	Erythema Score	Edema Score	Comments
R4803 Male	4 hr	2	2	SL-1, IT
	24 hr	2	3	SL-3
	48 hr	2	3	SL-4
	72 hr	2	3	SL-4
	7 days	2	1	DES
	10 days	1	0	DES
	14 days	1	0	DES
R4809 Male	4 hr	2	2	SL-1, IT
	24 hr	2	3	SL-2
	48 hr	2	3	SL-3
	72 hr	2	3	SL-4
	7 days	2	2	DES
	10 days	2	0	DES
	14 days	2	0	DES
R4810 Male	4 hr	2	2	SL-2, IT
	24 hr	2	3	SL-4, BLA-1
	48 hr	2	2	SL-4, BLA-1
	72 hr	2	3	SL-4, BLA-1
	7 days	2	2	DES
	10 days	2	1	DES
	14 days	1	0	DES

Table 90. Continued.

Animal # Sex / Wt	Scoring Interval	Erythema Score	Edema Score	Comments
R4818 Male R4807 Male	4 hr	2	2	IT
	24 hr	2	3	SL-4
	48 hr	2	2	SL-4
	72 hr	2	2	SL-4
	7 days	1	0	DES
	10 days	0	0	DES
R4807 Male	4 hr	2	2	SL-1, IT
	24 hr	2	3	SL-4
	48 hr	2	3	SL-4
	72 hr	2	3	SL-4
	7 days	2	1	DES
	10 days	2	1	DES
	14 days	1	0	DES
R4811 Male	1 hr	2	2	IT
	24 hr	2	3	SL-4
	48 hr	2	2	SL-4
	72 hr	2	2	SL-4
	7 days	2	1	DES
	10 days	0	0	DES
	14 days	1	0	DES

Table 91. Results of skin irritation testing with a 1-hour exposure to the optimized gel formulation.

Animal # Sex	Scoring Interval	Erythema Score	Edema Score	Comments
R4803 Male	1 hr	1	1	
	24 hr	2	3	BLA-1, SL-2
	48 hr	2	2	BLA-1, SL-2
	72 hr	2	2	BLA-1, SL-2
	7 days	1	1	DES
	10 days	1	0	DES
	14 days	1	0	DES
R4809 Male	1 hr	2	1	
	24 hr	2	3	SL-3, BLA-1
	48 hr	2	2	SL-3
	72 hr	2	2	SL-3
	7 days	1	1	DES
	10 days	0	0	DES
	14 days	0	0	DES
R4810 Male	1 hr	1	1	
	24 hr	2	2	SL-3, BLA-1
	48 hr	2	2	SL-4, BLA-1
	72 hr	2	2	SL-4, BLA-1
	7 days	2	1	DES
	10 days	2	1	DES
	14 days	1	0	DES

Table 91. Continued.

Animal # Sex / Wt	Scoring Interval	Erythema Score	Edema Score	Comments
R4818 Male	1 hr	2	1	
	24 hr	2	2	SL-3
	48 hr	2	2	SL-3
	72 hr	2	2	SL-2
	7 days	1	0	DES
	10 days	0	0	DES
R4807 Male	1 hr	1	1	
	24 hr	2	3	SL-4, BLA-1
	48 hr	2	3	SL-4
	72 hr	2	3	SL-4, BLA-1
	7 days	2	1	DES
	10 days	1	1	DES
	14 days	1	0	DES
R4811 Male	1 hr	1	1	
	24 hr	1	2	SL-1
	48 hr	1	1	DES
	72 hr	1	1	DES
	7 days	1	0	DES
	10 days	0	0	DES

Tables 92-97. The surfactant based clear gel product was found to be very severely irritating to the eyes based on the conditions of the test. Rinsing did not appear to alleviate or shorten the duration of irritation. The formulation of citronellyl acetate in the clear gel vehicle did not appear to improve the potential for severe ocular irritation with contact.

SUMMARY OF EXPERIMENTATION FOR ULTIMATE VEHICLE OPTIMIZATION:

Two new *in vitro* efficacy assessment techniques were implemented to provide more meaningful insight into possible *in vivo* success of a formulation. Actual testing with the methods showed they were more capable of identifying and distinguishing formulations with unacceptable *in vitro* efficacy than the ASTM methods. The modified ovicidal assessment using human hair wigs demonstrated the importance of simulating *in vivo* application techniques especially when assessing the number of eggs that remained undeveloped after treatment. Using possibly resistant lice collected from human subjects for pediculicidal assessments demonstrated a significant ability to distinguish formulations that most likely would fail *in vivo* studies due to resistance issues. In addition, this technique also can distinguish possible failures due to drug absorption or penetration issues as the lice collected from the field tend to be more rigorous and possess more durable exoskeletons.

Formulation studies identified a clear gel vehicle as the most preferred drug delivery system for citronellyl acetate. Using the modified *in vitro* efficacy assessment techniques to test numerous different clear gel compositions, one formulation was identified to possess maximum *in vitro* pediculicidal and ovicidal efficacy under all test conditions. Results of pre-clinical toxicity assessments indicated this same formulation resulted in much less skin irritation minimizing the chance of the most common potential side effects of treatment. Unfortunately, it was still severely irritating to the eyes and precautions should be taken to avoid ocular contact.

The final optimization of the product resulted in yet another provisional patent filing. On May 19, 2000, a provisional patent, US Serial # 60/205843, entitled "Pest

Table 92. Individual ocular irritation scores for the no rinse group.

Animal # Sex	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
R4251 Male	1 hr	1	4	20	1	5	2	2	3	14	39
	24 hrs	2	4	40	1	5	3	2	2	14	59
	48 hrs	2	4	40	1	5	3	2	1	12	57
	72 hrs	2	4	40	1	5	2	2	0	8	53
	7 days	2	3	30	0	0	2	1	0	6	36
	10 days	2	2	20	0	0	2	1	0	6	26
	14 days	1	1	5	0	0	1	1	0	4	9
	21 days	0	0	0	0	0	0	0	0	0	0
R4253 Female	1 hr	2	4	40	1	5	2	2	3	14	59
	24 hrs	2	4	40	1	5	3	2	2	14	59
	48 hrs	2	4	40	1	5	3	2	2	14	59
	72 hrs	3	4	60	1	5	3	2	1	12	77
	7 days	3	3	45	1	5	2	1	0	6	56
	10 days	2	3	30	0	0	2	1	0	6	36
	14 days	2	3	30	0	0	1	1	0	4	34
	21 days	2	3	30	0	0	0	0	0	0	30

Table 92. Continued.

Animal # Sex	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
R4257 Female	1 hr	1	3	15	1	5	1	2	3	12	32
	24 hrs	2	4	40	1	5	3	2	2	14	59
	48 hrs	2	4	40	1	5	3	2	1	12	57
	72 hrs	3	4	60	1	5	3	2	1	12	77
	7 days	3	4	60	1	5	2	2	1	10	75
	10 days	2	3	30	0	0	2	1	0	6	36
	14 days	2	3	30	0	0	1	1	0	4	34
	21 days	1	2	10	0	0	0	0	0	0	10
R4259 Female	1 hr	1	2	10	1	5	2	2	3	14	29
	24 hrs	2	4	40	1	5	2	2	2	12	57
	48 hrs	2	4	40	1	5	2	2	0	8	53
	72 hrs	2	4	40	1	5	3	2	1	12	57
	7 days	0	0	0	0	0	1	1	0	4	4
	10 days	0	0	0	0	0	1	0	0	2	2
	14 days	0	0	0	0	0	0	0	0	0	0

Table 92. Continued.

Animal # Sex	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
R4262 Female	1 hr	1	3	15	1	5	2	2	3	14	34
	24 hrs	2	4	40	1	5	2	2	3	14	59
	48 hrs	2	4	40	0	0	2	2	1	10	50
	72 hrs	2	3	30	0	0	2	2	0	8	38
	7 days	0	0	0	0	0	2	1	0	6	6
	10 days	0	0	0	0	0	1	1	0	4	4
	14 days	0	0	0	0	0	1	0	0	2	2
	21 days	0	0	0	0	0	0	0	0	0	0
R4263 Female	1 hr	1	4	20	1	5	2	2	3	14	39
	24 hrs	2	4	40	1	5	2	2	2	12	57
	48 hrs	2	4	40	1	5	3	2	1	12	57
	72 hrs	3	4	60	1	5	3	2	1	12	77
	7 days	2	3	30	0	0	2	1	0	6	36
	10 days	2	3	30	0	0	2	1	1	8	38
	14 days	1	1	5	0	0	0	0	0	0	5
	21 days	1	1	5	0	0	0	0	0	0	5

Table 93. Mean ocular scores for no rinse group.

Interval	Mean Ocular Score	Interval	Mean Ocular Score
1 hr	38.67	7 days	35.50
24 hrs	58.33	10 days	23.67
48 hrs	55.50	14 days	14.00
72 hrs	63.17	21 days	7.50

Table 94. Summary of examination findings for the no rinse group.

Interval	Animal # Comments – Fluorescein Exam Findings (Secondary Ocular Findings)					
	R4251	R4253	R4257	R4259	R4262	R4263
1 hr						
24 hrs	FAO	FAO	FAO (BLL)	FAO	FAO	FAO
48 hrs	FAO	FAO	FAO	FAO	FAO	FAO
72 hrs	FAO	FAO	FAO	FAO	FAO	FAO
7 days	FAO (VAS-1)	FAO (VAS-3)	FAO (VAS-3)			FAO (VAS-1)
10 days	FAO (VAS-1)	FAO (VAS-2)	FAO (VAS-1)			FAO (VAS-1)
14 days	FAO	FAO (VAS-1)	FAO (VAS-1)			FAO (VAS-1)
21 days		FAO	FAO			

Table 95. Individual ocular irritation scores for the rinsed group.

Animal # Sex	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
R4264 Female	1 hr	1	4	20	1	5	2	2	3	14	39
	24 hrs	2	4	40	1	5	3	2	2	14	59
	48 hrs	3	4	60	1	5	3	2	2	14	79
	72 hrs	3	4	60	1	5	3	2	1	12	77
	7 days	2	4	40	1	5	2	2	1	10	55
	10 days	2	4	40	1	5	2	1	1	8	53
	14 days	2	3	30	0	0	2	1	1	8	38
	21 days	2	2	20	0	0	1	1	0	4	24
R4265 Female	1 hr	2	4	40	1	5	2	2	2	12	57
	24 hrs	3	4	60	1	5	3	2	1	12	77
	48 hrs	3	4	60	1	5	3	2	1	12	77
	72 hrs	3	3	45	1	5	2	1	0	6	56
	7 days	2	2	20	0	0	1	1	0	4	24
	10 days	2	1	10	0	0	1	0	0	2	12
	14 days	1	1	5	0	0	0	0	0	0	5
	21 days	1	1	5	0	0	0	0	0	0	5

Table 95. Continued.

Animal # Sex	Interval	Cornea			Iris		Conjunctiva				Total
		O	A	OxAx5	I	I x 5	R	S	D	(R+S+D)2	
R4266 Female	1 hr	1	4	20	1	5	2	2	2	12	37
	24 hrs	2	4	40	1	5	2	2	2	12	57
	48 hrs	2	4	40	1	5	2	2	2	12	57
	72 hrs	3	4	60	1	5	2	2	0	8	73
	7 days	1	2	10	0	0	0	1	0	2	12
	10 days	1	1	5	0	0	0	1	0	2	7
	14 days	1	1	5	0	0	0	0	0	0	5
	21 days	1	1	5	0	0	0	0	0	0	5

Table 96. Mean ocular scores for rinsed group.

Interval	Mean Ocular Score
1 hr	44.33
24 hrs	64.33
48 hrs	71.00
72 hrs	68.67
7 days	30.33
10 days	24.00
14 days	16.00
21 days	11.33

Table 97. Summary of examination findings for the rinsed group.

Interval	Animal # & Comments – Fluorescein Exam Findings (Secondary Ocular Findings)		
	R4264	R4265	R4266
1 hr	(BUL)	FAO	FAO
24 hrs	FAO (BUL)	FAO	FAO
48 hrs	FAO	FAO	FAO
72 hrs	FAO	FAO	FAO
7 days	FAO (VAS-1)	FAO (VAS-1)	FAO VAS-1)
10 days	FAO (VAS-1)	FAO	FAO
14 days	FAO (VAS-1)	FAO	FAO
21 days	FAO (VAS-1)	FAO	FAO

Treatment Compositions" was filed. Table 98 summarizes the final formulation optimized for potential safety and efficacy, and Table 99 summarizes its method of manufacture.

INITIAL ATTEMPTS TO ELUCIDATE CITRONELLYL ACETATE'S MODE OF LETHAL ACTION TO HEAD LICE

BACKGROUND:

Due to the emergence of pesticide resistance, much concern has been raised about human head lice resistance to current therapy. Many authorities believe this resistance has formed due to the similar modes of action for current products, which act exclusively via interference with the central nervous system (CNS). Scientists have identified a gene responsible for knockdown resistance to DDT, and lice are also quickly developing resistance to other currently marketed products such as Rid™ and Nix™. As lice have proven to become quickly resistant to CNS agents, researchers and authorities are interested in evaluating possible future resistance to new pesticidal compounds.

Previous experiments have documented the ability of individual citronella oil constituents to kill lice. These experiments have identified that when citronellyl acetate was incorporated into an optimized clear gel delivery system, the resulting formulation possessed extremely lethal qualities. Additionally, *in vitro* ovicidal assessments have shown that citronellyl acetate is able to kill embryos inside the eggs prior to CNS development. This unique attribute of citronellyl acetate indicates that it possesses a much different mechanism of lethality than currently used products.

Extensive basic research has been performed in attempt to elucidate citronellyl acetate's possible mode(s) of lethal action. Literature review uncovered known modes of action for existing pesticides and those for other compounds that are chemically similar to citronellyl acetate. Most pesticides target different aspects of the CNS such as the voltage dependent Na^{+} channel, Ca^{+2} channels, Cl^{-} channels, acetylcholinesterase inhibition, and acetylcholine mimicking. However, additional classes exhibit activity through hormone mimicking and disruption of energy metabolism.

Table 98. Final optimized formulation with citronellyl acetate for treatment of human head lice.

Ingredient	% (w/w)
IPA	20.00
BHT	0.05
Steol CS-230	65.45
Pemulen® TR1	2.00
Citronellyl acetate	12.50

Table 99. Method of manufacture for ultimately optimized formulation.

Step #	Description
1	Add alcohol to an adequately sized mixing vessel and begin mixing
2	While mixing add BHT and mix until completely dissolved
3	While mixing, add citronellyl acetate and mix for 2 minutes
4	While mixing, add SteolCS-230 and mix for 2 minutes
5	While mixing, slowly add Pemulen to the vortex and mix until uniformly thickened

These mechanisms of action were previously summarized in Chapter 2 with the exception of that for an unconventional pesticide, limonene. Dr. George W. Ware of the University of Arizona has previously summarized limonene's use as a pesticide.[103] Limonene is a botanical product extracted from citrus peel. A specific form of limonene, *d*-limonene, has been shown to possess insecticidal qualities to typical pests such as fleas, lice, mites, and ticks, but is nontoxic to mammals. Interestingly, limonene's exact mode of pesticidal action is unknown, but is expected to be similar to that of pyrethrum since it appears to also affect the CNS, but not through acetylcholinesterase inhibition.

However, *d*-limonene has been extensively researched as a possible treatment of cancer where it has been discovered that it and other monoterpenes inhibit isoprenylation of G-Proteins.[140-146] Crowell, *et. al.*, specifically demonstrated structure-activity relationships of various different terpenes on isoprenylation inhibition.[140] They found that monohydroxyl, ester, and aldehyde substitution exhibited the highest activity. Following these were thiols that were more active than acids, diols, and epoxides (similar activity), all of which were more active than triols or unsubstituted ones. Figure 33 displays the specific structures tested in the hierarchy of the structure-activity relationships. According to the structure-activity relationship, citronellyl acetate should be expected to exhibit activity similar to the second most active group based on its similarity in structure to geraniol and identical functional group as perillyl acetate.

All cases have demonstrated that monoterpenes easily penetrate cell membranes and inhibit the isoprenylation of small G-proteins. The G-proteins possess isoprenyl anchors on their α and γ subunits. These lipid anchors interact with the membrane bilayer enhancing the membrane attachment of both the α and γ subunits. Lipid modifications include a palmitoyl (fatty acid) group on the α subunit and a farnesyl (polyisoprene) group on γ subunit, and the sites appear to be on the same surface of the protein. Figure 34 shows an example of the lipid anchors.

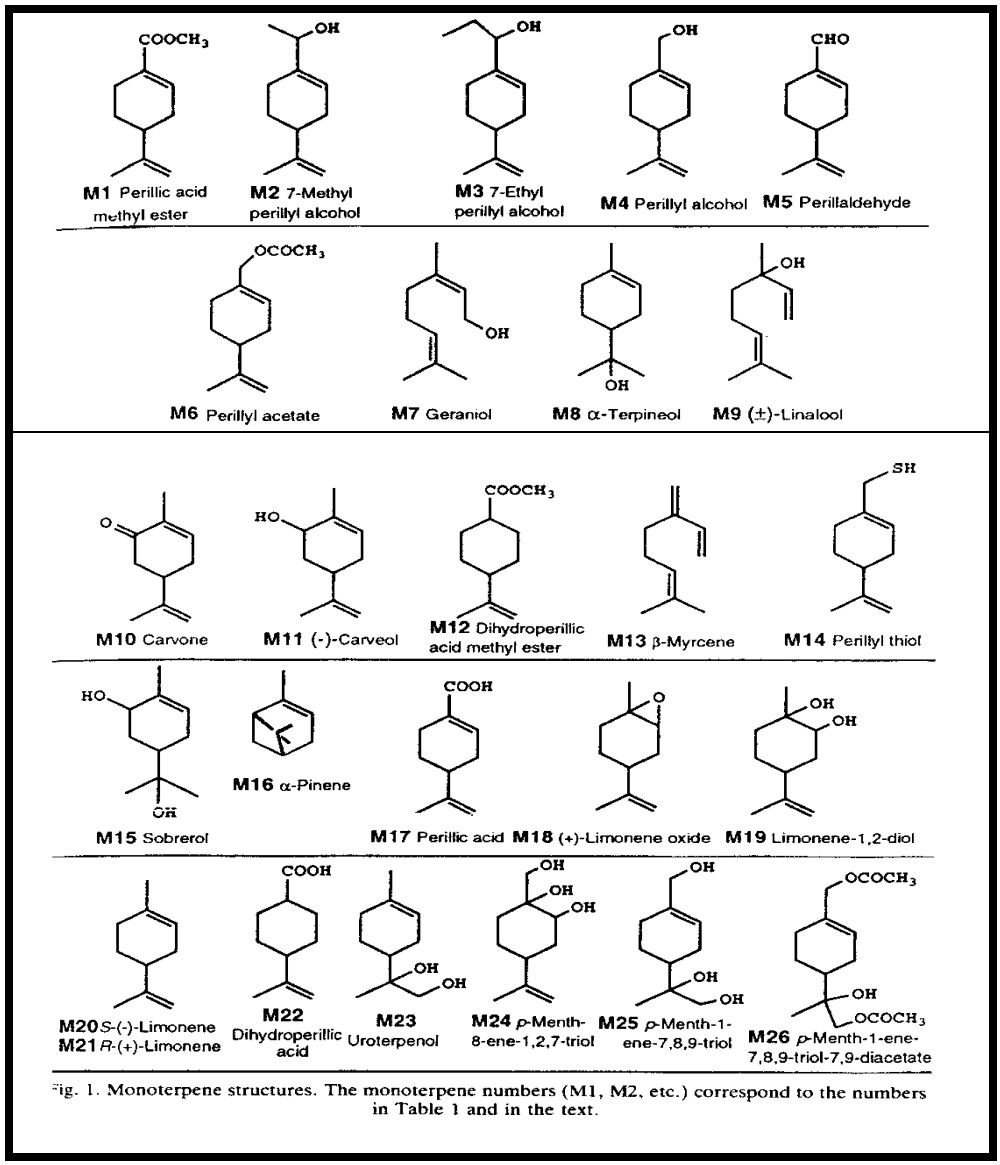


Fig. 1. Monoterpene structures. The monoterpene numbers (M1, M2, etc.) correspond to the numbers in Table 1 and in the text.

Figure 33. Hierarchy of structure-activity relationship on isoprenylation inhibition.

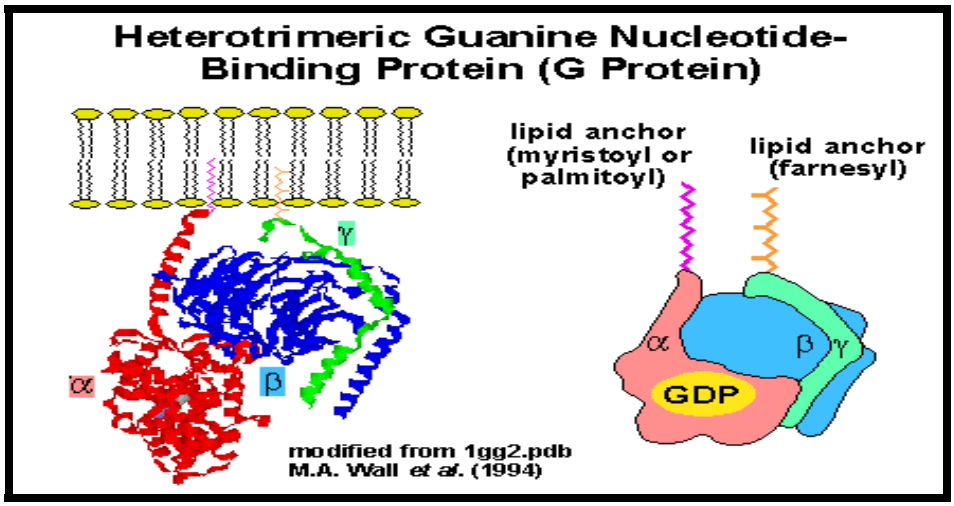


Figure 34. Example of lipid anchors of a G-protein.

Monoterpenes exhibit competitive binding to proteins that catalyze isoprenylation such as type I geranylgeranyl protein transferase (GGPTase). The competitive inhibition is stereochemistry specific, which explains why *d*-limonene is required for activity. This inhibition of isoprenylation of the G-protein's α and γ subunits has significant physiological implications.

The G-protein cycle is a critical pathway for GTP synthesis (similar cellular implications as ATP) and participates in all Ca^{+2} channel dependent physiological processes. The G-protein cycle and structure is demonstrated extremely effectively on the University of Kansas Medical Center website.[148,149] In the basal state, the G-protein "swims" in the plasma membrane with GDP bound to the α subunit until the first receptor site in the plasma membrane is activated. Before the receptor and protein can bind, the G-protein is anchored into the membrane through use of the isoprenyl groups on the α and γ subunits. Once bound, GDP is converted to GTP (similar energy cycle as the ATP cycle). The G-protein with bound GTP then diffuses away from the receptor, and the α subunit with bound GTP dissociates into the cytosol for interaction with its secondary effector while the βγ dimer remains anchored to the plasma membrane for interaction with its secondary effector.

The GTP on the α subunit is ultimately converted back to GDP and inorganic phosphate. After dissociation with the α subunit, the βγ dimer interacts with its effector, phospholipase C, which catalyses the hydrolysis of phosphatidylinositol biphosphate (Pt-Ins P2) to diacylglycerol (DAG) and inositol triphosphate (Ins-P3). The Ins-P3 diffuses into the cytosol activating the ligand gated Ca^{+2} channel of the endoplasmic reticulum (ER) allowing Ca^{+2} to flow into the cytosol. Most cells contain several protein kinases that are regulated, at least in part, by intracellular Ca^{+2}, and as levels increase their downstream responses are elucidated and modulated. Ultimately, the receptors are inactivated and the cycle begins to stall. Finally, the α subunit with bound GDP re-associates with its βγ dimer

subunit (still "swimming" in the plasma membrane), and the entire G-protein with bound GDP is available to begin the cycle again.

When the monoterpenes effectively inhibit the isoprenylation of the α and γ subunits, the isoprenyl anchors are not attached. Therefore, the protein is not able to anchor into the cell membrane and interact with the respective effectors. Thus, the entire G-protein cycle ceases to occur eliminating the production of GTP and blocking any Ca^{+2} mediated processes.

The review also uncovered a completely different possible mechanism of action. US Patent No. 6,130,253 demonstrated terpenes possess insecticidal qualities due to their ability to dissolve the cuticle of insects.[150] This lipid cuticle serves mainly to regulate water loss in the insect, and if disrupted or destroyed, the louse will quickly dehydrate and die. Specifically, the patent documents destruction of the cuticle of lice when exposed to certain terpenes in combination with citral. The authors used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) for inspection prior to and after exposure to the terpene blends. They revealed that when viewed using the methods, the cuticle before treatment appeared "dirty and uneven," but after exposure it appeared "denuded, leaving a pristine surface."

Based on the literature review three main hypotheses were drawn on the possible mode of citronellyl acetate's lethality to head lice. First, citronellyl acetate penetrates into the cells of the head louse and works on a molecular level to inhibit the isoprenylation of G-proteins resulting in depletion of GTP energy stores and locking the calcium channel, quickly causing paralysis and death. Second, the citronellyl acetate dissolves the lipid cuticle on the exoskeleton of the louse rendering it unable to regulate water loss causing quick and excessive dehydration resulting in ultimate death. Third, citronellyl acetate acts via a combination of both isoprenylation inhibition and destruction of the cuticle. Efficient and cost effective experiments were designed and performed to initially test the hypotheses.

MATERIALS:

Pure *R*- and *S*-citronellol were obtained from Sigma-Aldrich. All other materials used for preparation of the supplies were identical to those previously described. Samples for SEM were initially obtained in 70% ethanol, and were ultimately washed systematically in 80%, 95%, and 100% ethanol.

METHODS:

Formulations incorporating either pure *R*- or *S*-citronellol were prepared identically to the method previously summarized in Table 99. *In vitro* assessments were performed using the previously described method incorporating the laboratory lice and 5-second immersion technique, but only one replicate per formulation was used.

For evaluation of possible surface effects, lice and eggs were removed from children and treated identically as previously described for the modified *in vitro* method. Before applying the test product, one louse and two nits were transferred to a 70% ethanol solution for storage until future SEM preparation. Two lice and three eggs were collected after treatment (after 10-minute exposure and rinse) and also stored in 70% ethanol. On the day of SEM observation, the samples were dehydrated systemically by "washing" (soaking) them in aliquots of 80%, 95%, and 100% ethanol. The lice and eggs were allowed to soak in each aliquot for 10 minutes. The specimens were transferred to porous drying containers and were dried at the critical point using a Samdri –780A critical point drier (Tousimis Research Corp.) The dried samples were fixed on SEM mounts and gold-plated (30 second plating) using a Spi-Module plating device. The plated samples were then observed with a LEO982 SEM/X-Ray Digital Scanning Electron Microscope at 3.00 kV with various magnifications between X100 and X1000.

RESULTS AND DISCUSSION:

Two formulations were prepared with one of the chirally pure actives, *R*- or *S*-citronellol. Chirally pure citronellol was substituted for citronellyl acetate since it was readily available in the trade, and those of citronellyl acetate were not. The competitive

inhibition of the isoprenylation process has been proven to be stereochemistry specific. If citronellyl acetate's mode was via isoprenylation inhibition, then one enantiomer of the compound may exhibit lethal activity while the other not since the target binding site is chirally specific. However, if both enantiomers exhibited the same activity and symptoms, it would indicate the mode was via a surface effect on the cuticle. Even further, if both were active but resulted in different symptoms leading to death, this would indicate the compound acts via both mechanisms. Therefore, the formulations were prepared and tested for *in vitro* pediculicidal activity using lab lice and the 5 second immersion technique.

Interestingly, both formulations resulted in 100% activity. However, a significant difference in the symptoms of exposure and manner of death was observed between the two groups of lice. Immediately after rinsing, there was no difference between the groups. In both cases 3 to 4 lice were beginning to show movement, and some others were inactive but showed gut movement. After 60 minutes, physical signs of exposure began to differentiate. The lice exposed to *S*-citronellol were basically walking as normal, but 10 lice were showing tonic-clonic spasms similar to that seen for lice exposed to permethrin or other pyrethroids. Only 1 of the lice exposed to *R*-citronellol appeared alive and normal, 4 others were trying to walk, but the rest were completely immobilized. After 180 minutes, 4 four lice exposed to *R*-citronellol were lying down with "waggling" limbs and the rest remained completely immobilized. For the ones treated with *S*-citronellol, 2 were lying down with "waggling" limbs, 1 louse walking uncomfortably with stiff limbs, and the remainder were immobile.

While both pure enantiomers eventually exhibited 100% pediculicidal activity, the symptoms and physical characteristics after exposure varied drastically. These results indicate the feasibility of the theory that citronellyl acetate may exhibit lethal activity to lice via at least isoprenylation inhibition.

SEM was used in attempt to observe any possible surface destruction that the product may have exhibited. Because the formulation exhibits lethal activity in a very short time after exposure, a dramatic surface effect would be expected if it were the only mechanism for such lethality. Therefore, lice and eggs were collected before and after exposure to the gel formulation (with racemic citronellyl acetate), analyzed with SEM, and the images were compared to discern cuticle destruction. Figures 35-40 display various comparative surface images of an untreated louse with one after treatment with the gel product. No dramatic surface destruction was observed, but some images did reveal apparent cuticle dissolution and surface "erosion." Specifically Figures 35-37 show a much smoother surface around the dorsal column (principal point of thoracic muscular attachment, mainly the muscles that operate the legs) for the treated louse than the untreated. However, images of leg joints show very little or no effects on the tissue surfaces of the joints (Figure 38). Comparative images of eggs (Figures 39 and 40) also revealed slight evidence of surface effects. Figure 39 shows some slight differences in the banding of the seam between the operculum and rest of the egg. The image of the untreated egg reveals a "beaded" appearance to the band while the one after treatment does not (arrows in Figure 39). Figure 40 specifically reveals the difference in definition and height between the pores and also the difference in pore height.

SUMMARY:

Basic literature review uncovered two main possible modes of action for citronellyl acetate. These two modes were inhibition of G-protein isoprenylation and solvation of the exoskeleton's lipid cuticle. Cost and time effective experiments were designed and performed in attempt to begin to elucidate the actual mode or modes of action. While both chirally pure actives exhibited lethal activity, the resulting symptoms of exposure varied. SEM studies demonstrated that massive surface destruction of the cuticle was not evident, but some appearance of erosion and effect was documented.

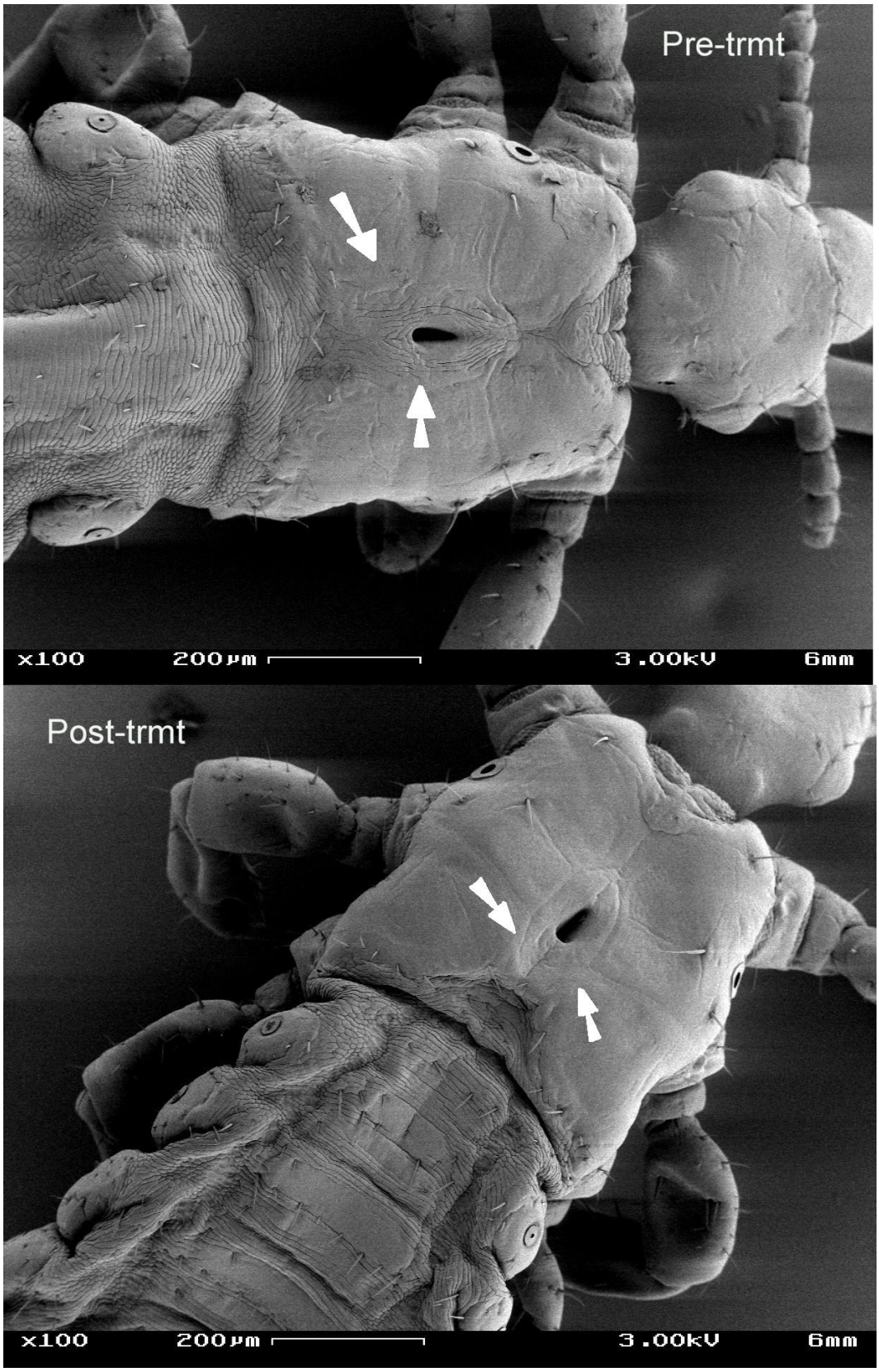


Figure 35. Comparative SEM image (top).

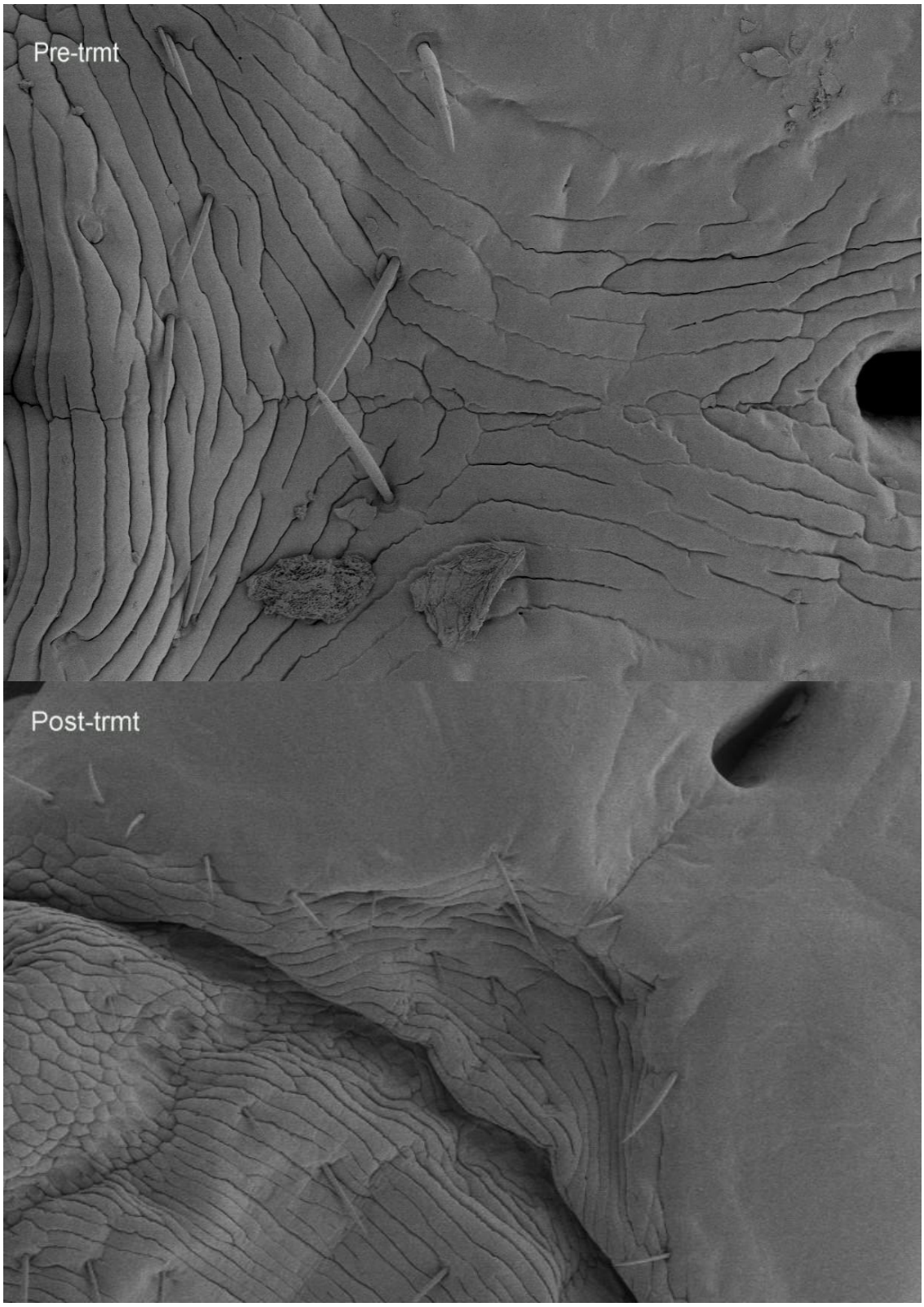


Figure 36. Further magnification of Figure 35 (top).

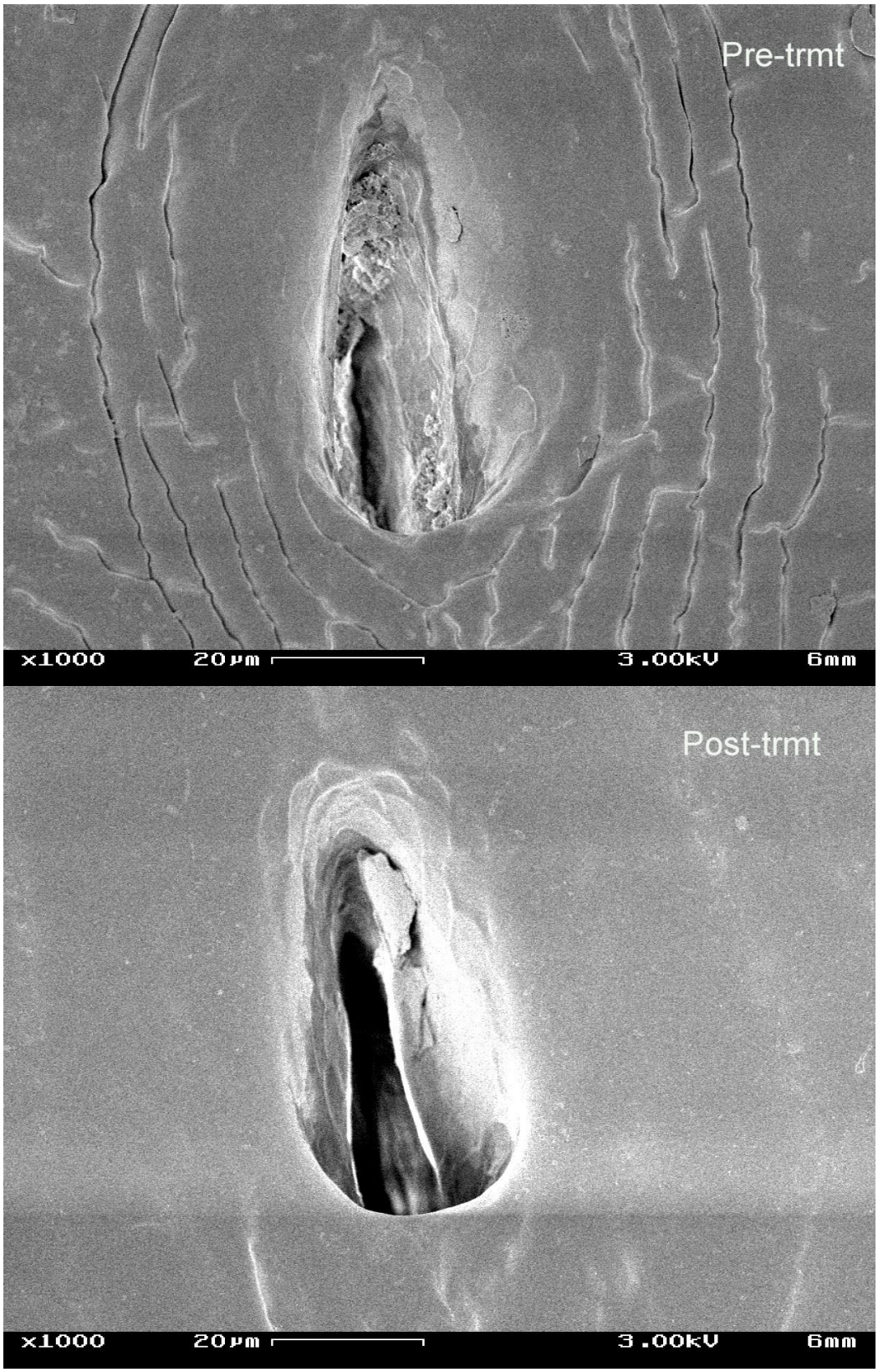


Figure 37. Comparison of dorsal column (further magnification, top).

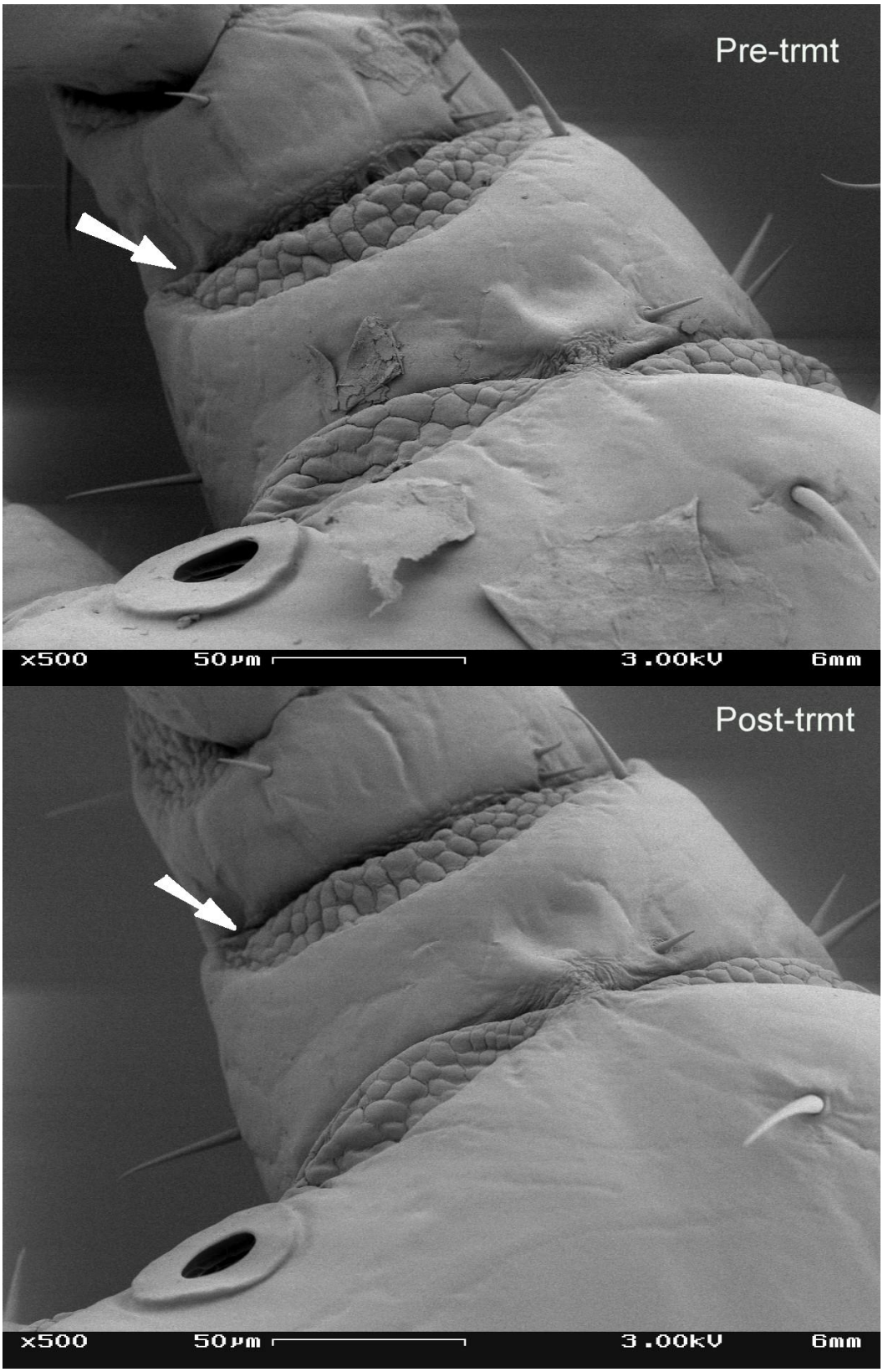


Figure 38. Comparative image of leg joints.

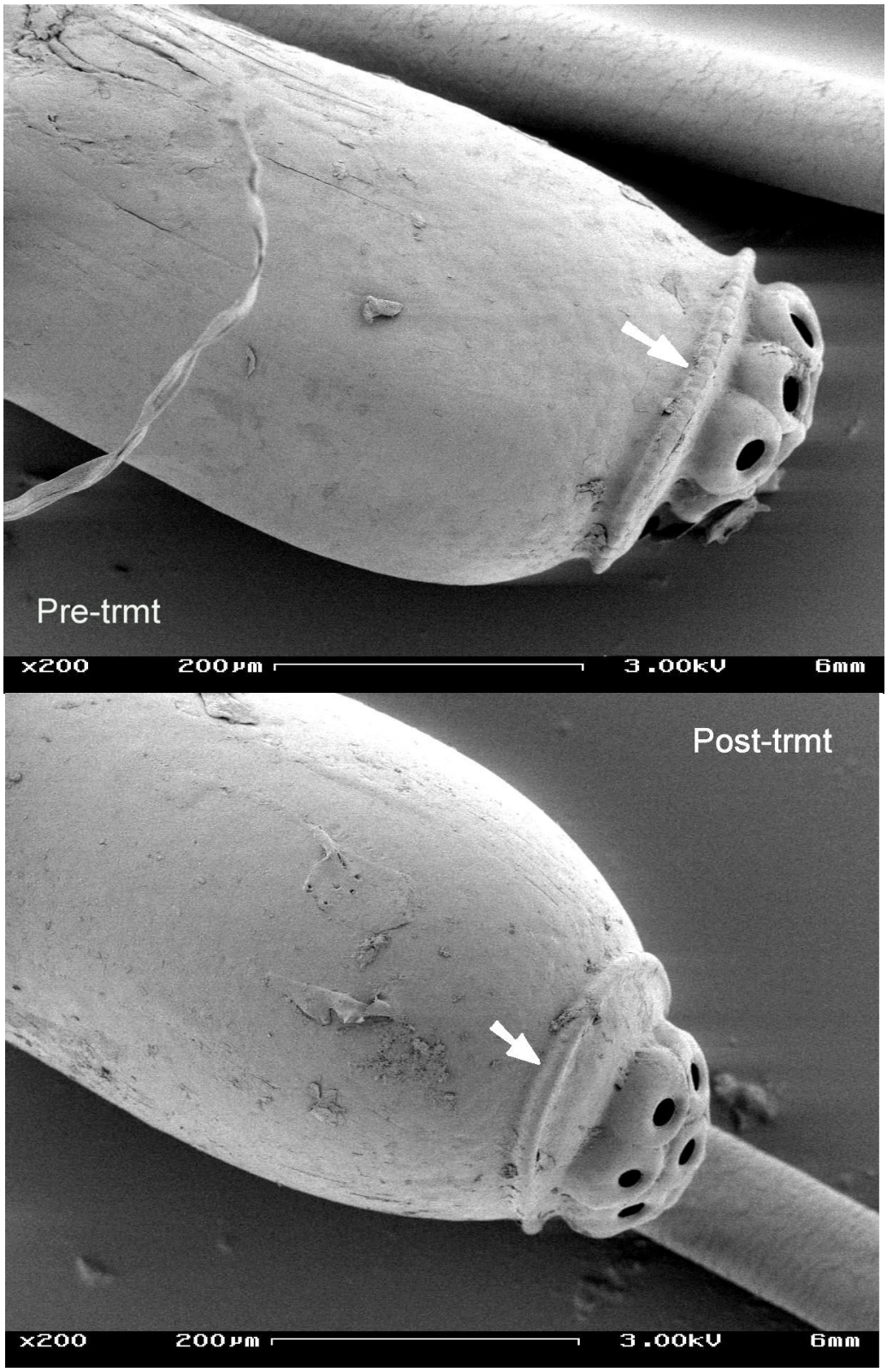


Figure 39. Comparison of eggs.

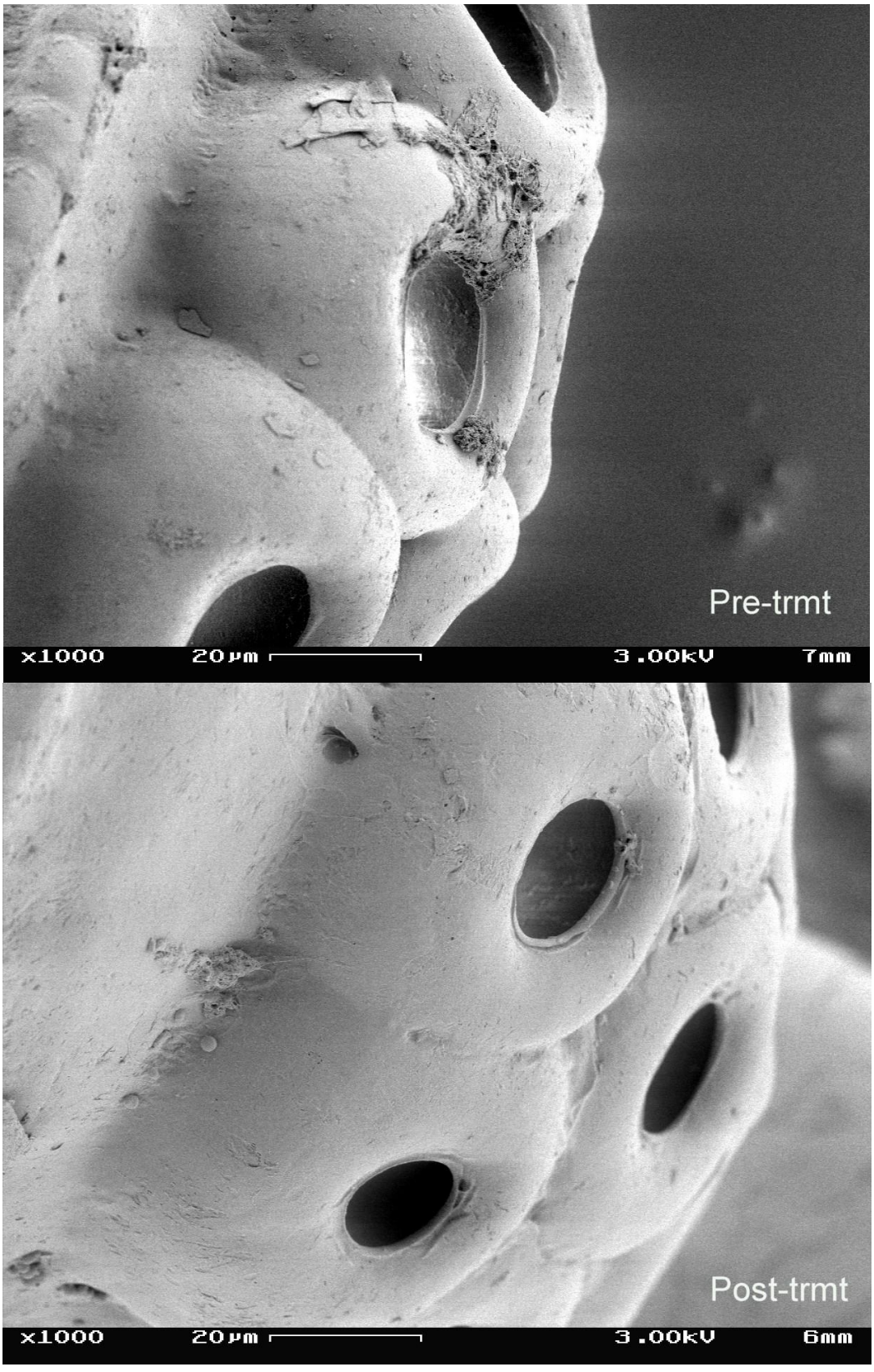


Figure 40. Further magnification of egg for comparison of operculums and pores.

The results of these initial experimentation indicated that citronellyl acetate acts by both hypothesized modes. This finding is significant since compounds that act via different modes of action and multiple modes of action are less likely to be susceptible to cross-resistance and/or ultimate resistance. However, caution is advised for drawing conclusions from these limited initial assessments, and additional experimentation is needed for ultimate confirmation of citronellyl acetate's modes of action.

CHAPTER IV

CONCLUSIONS

The recent documentation that human head lice are quickly developing resistance to currently available treatments has caused great alarm within the pharmaceutical industry and with clinicians, patients, and parents. For more than a decade, no new drugs or products have been approved with an indication for curing head lice infestations. This coupled with the emergence of unsafe and unfounded non-conventional treatments poses a great problem and risk to infested children. Therefore, a great need exists for a new, ethically proven treatment for the safe and effective eradication of head lice. Based on this need, the objectives of the research program were to screen pure citronella oil constituents for possible *in vitro* efficacy, identify a possible target compound, develop an initial delivery system for assessment, identify the lowest concentration of the target compound required for efficacy, assess the potential toxicity and risk of side effects with human use, ultimately optimize the formulation for maximized efficacy and minimized toxicity profiles, and attempt to elucidate the target compound's mode of action.

The experimental design of the screening process effectively identified the feasibility of using the pure citronella oil constituents, citronellyl acetate, citronellal, and citronellol, as new treatments for human head lice infestations. Citronellyl acetate emerged as the target drug compound for additional development and assessment based on its preferred physiochemical and *in vitro* efficacy properties.

Initial vehicle development experiments successfully identified an initial drug delivery system that maximized the *in vitro* pediculicidal qualities of citronellyl acetate and elicited its *in vitro* ovicidal qualities. As with most drug products, the vehicle greatly

affected the active's *in vitro* ovicidal qualities as well as the stability, efficacy, and toxicity profiles of the overall product. Stability studies identified decomposition of citronellyl acetate by acetic acid in the formulation, and identified the incompatibility of the product with HDPE packaging materials. Separation and isolation of the acetic acid and water into a second packaging component created an "add-mix" system that successfully solved the potential long-term stability issue, and the use of PET bottles proved to be compatible with the formulation.

The *in vitro* dose response study performed with the add-mix delivery vehicle successfully identified the 0.75% citronellyl acetate formulation was the lowest strength product that resulted in statistically significant pediculicidal activity. Additionally, the study demonstrated that 3.94% citronellyl acetate was required to possess 50% pediculicidal activity and 8.38% for 100% pediculicidal activity. Interestingly, however, efficacy did not fit linearly with dose. The same assessments revealed that increasing the exposure time from 10 minutes to 20 or 30 minutes did not substantially increase activity.

Based on the results of these initial experiments, US Patent No. 5,902,595 entitled "Pest Removing Composition" was issued on May 11, 1999, and was the first patent to reveal the insecticidal qualities of these GRAS status monoterpenes. A second application was filed demonstrating the ability of the formulation to exhibit statistically significant activity with lower concentrations of citronellyl acetate and is currently pending (PCT Application US00/21417).

Pre-clinical toxicology assessments successfully predicted potential toxicity issues with use of the add-mix drug product in humans and children. *In vitro* assessment of possible dermal penetration revealed no transdermal penetration of citronellyl acetate occurred with a 10-minute topical exposure. Acute oral and dermal toxicity testing demonstrated the add-mix formulation's extremely low toxicity, oral LD_{50}'s of 5000 mg/kg in mice and greater than 2000 but less 5000mg/kg in rats and a dermal LD_{50} of greater than 2000 mg/kg in rabbits. Potential human side effects of skin and eye irritation were also

successfully identified by the assessments since the add-mix formulation was classified as a severe irritant to both the skin and eyes of rabbits.

Based on the findings of the screening studies, initial vehicle development, and pre-clinical toxicity testing, an IND was filed with the FDA in order to proceed with human clinical trials. The results of these human clinical trials posed many obstacles for future use and approval of the add-mix product. First, due to the controlled, remote setting and extensive nit combing used for the initial human study, the placebo control (vehicle) demonstrated nearly the same activity as the products with citronellyl acetate. None of the studies identified a product that possessed any ovicidal activity or a 95% cure rate. Numerous side effects (contact site irritation) were noted when the product was applied to dry hair in the second human clinical study (XP002). Finally, the *in vivo* studies demonstrated very poor correlation with *in vitro* results.

Due to the lack of *in vivo* ovicidal activity, lack of 95% or greater clinical cure, and the high number of patients that experienced irritation, the add-mix product was determined to be undesirable for safe and effective use. Adding to the undesirable nature of the add-mix system was its thin consistency and the mixing requirement of the 2-component system that could both lead to poor compliance, even worse side effects, and misuse.

Therefore, efforts were re-focused on additional optimization of the drug delivery system to maximize efficacy while minimizing potential for side effects, and a new *in vitro* ovicidal assessment method was developed to improve correlation with, and prediction of, *in vivo* ovicidal performance. The new ovicidal assessment method incorporated an application technique most closely mimicking that of *in vivo* product application. By weaving tresses into a human hair wig, the product could be applied identically as in the field. By evaluating the level of embryonic development, valuable insight was gained into ability of a product to penetrate inside of the eggs. The new method resulted in a drastic improvement over the widely used ASTM method. While the new method still did not

produce well-correlated results for total ovicidal activity, developmental observations identified formulations unable to completely penetrate inside of the eggs and began to shed light on the possible mode of lethal action for citronellyl acetate.

Additional experimentation performed for improvement of the drug delivery system successfully produced a target formulation further optimized for potential safety and efficacy. Interestingly, although emulsion techniques were attempted, none of the resulting formulations possessed acceptable *in vitro* efficacy. Surfactant-based, clear gel formulations were identified as a preferred carrier for citronellyl acetate. Subsequently, another *in vitro* dose response study successfully identified that a 5.0% citronellyl acetate formulation was lowest strength product tested that resulted in statistically significant pediculicidal activity, and the 2.5% was the lowest for ovicidal activity. The study demonstrated that 3.3% and 9.0% citronellyl acetate concentrations were required for 50% and 100% pediculicidal activity, respectively, and 3.0% and 8.3% were required for 50% and 100% ovicidal activity, respectively. The study also identified that 11.1% citronellyl acetate was required to produced 100% undeveloped embryos. Primary skin and eye irritation tests identified that the optimized formulation was drastically less irritating to the skin (PII of 4.54, moderate irritant) as compared to the add-mix formulation (PII of 6.75, severe irritant). These additional optimization efforts resulted in another unique drug product for which another separate provisional patent application was filed (US Serial # 60/205843).

Yet another modified *in vitro* method was incorporated for ultimate assessment of the optimized product prior to proceeding towards human clinical trials. This pediculicidal assessment method incorporated treatment of actual human head lice suspected of possessing resistance to current treatments. The method clearly demonstrated the "knockdown" effect and differentiated the potential success of the surfactant-based gel from the potential failure of the other two propylene glycol-based gels.

Finally, an extensive literature search resulted in the identification of the two most probable modes of action for citronellyl acetate. Efficient and cost effective experiments

were designed and performed in attempt to begin to elucidate the mechanisms. The results of a chiral activity study and SEM observations indicate that citronellyl acetate most likely exhibits lethality due to both of the mechanisms identified, isoprenylation inhibition and surface destruction. However, additional testing is required for ultimate confirmation of the resulting indication.

Overall, all objectives of the research program were met. Citronellyl acetate was identified as a new target drug compound for potential treatment of human head lice infestations. Its incorporation into an optimized drug delivery system resulted in a potentially safe and effective drug product. Potential modes of action of the new drug compound were identified and initially elucidated. Also, a total of three patent applications were prepared, and one has already been issued during to the completion of the research goals.

For ultimate *in vivo* assessment and regulatory approval, the optimized formulation needs to be scaled to production size, manufactured, and analyzed for stability according to cGMPs. The previous IND application should be supplemented with all of the new Chemistry, Manufacturing, and Controls (CMC) information. Additional controlled human clinical trials should be performed to ultimately assess the safety and efficacy of the product. Finally, for confirmation on citronellyl acetate's possible modes of action, isoprenylation inhibition experiments (duplication of Crowell, *et. al.*, method) should be performed with chirally pure citronellol or citronellyl acetate, and numerous additional untreated and treated lice should be viewed using SEM for evaluation of surface destruction. Additionally, TEM may also be used to document the difference in cuticle thickness between the untreated and treated lice.

REFERENCES

1. Mumcuoglu, K. Y.; Zias, J. Head Lice, *Pediculus humanus capitis* (Anoplura: Pediculidae) from hair Combs Excavated in Israel and Dated from the First Century B.C. to the Eighth Century A.D. *Journal of Medical Entomology*. **1988**, *25(6)*, 545-47.
2. Burgess, I. F. Human Lice and their Management. *Advances in Parasitology*. **1995**, *36*, 271-342.
3. Brainerd, E. From eradication to resistance: Five continuing concerns about pediculosis. *Journal of School Health*. **1998**, *68(4)*, 146-150.
4. Parish, L. C. History of pediculosis. In *Cutaneous Infestations and Insect Bites*; Orkin, M., Maibach, H. I., Eds.; Marcel Dekker: New York, 1985; pp 159-162.
5. Dodd, C. S. Interventions for treating headlice. *The Cochrane Library*. **1999**, *3*, 1-38.
6. Taplin, D.; Meinking, T. Infestations. In *Pediatric Dermatology*, vol. 2; Schrachner, L. A., Hansen, R. C., Eds.; Churchill Livingstone: New York, 1988; pp 1465-1493.
7. Wickenden, J. Head lice, schools, teachers, and parents. *Health at School*. **1985**, *1*, 18-19.
8. Maunder, J. W. An update on head lice. *Health Visitor*. **1993**, *66(9)*, 317-318.
9. Nuttall, G. H. F. Studies on *Pediculus* I. The copulatory apparatus and the process of copulation in *Pediculus Humanus*. *Parasitology*. **1918**, *9*, 293-324.
10. Nuttall, G. H. F. The biology of *Phthirus pubis*. *Parasitology*. **1918**, *10*, 383-405.

11. Peacock, A. D. The structure of the mounthparts and mechanism of feeding in *Pediculus Humanus*. *Parasitology*. **1918**, *11*, 98-117.

12. Ferris, G. F. Contributions toward a monograph of the sucking lice. *Stanford University Publications: Biological Sciences*. **1935**, *2*, 529-608.

13. Buxton, P. A. *The louse*. Edward Arnold: London, 1947.

14. Maunder, J. W. The appreciation of lice. *Proceedings Royal Institution of Great Britian*. **1983**, *55*, 1-31.

15. Ubelaker, J. E.; Payne, E.; Allison, V. F.; Moore, D. V. Scanning electron microscopy of the human pubic louse, *Pthirus pubis* (Linnaeus, 1758). *Journal of Parasitology*. **1973**, *59*, 913-919.

16. Kadosaka, T.; Kaneko, K. [Studies on sucking lice (Anoplura) in Japan, part 6. A comparative observation of human lice eggs.] (in Japanese) *Japanese Journal of Sanitary Zoology*. **1985**, *36*, 343-348.

17. Burns, D. A.; Sims, T. A. A closer look at *Pthirus Pubis*. *British Journal of Dermatology*. **1988**, *118*, 497-503.

18. Nuttall, G. H. F. The biology of *Pediculus humanus*. *Parasitology*. **1917**, *10*, 80-185.

19. Lang, J. D. Biology and control of the head louse, *Pediculus humanus capitis* (Naoplura: Pediculicidae) in a semi-arid urban area. Ph.D. thesis, University of Arizona. 1975.

20. Chunge, R. N.; Scott, F. E.; Underwood, J. E.; Zavarella, K. J. A pilot study to investigate transmission of head lice. *Canadian Journal of Public Health*. **1991**, *82*, 207-208.

21. BLM. *The Head Lice Diagnosis Pocket Pack*. BLM Publications: Croydon, 1986.

22. Ibarra, J. How to detect head lice. *Health at School*. **1988**, *3*, 109-112.

23. Ibarra, J. "Bug busting": a strategy to combat head lice. *Professional Care of Mother and Child*. **1992**, *2*, 241-244.

24. Maunder, J. W. Updated community approach to head lice. *Journal of the Royal Society of Health*. **1988**, *108*, 210-212.

25. Roberts, C. Head lice. *Practice Nurse*. **1989**, *7*, 108-111.

26. Burgess, I. F.; Brown, C. M.; Burgess, N. A. Synergized permethrin mousse, a new approach to head lice eradication: efficacy in field and laboratory studies. *Clinical Therapeutics*. **1994**, *16*, 57-64.

27. Juranek, D. D. *Pediculus capitis* in school children. Epidemiologic trends, risk factors and recommendations for control. In *Cutaneous Infestations and Insect Bites*; Orkin, M., Maiback, H. I., Eds.; Marcel Dekker: New York, 1985; pp 199-211.

28. Maunder, B. Attitude to head lice – a more powerful force than insecticides. *Journal of the Royal Society of Health*. **1985**, *105*, 61-64.

29. Maunder, J. W. Human lice – biology and control. *Journal of the Royal Society of Health*. **1977**, *97*, 29-31.

30. Ashcroft, M. T. Racial differences in *Pediculus h. capitis* infestation in Guyana. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **1969**, *63*, 547.

31. Maunder, J. W.; Bain, I. L. Some influences on the micro-habitat on the prevalence, distribution and fecundity of human head lice (*Pediculus humanus capitis*). *Abstracts of the XVI International Congress of Entomology, Kyoto*. **1980**, 337.

32. Slonka, G. F.; McKinley, J. W.; McCroan, J. E.; Sinclair, S. P.; Schultz, M. G.; Hicks, F.; Hill, N. Epidemiology of an outbreak of head lice in Georgia. *American Journal of Tropical Medicine and Hygiene*. **1976**, *25*, 739-743.

33. Slonka, G. F.; Fleissner, M. L.; Berlin, J.; Puelo, J.; Harrod, E. K.; Schultz, M. An epidemic of *pediculosis capitis*. *Journal of Parasitology*. **1977**, *63*, 377-383.

34. Juranek, D. D. Epidemiology of lice. *Journal of School Health*. **1977**, *6*, 352-355.

35. Litt, J. Z. The quiddity of the head louse. *Archives of Dermatology*. **1978**, *114*, 1099.

36. Chunge, R. N. A study of head lice among primary schoolchildren in Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **1986**, *80*, 42-46.

37. Iwuala, M. O. E.; Onyeka, J. O. A. The incidence and distribution of head lice, *Pediculus humanus* var. *capitis* (Insecta, Anoplura) in primary and post-primary school pupils in Nsukka, East Central State, Nigeria. *Nigerian Medical Journal*. **1977**, *7*, 274-283.

38. Kwaku-Kpikpi, J. E. The incidence of the head louse (*Pediculus humanus capitis*) among pupils in Accra. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **1982**, *76*, 378-381.

39. Arene, F. O. I.; Ulaulor, A. L. Prevalence of head louse (*Pediculus capitis*) infestation among inhabitants of the Nigeria Delta. *Tropical Medicine and Parasitology*. **1985**, *36*, 140-142.

40. Jinadu, M. K. *Pediculosis humanus capitis* among primary school children in Ife-Ife, Nigeria. *Journal of the Royal Society of Health*. **1985**, *105*, 25-27.

41. Awahmukalah, D. S.; Dinga, J. S.; Nchako-Njikam, J. Pediculosis among urban and rural school children in Kumba, Meme division, south-west Cameroon. *Parassitologia*. **1988**, *30*, 249-256.

42. Ebomoyi, E. *Pediculosis capitis* among primary schoolchildren in urban and rural areas of Kwara State, Nigeria. *Journal of School Health*. **1988**, *58*, 101-103.

43. Dagnew, M. B.; Erwin, G. Epidemiology of common transmissible skin diseases among primary school children in north west Ethiopia. *Tropical and Geographical Medicine*. **1991**, *43*, 152-155.

44. Sinniah, B.; Sinniah, D.; Rajeswari, B. Epidemiology and control of human head louse in Malaysia. *Tropical and Geographical Medicine*. **1983**, *35*, 337-342.

45. Sinniah, B.; Chandra Shekhar; Ramphal, L.; Senan, P. Pediculosis among rural school children in Kelang, Selangor, Malaysia, and their susceptibility to malathion, carbaryl, perigen and kerosene. *Journal of the Royal Society of Health*. **1984**, *104*, 114-115, 118.

46. De Madureira, P. R. Pediculosis and ethnic groups. *International Journal of Dermatology*. **1991**, *30*, 524.

47. Mellanby, K. The incidence of head lice in England. *Medical Officer*. **1941**, *65*, 39-42.

48. Mellanby, K. Natural population of the head louse (*Pediculus humanus capitis*: Anoplura) on infected children in England. *Parasitology*. **1942**, *34*, 180-184.

49. Mellanby, K. The incidence of lice in England after four years of war. *Medical Officer*. **1943**, *70*, 205-207.

50. Coates, K. G. Control of head infestation in children. *Community Medicine*. **1971**, *126*, 148-149.

51. Donaldson, R. J. The head louse in England. Prevalence amongst school children. *Journal of the Royal Society of Health*. **1976**, *96*, 55-57.

52. Ewasechko, C. A. Prevalence of head lice (*Pediculus capitis* [De Geer]) among children in a rural, central Alberta school. *Canadian Journal of Public Health*. **1981**, *72*, 249-252.

53. Stano, G.; Attimonelli, D.; Moncada, G. [Epidemiological study of the spread of head lice amongst school children of the city of Bari.] (in Italian) *Igiene Moderna*. **1981**, *76*, 692-702.

54. Bharija, S. C.; Kanwar, A. J.; Singh, G.; Belhaj, M. S. *Pediculosis capitis* in Benghazi, Libya. A school survey. *International Journal of Dermatology*. **1988**, *27*, 165-166.

55. Lidror, R.; Lifshitz, Y. [A new method for louse control in Israel.] (in Hebrew) *Public Health (Israel)*. **1965**, *8*, 1-12.

56. Maguire, J.; McNally, A. J. Head infestation in school children: extent of the problem and treatment. *Community Medicine*. **1972**, *128*, 374-375.

57. Kim, T. K.; Pak, C. P.; Ho, S. Head louse infestation among students in Yongyang-gun Kyongsangbuk-to, Korea. *Korean Journal of Parasitology*. **1984**, *22*, 273-276.

58. Boyle, P. Pilot study of the prevalence of head lice infestation in a population of Saudi Arabian children. *Family Practioner*. **1987**, *4*, 138-142.

59. Suleman, M.; Fatima, T. Epidemiology of head lice infestation in school children at Peshawar, Pakistan. *Journal of Tropical Medicine and Hygiene*. **1988**, *91*, 323-332.

60. Andrews, J. R.; Tonkin, S. L. Scabies and pediculosis in Tokelau Island children in New Zealand. *Journal of the Royal Society of Health*. **1989**, *109*, 199-203.

61. Ibarra, J. Head lice in schools. *Health at School*. **1989**, *4*, 147-151.

62. Fan, P. C.; Chung, W. C.; Kuo, C. L.; Hsu, H. M.; Chow, C. Y. Present status of head louse (*Pediculus capitis*) infestation among school children in Yunlin County, Taiwan. *Kao Hsiung I Hsueth Ko Hseuh Tsa Chih*. **1990**, *7*, 151-159.

63. Mumcuoglu, K. Y.; Miller, J.; Gofin, R.; Adler, B.; Ben-Ishai, F.; Almog, R.; Kafka, D.; Klaus, S. Epidermiological studies on head lice infestation in Israel. *International Journal of Dermatology*. **1990**, *29*, 502-506.

64. Piotrowski, F. [Head infestations in children and adolescents in Gdansk voivodeship in 1979.] (in Polish) *Wiadomosci Parazytologiezne*. **1982**, *28*, 133-137.

65. Magra-Saenz de Buruaga, G.; Goiria-Ormazabal, J. I.; Lopez-Martinez, I.; Perez-Rodrigo, C.; Bonet-Romero, T.; Caturla-Latorre, J. [*Pediculosis capitis*: epidemiologic study of 23,624 schoolchildren in Bilbao.] (in Spanish) *Revista de Sanidad e Higiene Pública*. **1989**, *63*, 49-62.

66. Pai, K. S.; Park, M. S.; Lee, Y. S.; Kim, D. H.; Chung, K. S.; Lee, K. Y.; Kim, P. K.; Kim, K. Y.; Yong, T. S.; Ree, H. I. [The prevalence of head louse infestation among urban and rural children in Korea] (in Korean) *Kisaengchunghak Chapchi*. **1989**, *27*, 271-275.

67. Cambell, J. D. Peer relations in childhood. *Review of Child Development Research*. **1964**, *1*, 289-322.

68. Ingham, M. *Men*. Century: London, 1984.

69. Suleman, M.; Jabeen, N. Head lice infestation in some urban localities of NWFP, Pakistan. *Annals of Tropical Medicine and Parasitology*. **1989**, *83*, 539-547.

70. Hoffman, G. D. Epidemiology and control of *pediculosis capitis* infestation in the Federal Republic of Germany. *Journal of the Royal Society of Health*. **1983**, *103*, 88-92.

71. Mumcuoglu, K.; Miller, J.; Gofin, R.; Adler, B.; Ben-Ishai, F.; Almog, R.; Kafka, D.; Klaus, S. Head lice in Israeli children: parents' answers to an epidemiological questionnaire. *Public Health Reviews*. **1992**, *18*, 335-344.

72. Lindsay, S. W.; Peock, S. Insecticides against headlice in Glasgow. *Journal of the Royal Society of Health*. **1993**, *113*, 181-183.

73. Hoeppli, R. *Pediculus humanus* and phthiriasis in early Chinese and European medicine. In *Parasites and Parasitic Infections in Early Medicine and Science*; University of Malaya Press: Singapore, 1959; pp 342-359.

74. Busvine, J. R. The comparative toxicity of various contact insecticides to the louse *Pediculus humanus* (L.) and the bed bug (*Cimex lectularius* L.). *Annals of Applied Biology*. **1946**, *33*, 271-279.

75. Scobbie, E. B. S. Substances used in the treatment of *pediculosis capitis*. *British Medical Journal*. **1945**, *i*, 409-412.

76. Peters, B. A. Bile salt as a vehicle for a pediculicide. *British Medical Journal*. **1922**, *i*, 264.

77. Kambu, K.; Di Phanzu, N.; Coune, C.; Wauters, J. N.; Angenot, L. Contribution to the study of the insecticidal and chemical properties of Eucalyptus saligna of Zaire. *Plant Medicines and Phytotherapy*. **1982**, *16*, 34-38.

78. Corlette, C. E. Heliotropine; the best pediculicide. *Medical Journal of Australia*. **1925**, *i*, 185-186.

79. Greene, E. M. Pediculosis in Boston's public schools. *Boston Medical and Surgical Journal*. **1898**, *138*, 70-71.

80. Thevasagayam, E. S.; Perera, W. S.; Chow, C. Y. Head lice in Ceylon. In *Mimeographed Document No. WHO/Insecticides/17*; World Health Organization: Geneva, 1953.

81. Hatsushika, R.; Miyoshi, K. Human head louse, *Pediculus humanus capitis* (Anoplura), infestation. *Kawasaki Medical Journal*. **1983**, *9*, 29-34.

82. Brinck-Lindroth, G.; Lundqvist, L.; Nilsson, A. Control of the human head louse with disulfiram and benzyl benzoate emulsions in a laboratory study. *Acta Demato-Venereologica*. **1984**, *64*, 325-330.

83. Dubbreuilh, W.; Beille, L. [*Animal Parasites of the Skin*.] (in French) Masson: Paris, 1895.

84. Jamieson, W. A. The treatment of *pediculosis vestimentorum*. *Journal of Dermatology*. **1895**, *7*, 248-249.

85. Nuttall, G. H. F. Combating lousiness among soldiers and civilians. *Parasitology*. **1918**, *10*, 411-586.

86. Busvine, J. R.; Buxton, P. A. A new method of controlling the head louse. *British Medical Journal*. **1942**, *ii*, 464-466.

87. Alexander, J. O'D. In *Arthropods and Human Skin*; Springer: New York, 1984; pp 29-55.

88. Casella, J. P.; Baxter, G. F.; Hay, J. Crab louse infestation: are "blue spots" caused by "blue ointment"? *The Entomologist.* **1991**, *110*, 131-133.

89. Ashkenazi, I.; Abraham, F. A. [Phthiriasis palpebrarum] (in Hebrew) *Harefuah.* **1989**, *117*, 33-34.

90. Mantegna, M.; Marasa, L.; Amato, G. [From pthtiriasis to erthroderma.] (in Italian) *Annali Tialiano di Dermatologia clinica e Sperimentale.* **1982**, *36*, 279-286.

91. Puzatov, A. K.; Orlik Giu; Chausovski, B. S. [Toxicoderma following the use of grey ointment.] (in Russian) *Vrachebnoe Delo.* **1990**, *ii*, 94.

92. Soboleva, N. I. [Employment of preparation 'K' for pediculosis control.] (in Russian) *Zhurnal Mikrbiologii, Épidemiologii I Immunobiologii.* **1944**, *i*, 64-66.

93. Lenz, A. [On a new method of treating head lice with sulphur dioxide.] (in German) *Münchener Medizinische Wochenschrift.* **1921**, *68*, 1252-1253.

94. David, W. A. L. Fumigation as a method of controlling the body louse *Pediculus humanus corporis*, De Geer. Parts I and II. *Bulletin of Entomological Research.* **1944,** *35*, 79-89.

95. Busvine, J. R. The toxicity of some common fumigants to body lice. *Bulletin of Entomological Research.* **1943**, *34*, 19-26.

96. Buxton, P. A. The use of the new inecticide DDT in relation to the problems of tropical medicine. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* **1945**, *38*, 367-393.

97. Sinniah, B.; Sinniah, D. Comparative study of DDT, carbaryl (Sevin) and Actellic (pirimiphos-methyl) in the treatment of *Pediculus humanus capitis* infestation. *Public Health.* **1983**, *97*, 3-7.

98. Rupes, V.; Ledvinka, J.; Chmela, J.; Pinterova, J. [Susceptibility to DDT and some other insecticides of head lice, *Pediculus capitis*, in Czechoslovakia.] (in Czech) *Folia Parasitologi.* **1984**, *31*, 169-175.

99. Courtiade, C.; Labrèze, C.; Fontan, I.; Taïeb, A.; Maleville, J. [Pediculosis of the scalp: study by questionnaire in four groups of school students of Bordeaux in 1990-1991.] (in French) *Annales de Dematologie et de Vénéréologie.* **1993**, *120*, 363-368.

100. Sundnes, K. O.; Haimanot, A. T. Epidemic of louse-borne relapsing fever in Ethiopia. *Lancet.* **1993**, *342*, 1213-1215.

101. Bloomquist, J. R. Lecture notes. Insecticides: Chemistries and Characterisitics. Last modified Feb. 23, 1999. Retrieved Oct. 5, 2000. <http://ipmworld.umn.edu/chapters/bloomq.htm>.

102. Culver, C. A.; Malina, J. J.; Talbert, R. L. Probable anaphylactoid reaction to a pyrethrin pediculocide shampoo. *Clinical Pharmacology.* **1988**, *7*, 846-849.

103. Ware, G. W. Leture notes. An Introduction to Insecticides (3rd edition). Last modified Thursday, 23 December, 1999. Retrieved Sept. 24, 2000. <http://ipmworld.umn.edu/chapters/ware.htm>

104. Taplin, D.; Meinking, T. L.; Castillero, P. M.; Sanchez, R. Permethrin 1% creme rinse for the treatment of *Pediculus humanus* var. *capitis* infestation. *Pediatric Dermatology.* **1986**, *3*, 344-348.

105. Brandenberg, K.; Deinard, A. S.; Di Napoli, J.; Englender, S. J.; Orthoefer, J.; Wagner, D. 1 percent permethrin creme rinse vs. 1 percent lindane shampoo in treating pediculosis capitis. *American Journal for Diseases of Childhood.* **1986**, *140*, 894-896.

106. Bowerman, J. G.; Gomez, M. P.; Austin, R. D.; Wold, D. E. Comparative study of permethrin 1% creme rinse and lindane shampoo for the treatment of head lice. *Pediatric Infectious Disease Journal.* **1987**, *6*, 252-255.

107. Carson, D. S.; Tribble, P. W.; Weart, C. W. Pyrethrins combined with piperonyl butoxide (RID) vs. 1% permethrin (NIX) in the treatment of head lice. *American Journal for Diseases of Childhood*. **1988**, *142*, 768-769.

108. Di Napoli, J. B.; Austin, R. D.; Englender, S. J.; Gomez, M. P.; Barret, J. F. Eradication of head lice with a single treatment. *American Journal of Public Health*. **1988**, *78*, 978-980.

109. Haustein, U. F. [Pyrethrin and pyrethroid (permethrin) in the treatment of scabies and pediculosis.] (in German) *Hautartz*. **1991**, *42*, 9-15.

110. Andrews, E. B.; Joseph, M. C.; Magenheim, M. J.; Tilson, H. H.; Doi, P. A.; Schultz, M. W. Postmarketing surveillance study of permethrin creme rinse. *American Journal of Public Health*. **1992**, *82*, 857-861.

111. Maunder, J. W. Resistance to organochlorine insecticides in head lice , and trials using alternative compounds. *Medical Officer*. **1971**, *125*, 27-29.

112. Cole, M. M.; Burden, G. A. Phosphorus compounds as ovicides and adulticides against body lice. *Journal of Economic Entomology*. **1956**, *49*, 747-750.

113. Barnes, W. W.; Eldridge, B. F.; Greenberg, J. H.; Vivona, S. A field evaluation of malathion dust for the control of body lice. *Journal of Economic Entomology*. **1962**, *55*, 591-594.

114. Maunder, J. W. Use of malathion in the treatment of lousy children. *Community Medicine*. **1971**, *126*, 145-147.

115. Clark, P. H.; Cole, M. M. Resistance of body lice to carbaryl. *Journal of Economic Entomology*. **1967**, *60*, 398-400.

116. Moore, W.; Hirschfelder, A. D. An investigation of the louse problem. *Research Publications of the University of Minnesota*. **1919**, *8(4)*, 1-86.

117. Buxton, P. A. The biology of the body louse (*Pediculus humanus corporis*: Anoplura) under experimental conditions. *Parasitology*. **1940**, *32*, 303-312.

118. Culpepper, G. H. The rearing and maintenance of a laboratory colony of the body louse. *American Journal of Tropical Medicine*. **1944**, *24*, 327-329.

119. Culpepper, G. H. Factors influencing the rearing and maintenance of a laboratory colony of the body louse. *Journal of Economic Entomology*. **1946**, *39*, 472-474.

120. Culpepper, G. H. Rearing and maintenance of a laboratory colony of the body lice on rabbits. *American Journal of Tropical Medicine*. **1948**, *28*, 499-504.

121. Smith, C. N.; Eddy G. W. Techniques for rearing and handling body lice, oriental rat fleas, and cat fleas. *Bulletin of World Health Organization*. **1954**, *10*, 127-137.

122. Cole, M. M. Body lice. In *Insect Colonization and Mass Production*; Smith, C. N. Ed.; Academic Press: New York, 1966; pp 15-24.

123. ASTM. Standard testing methods for determining the effectiveness of liquid, gel, cream or shampoo insecticides against human lice, E938-83 (91). American Society for Testing and Materials: Philadelphia, 1991.

124. ASTM. Standard testing methods for determining the effectiveness of liquid, gel, cream or shampoo insecticides against human louse eggs, E1517-93. American Society for Testing and Materials: Philadelphia, 1993.

125. Kyle, D. R. Comparison of phenothrin shampoo and malathion lotion in the treatment of head louse infestation. *Journal of the Royal Society of Health*. **1990**, *110*, 62-63.

126. Miller, T. A. Lecture notes. Chapter 13: Insecticide Resistance. Last modified Sept. 18, 1998. Retrieved Nov. 4, 2000. <http://wcb.ucr.edu/wcb/schools/CNAS/ntm/tmiller /1/ modules/page31.html>.

127. Mumcuoglu, K. Y.; Hemingway, J.; Miller, J.; Ioffe-Uspensky, I.; Klaus, S.; Ben-Ishai, F.; Galun, R. Permethrin resistance in the head louse *Pediculus capitis* from Israel. *Medical and Veterinary Entomology*. **1995**, *9*, 427-432.

128. Shono, T. Pyrethroid resistance: importance of the kdr type mechanism. *Journal of Pesticide Science*. **1985**, *10*, 141-146.

129. WHO. Vector resistance to pesticides. In *Fifteenth report of the expert committee on vector biology and control. Technical Report Series, 818*; World Health Organization: Geneva, 1992.

130. Williamson, M. S.; Denholm, I.; Bell, C. A.; Devonshire, A. L. Knockdown resistance (kdr) to DDT and pyrethroid insecticides maps to a sodium channel gene locus in the housefly (*Musca domestica*). *Molecular and General Genetics.* **1993**, *240*, 17-22.

131. Juvin, P.; Moreau, P. US Patent No. 4,518,593. 1985.

132. United States Code of Federal Regulation. 21 CFR 172.515. 1998.

133. Anonymous. Carcinogenesis Studies of Food Grade Geranyl Acetate (71% Geranyl Acetate, 29% Citronellyl Acetate) (CAS No. 105-87-3) in F344/N Rats and B6C3F1 Mice (Gavage Study). *Govt Reports Announcements & Index (GRA&I).* **1988**, 12.

134. United States Department of Health and Human Services. Guide for the Care and Use of Laboratory Animals. *DHHS Publication No. (NIH) 86-23.* **1985**.

135. Draize, J. H. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics. *The Association of Food and Drug Officials of the United States*: **1959**, 49-51.

136. US Environmental Protection Agency. Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals-Addendum 3 on Data Reporting. 1988.

137. Kay, J. H.; Calandra, J. C. Interpretation of Eye Irritation Tests. *Journal of the Society of Cosmetic Chemists.* **1962**, *13*, 281-289.

138. Franz, T. J. The finite dose technique. *Current Problems in Dermatology.* **1978**, *7*, 58-68.

139. Sun, Y.; Liu, J.; Kimbleton, E.; Wang, J. US Patent 5,993,787. 1999.

140. Crowell, P. L.; Ren, Z.; Lin, S.; Vedejs, E.; Gould, M. N. Structure-activity relationships among monoterpene inhibitors of protein isoprenylation and cell proliferation. *Biochemical Pharmacology.* **1994**, *47(8)*, 1405-15.

141. Crowell, P. L.; Chang, R. R.; Ren, Z.; Elson, C.; Gould, M. N. Selective inhibition of isoprenylation of 21-26-kDa proteins by the anticarcinogen d-limonene and its metabolites. *Journal of Biological Chemistry.* **1991**, *266(26)*: 17679-17685.

142. Ren, Z.; Gould, M. N. Modulation of small G protein isoprenylation by anticancer monoterpenes in in situ mammary gland epithelial cells. *Carcinogenesis.* **1998**, *9(5)*, 827-832.

143. Schulz, S.; Buhling, F.; Ansorge, S. Prenylated proteins and lymphocyte proliferation: inhibition by d-limonene related monoterpenes. *European Journal of Immunology.* **1994**, *24(2)*, 301-307.

144. Uribe, S.; Rangel, P.; Peña, A. Molecular association enhance the toxic effects of non-substituted monoterpene suspensions on isolated mitochondria. *Xenobiotica.* **1991**, *21(5)*, 67-688.

145. Popják, G.; Hadley, C. Inhibition of liver prenyltransferase by citronellyl and geranyl phosphonate and phosphonylate. *Journal of Lipid Research.* **1985**, *26*, 1151-1159.

146. Hardcastle, I. R.; Rowlands, M. G.; Barber, A. M.; Grimshaw, R. M.; Mohan, M. K.; Nutley, B. P.; Jarman, M. Inhibition of protein prenylation by metabolites of limonene. *Biochemical Pharmacology.* **1999**, *57*, 801-809.

147. Helmkamp, G. M.; Rawitch, A. B.; Grant, J. R. G-proteins (interactive models). Retrieved Aug. 12, 2000. <http://www.kumc.edu/research/medicine/biochemistry/bioc800/gprotfr.html>

148. Helmkamp, G. M.; Grant, J. R. G-Signal Animation. Retrieved Aug. 18, 2000. <http://www.kumc.edu/research/medicine/biochemistry/bioc800/g-signal.html>

149. Helmkamp, G. M.; Grant, J. R. G-PLC Animation. Retrieved Aug. 18, 2000. <http:// www.kumc.edu/research/medicine/biochemistry/bioc800/g-plc.html>

150. Franklin, L. U.; Cunnington, G. D.; Young, D. E. US Patent No. 6,130,253. 1999.