Practice Oriented Results on Use and Production of Neem-Ingredients and Pheromones

Neem-Ingredients: Toxicological Results and Possible Medical Uses

Organized by:
H. Kleeberg
(Ed.)

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Practice Oriented Results on Use and Production of Neem-Ingredients and Pheromones VII

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<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Welcoming address</td>
<td></td>
</tr>
<tr>
<td><strong>Azadirachtin - A Review of its Mode of Action in Insects</strong></td>
<td>1</td>
</tr>
<tr>
<td>Jennifer A. Mordue</td>
<td></td>
</tr>
<tr>
<td><strong>A Comparison of the Effects of Azadirachtin A on Cultured Insect and</strong></td>
<td>5</td>
</tr>
<tr>
<td>Mammalian Cells</td>
<td></td>
</tr>
<tr>
<td>A. Jabbar, Robin H. C. Strang</td>
<td></td>
</tr>
<tr>
<td><strong>AzadirachtinA-content and Bio-efficacy in Hair Treated with</strong></td>
<td>9</td>
</tr>
<tr>
<td>NeemAzal-formulations</td>
<td></td>
</tr>
<tr>
<td>Ralf Troß, Vera Bernauer-Jacob, Edmund Hummel, Hubertus Kleeberg</td>
<td></td>
</tr>
<tr>
<td><strong>Toxicological Results of NeemAzal-technical and NeemAzal-formulations</strong></td>
<td>21</td>
</tr>
<tr>
<td>Robert R. Stewart</td>
<td></td>
</tr>
<tr>
<td><strong>Neem-Therapie der kindlichen Scabies</strong></td>
<td>27</td>
</tr>
<tr>
<td>Franz-Josef Knust</td>
<td></td>
</tr>
<tr>
<td><strong>Erste Ergebnisse mit NeemAzal-Formulierungen gegen Flöhe</strong></td>
<td>31</td>
</tr>
<tr>
<td>Vera Bernauer-Jacob</td>
<td></td>
</tr>
<tr>
<td><strong>NeemAzal in der Anwendung bei Pferd und Esel</strong></td>
<td>37</td>
</tr>
<tr>
<td>Ellen Freudenstein</td>
<td></td>
</tr>
<tr>
<td><strong>Anti Bacterial Activity of Neem Extract: Determination of Minimum</strong></td>
<td>39</td>
</tr>
<tr>
<td>Active Concentration against Dermatological Bacterial (<strong>Staphylococcus</strong></td>
<td></td>
</tr>
<tr>
<td>and <strong>Pseudomonas sp.</strong>)</td>
<td></td>
</tr>
<tr>
<td>Rigobert T. Yaméogo</td>
<td></td>
</tr>
<tr>
<td><strong>Effect of Azadirachta indica (Neem) Extract on Paracetamol Induced</strong></td>
<td>47</td>
</tr>
<tr>
<td>Hepatotoxicity</td>
<td></td>
</tr>
<tr>
<td>Nahed Abdel-Maksoud, Madeha M. Zakhary, Manal M. Shehata, Amira M. El-</td>
<td></td>
</tr>
<tr>
<td>Noweihi, Adel A. Gomaa, and I.M. Kelany</td>
<td></td>
</tr>
</tbody>
</table>
Dear ladies and gentlemen,

after we have organized 6 workshops predominantly on Neem, which concentrated our attention on plant protection and pest control, we have now invited you for a totally different topic, namely the "Toxicological Properties and Possible Medical Uses of Neem-Ingredients". Due to the various traditional (Indian) medical applications of Neem-extracts, it seems interesting to have a special discussion on this subject. Although, we are a small group of participants, I hope that we will have an interesting time, plenty of discussions and new incitements during the short period of our workshop.

Wetzlar, June 20. 1997

Hubertus Kleeberg
AZADIRACHTIN - A REVIEW OF ITS MODE OF ACTION IN INSECTS

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Azadirachtin, from the neem tree *Azadirachta indica* (A Juss), is the main biologically active component of neem insecticides (for the latest major work on neem see Schmutterer, 1995). Azadirachtin is a tetranaortriterpenoid plant limonoid with potent insect antifeedant and growth disrupting properties. It was isolated from the seeds of *Azadirachta indica* by Butterworth and Morgan (1968) and its full structural determination was completed some 17 years later. Nakanishi and co-workers presented the first structure proposal (Zanno *et al.*, 1975), the correct structure appeared in papers submitted in 1985 by Broughton *et al.* and Kraus *et al.* (Kraus *et al.*, 1985; Broughton *et al.*, 1986), and the full details were finally described by all groups in 1987 (Bilton *et al.*, 1987; Kraus *et al.*, 1987a; Turner *et al.*, 1987).

CHEMISTRY

Azadirachtin, also termed Azadirachtin A (Rembold, 1989) is the major component of neem seeds. 3-Tigloylazadirachtol (azadirachtin B) is present at concentrations up to 20% of that of azadirachtin, other azadirachtins (C-L) occur at much lower concentrations. For a recent comprehensive review of the chemistry of azadirachtin see Ley *et al.* (1993) and Kraus, (1995). The biosynthesis of azadirachtin is thought to involve tirucallol, a tetracyclic triterpenoid, and a series of oxidation and rearrangement reactions which produce finally, the tetranaortriterpenoid azadirachtin family (Ley *et al.*, 1993). In addition to the azadirachtin group of compounds in seeds several other families of tetranaortriterpenoids are present. Examples of these are nimbin and salannin which show moderate to good antifeedant effects against many insects although are not very physiologically active. Azadiradione and nimbolide are other triterpenoids which have some insect antifeedant activity and are effective against a number of bacteria. They occur in minute quantities in some neem insecticides and are important in that they have been implicated as the principal cytotoxic component of neem seed insecticide preparations (see Kraus 1995 for review) Cohen *et al.*, 1996.

BIOLOGICAL EFFECTS

Azadirachtin has strong antifeedant activity against many insect species, which is supplemented also by marked insect growth regulatory (IGR) and sterility effects. While the antifeedancy effects may vary between different insect species the IGR and sterility effects of azadirachtin are highly consistent (Mordue Luntz & Blackwell, 1993).

a) Antifeedancy

Investigations of the antifeedancy mode of action, using azadirachtin and both natural and synthetic analogues, have revealed that both polyphagous and oligophagous species of insect are behaviourally sensitive to azadirachtin with the most sensitive species being able to differentiate extremely small changes in the parent molecule.

Antifeedancy effects fall into two different categories. Firstly, there are primary antifeedancy (gustatory) effects where azadirachtin is being perceived at the...
chemoreceptor level by the insect taste receptors on the feet and mouthparts. Azadirachtin stimulates the deterrent cells and inhibits the sugar (phagostimulatory) cells in these receptors (Simmonds & Blaney, 1994) which leads to a behavioural avoidance of foodplants treated with azadirachtin. Secondly, there are secondary antifeedancy effects (anorexia) brought about by the physiological toxic effects of azadirachtin after ingestion or uptake. The primary antifeedancy effects of azadirachtin vary markedly between different insect species depending upon differences in chemoreceptor sensitivity and central nervous integration mechanisms. Lepidopterous larvae are the most behaviourally sensitive Order of insects to azadirachtin, Coleoptera, Hemiptera and Hymenoptera have moderate behavioural sensitivity to azadirachtin whereas the Orthoptera have a large range of sensitivities depending on species, for example, the desert locust, Schistocerca gregaria is deterred in feeding bioassays at 0.001 ppm quantities of azadirachtin whereas the North American plains grasshopper Melanoplus sanguinepes is not deterred by concentrations as high as 1000 ppm (Table 1).

<table>
<thead>
<tr>
<th>Insect Order</th>
<th>ED50 (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepidoptera</td>
<td>&lt; 0.001 - 50</td>
</tr>
<tr>
<td>Coleoptera</td>
<td>100 - 500</td>
</tr>
<tr>
<td>Hemiptera</td>
<td>100 - 500</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>100 - 500</td>
</tr>
<tr>
<td>Orthoptera</td>
<td>0.001 - &gt;1000</td>
</tr>
</tbody>
</table>

**b) Physiological effects (IGR/sterility)**

The physiological effects of azadirachtin are more consistent between species, with the average ED50 being approximately 2μg azadirachtin/g body weight. These effects also are of two types: firstly, indirect effects are modulated via the insects' endocrine system and secondly, direct effects occur where azadirachtin is acting directly on specific tissues. In the former, a blockage of release of the neurosecretory hormones from the brain leads to a blockage or delayed release of the moulting hormone β-ecdysone and juvenile hormone (Mordue (Luntz) & Blackwell, 1993). These two hormonal systems together act in concert to induce the moult and metamorphosis to the adult stage. In the adult the hormones are also involved in egg development. Disruption of these morphogenetic hormones results in the typical insect growth regulatory effects seen in azadirachtin treated insects i.e. slowing of growth, delayed moulting, moult abnormalities and an inability to complete ecdysis (shed the cast skin). In adults, it is also responsible for the inhibition of ovarian development, the resorption of eggs, reduced fecundity and sterility. In the latter the direct effects of azadirachtin can be seen in those tissues with rapidly dividing cells e.g. wing buds, testes, where azadirachtin blocks cell division at prometaphase I; also in muscles, where a flaccid paralysis is seen; and the midgut, where specific digestive enzymes are not produced.
Both the behavioural and physiological actions of azadirachtin work in concert together and crop protection results from a combination of feeding deterrence, growth and moulting aberrations and reduced fecundity in a manner which is specific to each species. *S. gregaria* for example will choose to starve to death rather than ingest barley seedlings sprayed with azadirachtin, the aphid *Myzus persicae* is not deterred at all by azadirachtin concentrations less than 100 ppm, however numbers of progeny are significantly reduced by concentrations as low as 5 ppm (Mordue (Luntz) et al, 1996).

c) **Biochemical effects**

Specific binding studies using tritiated dihydroazadirachtin as the binding ligand are beginning to reveal the basic cellular lesion of azadirachtin (Nisbet et al, 1997). The binding characteristics of the ligand to homogenates of *S. gregaria* testes and to an insect cell line (*Spodoptera* Sf9 cells) have revealed specific, time dependent, saturable binding to proteins within the cells either at the sperm tails of developing sperm, or in the nuclei of the Sf9 cells. Analysis of the saturation characteristics has indicated one population of binding sites. Once at a maximum level of binding, dissociation of the radioligand: membrane complex in the presence of unlabelled parent compound is incomplete i.e. binding is semi-permanent. The competitive binding of different analogues of azadirachtin to these binding sites revealed similar orders of potency to those found with antifeedancy bioassays or IGR effects which strongly suggests a causal link between the ability to bind membrane proteins specifically and the ability of the molecule to exert biological effects. Further studies will enable the characterisation and identification of the binding proteins which will lead to a resolution of its cellular mode of action.

**CONCLUSIONS**

A detailed understanding of the biological activity of azadirachtin and its analogues against different pests has now been achieved. Biochemical mode of action studies are beginning to shed light on the cellular action. Such studies are providing essential background knowledge for the successful introduction of neem based products into the insect control market.


A comparison of the effects of azadirachtin A on cultured insect and mammalian cells.

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Introduction.

Rembold and Annadurai (1993) demonstrated that the growth and replication of the insect cell line, Sf9, derived from ovarian cells of Spodoptera frugiperda, was strongly inhibited by the presence of low concentrations of azadirachtin A added to the medium. Moreover, they observed that different neem terpenoids had different potencies in a way which mimicked their effects on the whole living insects. These studies not only give hope that the effect of neem terpenoids may be studied in cell culture, but also that the action of the compounds lies at the cellular level.

In practical terms, one of the potential advantages of neem terpenoids as insecticides is their low toxicity towards mammals and vertebrates in general. It is a reasonable hypothesis that if the effect against insects can be shown at the cellular level, then the effect, (or lack of it), against mammals may also be evident at the cellular level.

The aim of the work reported here was to use cultures of insect and mammalian cells to extend the work of Rembold and Annadurai to compare the effect of pure azadirachtin A on different cell lines.

Materials and Methods.

Cell lines.

The following cell lines were employed: Sf9 (derived from the ovarian cells of Spodoptera frugiperda; L929 (derived from mouse fibroblast); P9 (a liver cell line transformed by means of SV40 virus); MCF 7 (derived from human breast cancer). All were maintained under sterile conditions in appropriate media, and growth conditions, and were recultured on a weekly basis.

For growth experiments the cells were allowed to grow in the wells of a multi-well plate with 8 replicate for each set of conditions. 100μl of medium containing 20-100x10^3 cells.ml⁻¹ were introduced into each well, and fresh medium added. The cells were then allowed to replicate in the presence and absence of azadirachtin A for 5 days, and the number of viable cells present estimated as described below.
Estimation of cell number by colorimetry.

The method used was based on the capacity of living cells to reduce the dye 3-(4,5-dimethylthiazol-2-yl)-(2,5-diphenyltetrazolium bromide) (MTT) to form a water-insoluble formazan dye (Mosmann, 1983).

At the end of the growth period, 50μl of a solution of MTT was added to the wells, and the colour allowed to develop for 15-30 min. depending on the number of cells present. After the insoluble dye had settled, the supernatant of cells and medium was aspirated off by means of suction, and the formazan dissolved in 200μl of dimethyl sulphoxide. The amount of reduced formazan was then estimated at 540nm by means of an ELISA plate reader.

Preliminary experiments involving counting cells by means of a haemocytometer had indicated that there was a linear relationship between cell numbers and the colour developed over a range of from 0.2x10^3 to 25x10^3 cells/ well.

Azadirachtin A.

The terpenoid was purified from Indian neem seeds by the usual methods of solvent extraction, partition, and column chromatography. It was finally recrystallised from tetrachloromethane. Its purity was assessed as >95% by reverse phase HPLC and NMR spectroscopy.

The terpenoid was dissolved in a range of concentrations in 10% ethanol, and aliquots of the solutions added to wells so that the final concentration of ethanol was 1%, and the range of final concentrations of azadirachtin A was from 1x10^{-14}M to 1x10^{-3}M. Control wells contained only the ethanol in the medium.

Results.

The effects of a wide range of concentrations of azadirachtin are shown in Fig. 1. They confirm that the cells of the insect line Sf9 are very sensitive to the neem terpenoid. The growth of the cells is severely inhibited with an EC50 of 10^{-10}M.

In sharp contrast to this result, the mammalian cell lines used were little affected by the presence of azadirachtin in the medium until the concentrations were very high indeed. It was only at concentrations in excess of 1x10^{-4}M that the cell growth was noticeably reduced. No significant differences could be detected in the response of the three cell lines used, all of which had been derived from different mammalian tissue types.
Fig. 1. Comparison of the effects of azadirachtin A on the growth of insect and mammalian cultured cells.

Azadirachtin in the growth medium
(log 10 molar concentration)
Discussion.

The results using the line of cells derived from the ovary of *Spodoptera frugiperda* confirm and extend those previously reported by Rembold and Annadurai (1993). They show that the replication of the insect cells is inhibited, and it has been possible to give an exact EC50 of $10^{-10}$M due to the ease of the method which allows for a large number of simultaneous replicates and eliminates the laborious process of counting the cells. The use of the formazan method also removes the uncertainty of numbers of dead and viable cells, as the colour development depends on the presence of intact, functional mitochondria.

The present authors have used the method to compare the effectiveness of a range of other neem terpenoids and their derivatives in inhibiting the growth of Sf9 cells (Jabbar and Strang, unpublished results) The order of potency of effect is very similar to that which has been established for whole living insects by, for instance, the *Epilachna* bioassay of Rembold and Puhlmann (1993). This gives added weight to the contention that the effects of the neem terpenoid which disrupt the growth of the developing insect can be studied in cell culture.

The other result of interest reported here is the lack of sensitivity to azadirachtin shown by three cell lines derived from mammalian tissues. It is, of course dangerous to extrapolate too much from a limited number of transformed mammalian cell types, but the results are certainly consistent with the fact that the high toxicity of azadirachtin is limited to invertebrate species, and that this difference is apparent at the cellular level.

References.


AzadirachtinA-content and Bio-efficacy in Hair Treated with NeemAzal-Formulations

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Summary: Bioefficacy data and analytical results are compared for NeemAzal-formulations on felt, hair of dogs and human hair after applications against textile pests, flea and lice respectively. Due to the dry surrounding conditions of the stored textiles the degradation of AzadirachtinA is very slow and the biological activity lasts for at least half a year. In dogs coat AzadirachtinA is present due to incomplete flushing of the hair at intermediate levels and remains for several days. This may lead to several days protection against new infestation. In human hair the Neem-ingredient is washed off practically completely after the application. Still the short contact of lice with the active ingredient seem to be sufficient for a satisfactory control after 3 applications.

Introduction

Neem-products are traditionally used in India for several medical and cosmetic purposes. Preparations of different parts of the tropical Neem tree (Azadirachta indica A. Juss) are used as an antiseptic, against endo- or ectoparasites, for cure of dandruff or simply as a hair or skin conditioner/strengthener (Ketkar & Ketkar, 1993).

In addition to the traditional experience, many scientific reports show that Azadirachtin containing preparations of Neem-seeds are comparable in their efficacy to synthetic chemicals (2, 3). However, various toxicological investigations show that Neem-ingredients are safe to humans and mammals (see: Stewart, this volume) and act through their specific influence (see: Jabbar & Strang, this volume) on the hormonal system of the insects concerned (see: J Mordue, this volume).

NeemAzal is a specific, standardized extract of the seeds of the Neem tree and concentrates the active ingredient which is responsible for the effects on insects, namely Azadiracthins - which are a group of Tetranortriterpenoids - with a similar chemical structure. The most common representative of this group is AzadirachtinA, which is contained in NeemAzal to 34% in an average (4, 5). The other structurally determined Azadiracthins comprise about 17% and other analytically determined Limonoids (like Salannin, Nimbin) about 6% of the dry weight of NeemAzal.
Furthermore NeemAzal contains about 5% nonpolar material (like esters of fatty acids), about 5% hydrate water, not further characterized low molecular weight material like peptides, (oligo-)-saccharides and traces of salts.

For different purposes appropriate formulations have been developed. The intended areas of application and the content of AzadirachtinA are summarized in table 1.

With the exception of Neem-Extract-ointment the NeemAzal-formulations have a shelf live of about 2 years under proper (cool) storage conditions.

**Table 1:** The intended area of application and the AzadirachtinA (AzA) content of different formulations of NeemAzal (≤ 34% AzadirachtinA) is summarized.

<table>
<thead>
<tr>
<th>formulation</th>
<th>intended area of application</th>
<th>AzA-cont. %</th>
<th>remarks, other possible areas of application</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeemAzal-T/S</td>
<td>plant protection</td>
<td>1%</td>
<td>under registration in BRD</td>
</tr>
<tr>
<td>NeemAzal-T</td>
<td>plant protection</td>
<td>5%</td>
<td>registered in Switzerland</td>
</tr>
<tr>
<td>NeemproTex:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>akut</td>
<td>textile protection</td>
<td>0.3%</td>
<td>animal surrounding (pets), domestic mites?</td>
</tr>
<tr>
<td>permanent</td>
<td>textile protection</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Neem-Extract:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-BD</td>
<td>textile protection</td>
<td>2%</td>
<td>(spot on) animal ectoparas.</td>
</tr>
<tr>
<td>-ointment</td>
<td>skin care</td>
<td>0.7%</td>
<td>ectoparas. (human, animal)</td>
</tr>
<tr>
<td>-shampoo</td>
<td>hair care</td>
<td>1%</td>
<td>ectoparas. (human, animal)</td>
</tr>
<tr>
<td>NeemAzal-W</td>
<td></td>
<td>10-30%</td>
<td>mosquitoes</td>
</tr>
</tbody>
</table>

1) corresp. to 2% NeemAzal, other concentrations are currently under test.

On behalf of the lability of the Azadirachtins it is a technical problem to formulate Neem extracts in a manner which assures an as long as possible shelf-life on the one hand and does not prevent the rapid degradation in non-target areas like water, soil etc. For example, the formulation NeemAzal-T/S, which is currently tested intensively for plant protection, decomposes on plant leafs, in water or in soil with a half life of 1 or a few days (5, 6).

For the control of ectoparasites, a compromise has to be found between the rapid degradation of the active ingredient, possible modes of application, and a reasonable shelf-life of the product, which permits marketing.
Materials and Methods

Natural Textiles

Tests with the formulation NeemAzal-BD (2% AzadirachtinA) and NeemAzal-WF (1% AzadirachtinA) were carried out in Petri-dishes (plastic, diameter: 9cm) in the laboratory under room-conditions (20±2°C) with the Webbing Clothes Moth Tineola bisselliella and the carpet beetle Anthrenus vorax, respectively. The insects fed on squares of black felt (4.5 x 4.5 cm, thickness 2mm) which was untreated (control) or treated on both sides with two different concentrations of the two formulations: NeemAzal-BD: 56 (BD-1) and 334 (BD-2) mg AzA/m²; NeemAzal-WF: 110 (WF-1) and 640 (WF-2) mg AzA/m². NeemAzal-WF is a formulation, which is very similar to the new formulation „NeemProTex permanent“ and was applied directly by spraying the appropriate amount per unit area. NeemAzal-BD was used as a 1 and 5 % aqueous spraying solution, respectively.

After treatment the felt was dried under room-conditions and cut into pieces of 4.5 x 4.5 cm. On day 0, 30, 60, 150, and 210 felt pieces were transferred into the Petri-dishes and 5-10 young larvae of the respective pests were added with a fine brush on each piece of felt (2 to 4 repetitions). Assessments were carried out at intervals of approximately 30 days. Efficacy, the number of stripped off exuviae, the amount of feeding tunnels (of Tineola bisselliella) as well as the amount of excrements and debris (of Anthrenus vorax) were determined for the different formulations. For analytical purposes felt pieces which had been kept under room conditions for the above mentioned periods of time were deep frozen (below -15°C) and analyzed after about one year after the treatment of the felt. The analytical determination of the AzadirachtinA-content of felt was carried out by comparison of the HPLC areas at the retention time of AzadirachtinA of the felt extracts with AzadirachtinA-standard solutions. The felt used had a weight of 600 g/m² and consists of 80% wool and 20% cotton.

Human Hair and Head Lice

The hair of the test-persons was shampooed carefully with a given amount of the formulation NeemAzal-FT. The Neem-extract was allowed to act for 5-10 minutes after which the hair was rinsed thoroughly with water. The amount of this flushing solution was measured with a volumetric beaker. Samples of dry hair were taken at random from the test persons before, 1 hour after and 13 days after the treatment. The hair samples and the washing solution were investigated analytically with respect to AzadirachtinA (see above).

Dogs and Flea

Dogs infested with 100 flea (see: Bernauer-Jacob & Schein, this volume) have been treated with the NeemAzal-shampoo on day 3, 5 and 9 after severe infestation with flea.
was obvious. The dogs were shampooed with 10, 50 and 50 ml of the shampoo, respectively. The infestation was assessed daily after the treatment and categorized into 4 groups: very strong (300 relative units), strong (200 relative units), weak (100 relative units) and non infested (0 relative units). Samples of the hair were investigated analytically with respect to AzadirachtinA (see above).

Results

For the understanding of the mode of action of NeemAzal-formulations and the dependence of the efficacy with respect to pest insects the AzadirachtinA content was determined analytically in different biotests. From three very different test-conditions first results are available (purpose of the test, test-system, conditions etc.):

1.) Textile protection, felt, study of time-dependence
2.) Control of human head lice, human hair, residues after rinsing of the hair
3.) Control of animal ectoparasites, dogs, efficacy of treatment

The results of these studies are described one after the other.

Natural textiles

The number of deleted exuviae of *T. bisselliella* is a good indication for moulting. The results show a clear inhibition of the development of the insects by the different NeemAzal-formulations (see fig. 1.). From the different tests it can be concluded that the Neem ingredients remain active for at least 210 days in this test.

![Graph showing cumulative number of exuviae](image)

**Figure 1:** Cumulative number of exuviae of the larvae of *Tineola bisselliella* after treatment of felt with NeemAzal-formulations and introduction of young larvae after certain time intervals of storage of the felt under room conditions.
At all storage times of the felt only the larvae of the control developed normally. The number of head capsules usually increases asymptotically till pupation. On treated felt the inhibition of the development of the larvae is clearly obvious after the first assessment date already.

60 days after treatment of the felt, a clear concentration dependence of the number of collected exuviae is observed. The inhibition of the development of the larvae continues in a similar manner until at least 210 days after treatment with NeemAzal-formulations. at all concentrations, the smallest amount of active ingredient which had been applied is sufficient to show a clear reduction of the larval development in comparison to the control at all times.

Larvae of Anthrenus vorax show a similar reduction in the number of exuviae with time and concentration of the active ingredient (see fig 2).

Figure 2: Cumulative number of exuviae of larvae of Anthropus vorax after treatment of felt with NeemAzal-formulations and introduction of young larvae after certain time intervals of storage of the felt under room conditions.

Due to the fact that larvae of T. bisselliella as well as of A. vorax were not always in the same developmental stage (usually L2-L3 was used) at their introduction into the test chamber, the relative reduction of the developmental inhibition changes to some extent.
Figure 3: Amount of excrements and debris, exuviae and mortality of *Anthrenus vorax* 50 days after introduction of young larvae to wool treated with NeemAzal-formulations

In fig. 3, the results of a test where pieces of wool were infested with 50 larvae of *A. vorax* are summarised. The insects were allowed to feed for 55 days under room conditions. The number of exuviae as well as the weight of excrements and debris is clearly reduced already at the lowest concentration (1.8 mg NeemAzal/g wool). The mortality of the larvae is 100% for all treatments.

Figure 4: Time dependence of the amount of AzadirachtinA on felt stored under room conditions for nearly one year.
It was the purpose of the parallel analytical investigation of the AzadirachtinA content in the felt samples to understand this long term effect of the NeemAzal-formulations. In figure 4 the analytically determined amount of AzadirachtinA is given for different storage times of the felt under room conditions. In view of the usual fast degradation of Azadirachtins, the lack of degradation of AzadirachtinA in the felt samples may be explained by the dry environment, to which the active ingredient is exposed on the felt pieces. On behalf of the non-toxic nature of the active ingredient and its rapid degradation in aqueous environments the use of NeemAzal-formulations for the control of natural textiles seems to have considerable commercial potential.

Human Hair and Head Lice

Neem-Extract-Shampoo is usually used in a traditional manner (see above). From the response letters of several people who had used Neem-Extract-Shampoo for the control of head lice, it becomes clear that this area of application has a high demand in the public due the lack of non-toxic alternatives in the market.

The responses (see table 2) show that Neem-Extract-Shampoo can control head lice efficiently after 2-3 applications. The shampoo was distributed carefully in the infested hair together with some water on day 1, 3, 10 and 13. The results are given in table 2.

Table 2: Results of the treatment of children and adults infested by head lice with Neem-Extract-Shampoo (according to information of response letters).

<table>
<thead>
<tr>
<th>treatment number</th>
<th>freedom from lice and nits after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. treatment</td>
</tr>
<tr>
<td></td>
<td>child</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

From a total number of 49 response-letters (42 children, 7 adults) only 3 persons felt that the efficacy of the application of the Neem-Extract-Shampoo was unsatisfactory due to the presence of lice/nits after 3 applications. These 3 persons belonged to the same family and obviously were infected together. In few cases, the persons did not continue to apply the shampoo after the second treatment, since they felt that these applications were sufficiently successful. Some persons (24 %) were satisfied with the results after the second treatment, however, most of them continued the treatment in order to assure its success. Only 2 persons found the effect of the shampoo satisfactory after the fourth treatment.
Several other persons used the shampoo against fleas or mites of their cats and dogs. From 16 response-letters, only one person found the efficacy of the shampoo as unsatisfactory in this area.

In order to understand the mode of action of the shampoo better, three persons were treated by thorough distribution of the shampoo in their hair. Due to the considerable differences in the lengths of the hair of the test-persons (see table 3) the amount of shampoo used and consequently the amount of AzadirachtinA applied per person varied. As expected, no AzadirachtinA could be detected analytically in hair samples taken before the treatment.

After 5 minutes the hair was rinsed with plenty of tap water. The amount of this water its AzadirachtinA concentration and the amount of AzadirachtinA which consequently had been washed off per person is given in table 3. In an average only 3 ppm of AzadirachtinA are present in the hair directly after the application (see also fig. 5).

Thirteen days after the application, another sample of hair was taken and analyzed. At a determination limit of about 2 mg AzadirachtinA/kg hair no AzadirachtinA could be detected.

Table 3: Test conditions and AzadirachtinA-content after application of Neem-Extract-Shampoo to hair. (AzA: AzadirachtinA; b.d. below limit of determination)

<table>
<thead>
<tr>
<th>Test code</th>
<th>length of hair (cm)</th>
<th>treatment ml shampoo</th>
<th>mg AzA per person</th>
<th>treatment mg AzA/kg hair</th>
<th>before 1 hour after</th>
<th>13 days after</th>
<th>rinse water l/person</th>
<th>mg AzA per person</th>
<th>% of AzA applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>M 35</td>
<td>25</td>
<td>222</td>
<td>b.d.</td>
<td>b.d.</td>
<td>6,9</td>
<td>183</td>
<td>82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K 50</td>
<td>25</td>
<td>222</td>
<td>b.d.</td>
<td>3</td>
<td>10,4</td>
<td>144</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H 5</td>
<td>15</td>
<td>133</td>
<td>b.d.</td>
<td>5,5</td>
<td>6,2</td>
<td>105</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>average:</td>
<td>192</td>
<td>b.d.</td>
<td>3</td>
<td>b.d.</td>
<td>8</td>
<td>144</td>
<td>75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From these results it can be concluded that obviously about 75% of the active ingredient are rinsed off in this test and only about 2% of the AzadirachtinA applied was present in the hair directly after the treatment. This amount degrades in the subsequent days so that no AzadirachtinA can be detected 13 days later.
Thus it may be concluded that the direct contact of the ectoparasites with high concentrations of AzadirachtinA as it is present during the application (shampooing i.e. about 1 volume of shampoo and 5 volumes of water, corresponding to about 1500 ppm AzadirachtinA in the "shampoo solution") may influence the ectoparasites severely. The small amount of active ingredient present after rinsing the hair with water may have an additional positive effect on the control of the ectoparasites.

**Dogs and Flea**

Different tests for the control of flea (*Ctenocephalides felis*) on dog are described by Bernauer-Jacob and Schein (see this volume). From these tests samples of hair of the treated and control dogs were investigated analytically with respect to AzadirachtinA.

Figure 6 shows a typical example of the decrease of the infestation with flea in the course of the 3 treatments and afterwards. On day 3, 5, and 9 the dogs were shampooed with 10, 50 and 50ml of NeemAzal-shampoo, respectively. The shampoo was rinsed off after 5, 10 and 10 minutes with some water. Care was taken that some of the shampoo remained in the coat. This was checked qualitatively by the turbidity of the water which run off. The infestation of the dogs with flea was categorized by relative units (no infestation: 0; slight infestation: 100; strong infestation: 200 and very strong infestation: 300 relative units).
Figure 6: Time dependence of the degree of infestation and the AzadirachtinA content in the coat of dogs. On day zero the dogs had been infested with 100 adult flea each. Applications of Neem-Extract-Shampoo were done on day 3, 5 and 9 after infestation.

A typical example of the time dependence of the infestation and the AzadirachtinA content (see figure 6) shows that the infestation continued after the first treatment and decreased strongly after the second and third treatment.

In the course of these treatments, the AzadirachtinA content in the hair samples increased from treatment to treatment, reaches a maximum after the third treatment and then drops down to a lower, but clearly measurable level at day 14. At this level, it remains at least for 10 more days. In the hair of three dogs, which had been analyzed from this experiment, the maximum amount of AzadirachtinA varied between about 100 to 1600 ppm of AzadirachtinA. This large variation is probably due to the residual amount of active ingredient in the hair which was intended but difficult to control. The time dependence of the Azadirachtin-content as well as the degree of infestation was very similar to that shown in figure 5 in all three cases investigated.

On day 15 of the experiment the control dogs were treated with 100 ml of shampoo. On the next day, no flea could be detected any more. The AzadirachtinA content increased to a plateau value of about 300 ppm where it remained at least for 6 days (see figure 7). These results indicate that the efficiency of the treatment as well as the AzadirachtinA-content in the hair of treated dogs parallels the amount of NeemAzal-Shampoo applied. Obviously, optimum results could be obtained with 50-100 ml of shampoo for the dogs (mostly "Beagles") used in this tests. Further experiments have to show whether the AzadirachtinA content, which obviously remains for some time after the treatment in the hair is sufficient to prevent new infestation with flea.
Figure 7: Time dependence of the degree of infestation and the AzadirachtinA content in the coat of a „control dog“ (compare fig. 6). On day zero the dog had been infested with 100 adult flea. One single application with 100 ml NeemAzal-Shampoo was done on day 15 after infestation.

Conclusions

At the first glance, the analytical results presented here together with the corresponding efficacy data are surprising in view of the well documented rapid degradation of AzadirachtinA under many conditions. Especially, the results for the protection of natural textiles (felt) indicate that the film of the Neem-ingredients which is expected to be formed after drying of the spraying solution on the tissue has similar properties with respect to the protection of AzadirachtinA as the formulation. Since Azadirachtin is present in the formulation only as a minor component, it may be expected that the formulation additives protect the labile active ingredient from various possibilities of degradation in the formed film. Under these dry conditions, AzadirachtinA is not much degraded and obviously is available to protect the textiles for a period of several months as indicated by the bioefficacy data.

In the coat of the dogs AzadirachtinA is present in meaningful amounts for at least 1-2 weeks. It is unclear how fast a degradation may occur afterwards. It can be expected that the Azadirachtin will be distributed some time after application in the coat randomly. It will be a matter of further investigations, whether the degradation of AzadirachtinA in the deeper, more humid, parts of the hair will be more rapid than in the presumably dryer outer parts.

In the case of applications of NeemAzal-Shampoo against human head-lice, the mode of application assures a very high concentration of the Neem-ingredients for a short time
after which about 75% is washed away. From the results presented in table 3 it is obvious that the difference between the amount of AzadirachtinA applied and rinsed off is not present in hair directly after the treatment. It can be speculated but has to be proven by further investigations whether the active ingredient is degraded to a meaningful extent by the conditions used for the application (rinsing with warm water). In any case, even the low amounts of AzadirachtinA which can be determined in the hair after the treatment disappear rapidly under normal conditions.

The obtained analytical information as well as the biological efficacy indicates that the modes of application served the intended purposes well: long term protection of natural textiles, (at least) several days presence of the active ingredient in the coat of dogs for their protection against flea, and rapid disappearance of AzadirachtinA in the case of the control of human head lice under normal conditions. These results together with the absence of toxicological risks (see Stewart, this volume) make various applications of the standardized and well investigated NeemAzal-formulations possible.

References
Toxicological results of NeemAzal Technical and NeemAzal Formulations

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Washington, D.C. 20036

Introduction

Neem based products have been used as pesticides in various regions of the world for many years. It was not until July 9, 1985, however, that the first pesticidal product containing azadirachtin as its active ingredient was registered by the U.S. Environmental Protection Agency for use to control insects. On February 17, 1993, EPA published the Final Rule providing for an exemption from the need of a tolerance for azadirachtin when used on all raw agricultural commodities at a use rate of 20 grams per acre.

Azadirachtin was determined by EPA to be a biochemical pesticide and subject to the reduced set of data requirements for biochernicals. The data ordinarily required to support the registration of a biochemical pesticide include, product chemistry, acute toxicity, a battery of three mutagenicity studies, a developmental toxicity study in one species, a 90-day subchronic feeding study, an acute avian toxicity study, an avian dietary study, a freshwater fish study, and an acute *Daphnia* toxicity study.

Product History

The Neem Products Division of E.I.D. Parry (INDIA) Ltd. is developing data to support the registration of its NeemAzal Technical and end-use formulations (especially NeemAzal-T/S, containing 1% azadirachtin A) as developed by Trifolio-M (Germany). Mammalian toxicity and non-target organism studies to fulfill the biochemical guidelines have been completed following the appropriate EPA guidelines. The studies have been conducted by Huntingdon Life Sciences Ltd., Huntingdon (England), BioChem GmbH, Karlsruhe (Germany) and Jai Research Foundation, Valvada (India) and sponsored by E.I.D. Parry Ltd., Chennai (India) and Trifolio-M, Lahnau (Germany).

Acute Toxicity

NeemAzal Technical exhibits minimal acute toxicity (Table 1). No mortality was observed at the highest doses tested in acute oral, dermal, and inhalation studies. The test concentration in the acute inhalation study was limited by the physical nature of the material. Slight eye irritation and some dermal sensitization was noticed.
Table 1 Acute Toxicity of NeemAzal Technical

<table>
<thead>
<tr>
<th>Toxicity Type</th>
<th>Toxicity Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral Toxicity</td>
<td>LD$_{50}$ &gt; 5 g/kg</td>
</tr>
<tr>
<td>Dermal Toxicity</td>
<td>LD$_{50}$ &gt; 2 g/kg</td>
</tr>
<tr>
<td>Inhalation Toxicity</td>
<td>LD$_{50}$ &gt; 0.72 mg/L (max. achievable conc.)</td>
</tr>
<tr>
<td>Eye Irritation</td>
<td>Slight conjunctival irritation</td>
</tr>
<tr>
<td>Dermal Irritation</td>
<td>Non-irritating</td>
</tr>
<tr>
<td>Skin Sensitization</td>
<td>Sensitizer</td>
</tr>
</tbody>
</table>

Acute toxicity studies conducted with the 1% formulation also show very low toxicity. The results (Table 2) show that the formulation is neither irritating nor a sensitizer and the inhalation LD$_{50}$ is much higher, reflecting largely the ability to achieve a higher ambient concentration in the test chamber.

Table 2 Acute Toxicity of NeemAzal Formulation

<table>
<thead>
<tr>
<th>Oral Toxicity</th>
<th>LD$_{50}$ &gt; 5 g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermal Toxicity</td>
<td>LD$_{50}$ &gt; 2 g/kg</td>
</tr>
<tr>
<td>Inhalation Toxicity</td>
<td>LD$_{50}$ &gt; 5.4 mg/L</td>
</tr>
<tr>
<td>Eye Irritation</td>
<td>Non-irritating</td>
</tr>
<tr>
<td>Dermal Irritation</td>
<td>Non-irritating</td>
</tr>
<tr>
<td>Skin Sensitization</td>
<td>Non-sensitizer</td>
</tr>
</tbody>
</table>
**Mutagenicity**

Potential mutagenic activity was investigated in both reverse and forward mutation assays and for chromosomal damage. The studies included *in vitro* and *in vivo* techniques. In each case the results were negative (Table 3).

**Table 3 Mutagenicity of NeemAzal Technical**

<table>
<thead>
<tr>
<th>Test Type</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ames Assay</td>
<td>Negative</td>
</tr>
<tr>
<td>Mammalian Cell Assay</td>
<td>Negative</td>
</tr>
<tr>
<td>Mouse Micronucleus</td>
<td>Negative</td>
</tr>
</tbody>
</table>

**Subchronic Toxicity**

The potential for subchronic toxicity of NeemAzal Technical was investigated by feeding rats treated chow for a period of 13 weeks. A group of 20 rats (10 of each sex) were fed one of five different treatment levels, 0, 100, 400, 1600, or 6400 ppm of Neem-Azal technical in the diet for a period of 13 weeks. At the end of the study period, gross necropsies were conducted, as well as histopathological examination of various tissues.

The effects noted during the study included:

- a slight decrease in body weight gain at 6,400 ppm,
- a slight decrease in water intake in the males at 6,400 ppm,
- longer activated partial thromboplastin times (APTT) and higher mean thrombotest (TT) values for the males treated with 6,400 ppm,
- elevated platelet values for females treated with 6,400 ppm,
- both sexes exposed to 1,600 and 6,400 ppm exhibited higher globulin and protein levels, and
- an increase in relative liver weights for the high-dose animals.

Upon histopathological examination, the following effects were reported:

- hepatocyte hypertrophy at 6,400 ppm,
- periportal fat deposition in females at 400 (minimal effect), 1,600 and 6,400 ppm,
- and thyroid follicular cell hypertrophy in females at 6,400 ppm.

**The NOEL was 100 ppm (7.7 and 9.4 mg/kg) and the LEL was 400 ppm (31.6 and 35.7 mg/kg) based on a higher incidence and degree of increased periportal fat deposition.**
Other Effects - Developmental Toxicity

The developmental toxicity study was conducted to assess the effects of NeemAzal Technical on the pregnant rat and developing conceptus. The test substance was administered by intragastric intubation at dosages of 50, 225 and 1,000 mg/kg/day to groups of 25 time-mated females, once daily, from Day 6 to Day 19 of pregnancy, inclusive. Another group of 25 time-mated females was similarly treated with vehicle (1% methyl cellulose) as a control. On Day 25 of pregnancy, the dams were killed and subjected to post mortem examination, litter parameters were determined and fetuses were examined for visceral or skeletal changes.

Treatment at 1,000 mg/kg/day was associated with lower body weight gain and reduced food consumption compared to the concurrent control group, during the first two days of treatment, and again during days 10 to 13 post coitum. All animals showed post-dosing salivation. Treatment at 225 mg/kg/day was associated with a slight reduction in food consumption during the first two days of treatment. Post-dosing salivation was observed in four of 25 treated animals. No maternal treatment related effects were observed at 50 mg/kg/day.

At 1,000 and 225 mg/kg/day, there was an equivocal increase in the incidence of interventricular septal defect (four fetuses / four litters affected) compared with the background control incidence. At 1,000 mg/kg/day, there was also an increase in fetuses with supernumerary ribs compared with controls. At 50 mg/kg/day, there was no obvious adverse effect on embryo-fetal survival or development.

The clear No-Observed-Adverse-Effect-Level (NOAEL) of NeemAzal Technical for maternal and developmental toxicity was 50 mg/kg/day.

Ecological Effects

Studies were conducted to assess the toxicity of NeemAzal Technical to non-target organisms.

An acute oral toxicity (LD₅₀) study was conducted with NeemAzal Technical using the bobwhite quail. Groups of five male and five female birds were given a single oral dose, by intubation, of either 1000, 2000 or 4000 mg NeemAzal Technical per kilogram of body weight. A similar sized control group was given the 1% methyl cellulose vehicle alone. Birds were observed for 14 days following dosing. Observations included mortality, clinical signs, body weight and food consumption. No mortalities occurred after dosing and all birds remained in good health throughout the post-treatment period. In addition, body weights and food consumption were unaffected by treatment and no abnormalities were observed at necropsy. Therefore, the acute oral LD₅₀ value of NeemAzal Technical to bobwhite quail was found to be greater than 4000 mg NeemAzal Technical/kg. The no observed effect level was 4000 mg NeemAzal Technical/kg.
A subacute dietary toxicity (LC50) study was conducted on NeemAzal Technical using bobwhite quail. Groups of ten young bobwhite quail were offered diet containing 1300, 2600 and 5200 ppm NeemAzal Technical. Two similar sized control groups were offered the basal diet alone. Test diets were introduced when the birds were ten days old and were offered to the birds for five days. Test diets were then replaced with basal diet and the birds observed for a further three days. Observations included mortality, clinical signs, body weight and food consumption. At the completion of the study, ten control birds and ten birds from the highest dose level were subjected to a gross post mortem examination. Other than two incidental deaths not related to test treatments, no mortalities occurred and all birds remained in good health for the duration of the study. Body weight and food consumption were similarly unaffected by treatment. Therefore, the dietary LC50 value for bobwhite quail was found to be greater than 5200 ppm NeemAzal Technical, placing it in the practically non-toxic EPA classification category. The no effect level was considered to be 5200 ppm NeemAzal Technical.

A freshwater acute study was conducted with NeemAzal-T/S on rainbow trout (Oncorhynchus mykiss) under OECD protocols (Grunert 1996a). Groups of ten fish were exposed to concentrations ranging from 50 to 800 mg/L NeemAzal-T/S (0.5 to 8 mg/L azadirachtin) for 96 hours. Test volumes were renewed once at 48 hours. Fish were observed for mortality and sublethal responses. Following 96 hours of exposure, the LC50 was calculated to be 160 mg NeemAzal-T/S per Liter (1.6 mg azadirachtin/L).

A similar acute toxicity study was conducted with NeemAzal-T/S and the freshwater carp (Cyprinus carpio) using OECD protocols (Jai Research 1996). Ten carp were exposed to NeemAzal-T/S at a concentration of 100 mg NeemAzal-T/S per Liter. Another ten fish were maintained as a control. Test and control solutions were changed daily in this static renewal study. No mortality was observed after 96 hours. Therefore, the 96-h LC50 was calculated to be greater than 100 mg NeemAzal-T/S per Liter (>1.0 mg azadirachtin/L).

The toxicity of NeemAzal-T/S (1% azadirachtin) to freshwater invertebrates was investigated under OECD protocols (Grunert 1996b). Twenty Daphnia magna were exposed under static conditions to seven nominal concentrations ranging from 87.8 mg/L to 1000 mg/L as NeemAzal-T/S, plus a control, for 48 hours. At the completion of the study, 50% of the Daphnia in the highest concentration (1000 mg NeemAzal-T/S per Liter) were immobilized and considered to be the 48-h EC50. This was equivalent to 6.572 mg azadirachtin/L (measured concentration). The no observable effect concentration (NOEC) was 197.5 mg NeemAzal-T/S per Liter.

An algal growth inhibition study was conducted on NeemAzal with Scenedesmus subspicatus, following OECD protocols (Grunert 1996c). Algae were exposed to NeemAzal-T/S (1% azadirachtin) for 72 hours under static conditions. Seven treatment concentrations ranging from 22.0 mg/L to 2494.4 mg/L, as NeemAzal-T/S, plus a control were employed. After 72 hours of exposure, no significant inhibition or stimulation of growth rate could be determined. Therefore, the EC50 was determined to be greater than 2494.4 mg NeemAzal-T/S per Liter (>8.9 mg azadirachtin/L).
Conclusion

Data generated on the toxicity and ecotoxicity of NeemAzal Technical and NeemAzal 1% demonstrate that the toxicity of azadirachtin to mammals and non-target organisms is low. Therefore the use of azadirachtin based products in agriculture is unlikely to result in food residues of concern. Likewise, azadirachtin can easily be formulated into products that can be used by consumers without significant risk.

Während die Behandlung von Kopfläusen (Pediculosis capitis) mit 1,4 %igem Neem-Extrakt in flüssiger Form schon lange bekannt ist und von uns seit Anfang 1994 mit recht gutem Erfolg durchgeführt wird, haben wir zunächst bei der Scabies-Behandlung eine 1%ige Neem-Salbe, die uns die Firma Trifolio M zur Verfügung gestellt hat, genutzt. Dabei wurde folgendes Behandlungsregime durchgeführt:

- Einmal tägliche Applikation der Neem-Salbe auf die betroffenen Hautareale. Da es sich meist bei unseren Patienten um recht generalisierte Scabieserkrankungen handelt, haben wir zur Gewährleistung einer ausreichenden Hautatmung den Rumpf jeweils abends gegen 19,00 h und die Extremitäten nebst Kopfbereich (soweit befallen) jeweils morgens gegen 7,00 h behandelt.


Zwei der Patienten mußten als Therapieversager angesehen werden und erhielten anschließend eine Behandlung mit Cotramiton. Bei diesen beiden Patienten konnte allerdings eine Re-Infektion durch weitere Familienangehörige nicht eindeutig ausgeschlossen werden.

Ab Juni 1996 haben wir die Behandlung der Scabies mit 2%iger Neem-Salbe, wiederum von der Firma Trifolio M hergestellt, durchgeführt. Entsprechend dem o.g. Behandlungs muster wurden insges. 9 Patienten im Alter von 2 Mon. bis 5 Jahren behandelt (es wurden nur die Patienten berücksichtigt, die die unten genannten Verlaufterkontrollen lückenlos erfüllten; sowohl in der ersten Behandlungsgruppe als auch in der zweiten hatten wir noch ca. 50 % mehr Patienten stationär und/oder ambulant in Betreuung, bei denen die Studienkriterien nicht erfüllt wurden, die aber etwa den entsprechenden Erfolg boten.).
Bei 8 Patienten konnten wir einen optimalen Therapieerfolg innerhalb von 4 bis 6 Tagen erreichen, während ein Kleinkind als Therapieversager angesehen werden mußte.

Als erfreulicher Nebeneffekt der Neem-Salben-Therapie zeigten die Patienten eine deutliche Rückläufigkeit der zu Therapiebeginn bestehenden Hautausschlag. Auch konnten die bei den bisherigen Therapieregimen häufig zu beobachtenden s.g. postscabioßen Exantheme nicht bzw. in wesentlich geringerem Ausmaße beobachtet werden. Dies dürfte auf die regelmäßige Applikation der rückfettenden Neem-Salbe bzw. Salbengrundlage sowie auf Nichtanwendung der sonst üblichen aggressiven Substanzen zurückzuführen sein.

Bei dem s.g. postscabioßen Exanthem, das neurodermitischen Effloreszenzen bzw. dem s.g. Skrophulos sehr ähnlich, handelt es sich ja in den meisten Fällen um lokale allergische Reaktionen einerseits auf die angewendeten Therapeutika, andererseits aber auch auf den Verursacher der Scabies. Unter Applikation entsprechender cortisonhaltiger Salben kam es meist zum Verschwinden dieser Auffälligkeiten.


....
Der Therapieerfolg trat bei bisher 4 behandelten Patienten innerhalb von 3 bis 6 Tagen auf. Die o.g. laborchemischen Kontrolluntersuchungen zeigten auch unter diesem verschärften Regime keine Zeichen einer Toxizität.


Der Hinweis auf die s.g. relativ hohe Hintergrundbelastung unserer Kinder mit Lindan und /oder Pyrethroiden darf kein weiterer Freibrief für die Anwendung dieser Substanzen bei der Behandlung von Ectoparasiten sein.


Last but not least sei angemerkt, daß im anglo-amerikanischen pädiatrischen Schrifttum wesentlich kritischer über die Nebenwirkungen der Behandlung von Scabies bzw. Ektoparasiten mit Lindan und Pyrethroiden bzw. mit Crotamiton als im deutschsprachigen Raum berichtet wird.

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   W.B. Saunders Company, Philadelphia, 1996
Erste Ergebnisse mit NeemAzal-Formulierungen gegen Flöhe

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Der Flohbefall war schon immer ein großes Problem der Menschheit. Die Pest war im 14-ten Jahrhundert verantwortlich für den Tod von 25% der Bevölkerung in Europa. Xenopsylla cheopis, der tropische Rattenfloh war der Überträger der Pest. Der Menschenfloh *Pulex irritans*, war im Mittelalter ebenfalls weit verbreitet.

Nun sind die Gefahren, die uns von den Flöhen unserer Haustiere drohen, sicherlich geringer, der Wunsch, diese Parasiten kontrollieren zu können mit gestiegenem Hygienebewußtsein aber größer denn je.


Bei og. Flöhen handelt es sich um sog. polyxene Flöhe, d.h. sie sind nur bedingt wirtsspezifisch und befallen auch Menschen und andere Haustiere.

Die Zunahme der Haustierhaltung und die damit verbundene enge Gemeinschaft von Mensch und Tier, machen eine Bekämpfung dieser Parasiten dringend notwendig.

Bei Hund und Katze verursachen die Flöhe durch ihren Biß eine Irritation der Haut. Die Tiere beginnen sich zu kratzen.

Durch verschiedene Allergene, die sich im Flohspeichel, befinden, kommt es sehr häufig zu sog. Flohbißallergien und durch den damit verbundenen Juckreiz oft zu Kratzekzemen mit sekundärer bakterieller Besiedlung.


Practice Oriented Results on Use and Production of Neem-Ingredients and Pheromones VII
H. Kleeberg (ed)
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- 31 -

Ein wirksames Flohbekämpfungsmittel muß also sowohl die adulten Flöhe auf dem Tier als auch seine Entwicklungsstadien in der Umgebung abtöten.

In den USA geben die Tierhalter jährlich eine Milliarde Dollar für die Flohbekämpfung aus.

Gerade in den letzten Jahren sind eine Menge neuer Wirkstoffe speziell für die Flohbekämpfung, auf dem Markt erschienen. Hier eine kurze Übersicht über bislang eingesetzte Mittel:

I Klassische Antiparasitika
1. Chlorierte Kohlenwasserstoffe
2. Phosphorsäureester
3. Carbamate
4. Avermectine
5. Pflanzliche Insektizide
6. Pyrethroide
7. Neue Insektizide mit neurotoxischer Wirkung (Fibronil, Imidacloprid)

II Alternative Antiparasitika
8. Repellentien
9. IGR = Insect Growth Regulators (Molting Inhibitors und Insect Development Inhibitors)
10. Mineralien
11. Biologische Methoden

Von den jeweiligen Wirkstoffgruppen gibt es verschiedene Formulierungen sowohl zur Behandlung am Tier wie zur Umgebungsbehandlung.

Aus meiner eigenen Praxis kann ich berichten, daß die Flohbehandlung mit den verschiedenen Wirkstoffen, besonders in den letzten Jahren z.T. sehr unbefriedigende Ergebnisse zeigte. Diese Problematik ist im Süden der USA bereits seit längerem bekannt:

- 32 -
Ursache hierfür sind die seit einiger Zeit immer wieder auftretenden Insektizidresistenzen verschiedener Flohpopulationen.

Diese Problematik und der immer wieder geäußerte Wunsch der Patientenbesitzer nach biologischen umweltverträglichen Parasitenvernichtungsmitteln haben mich auf die Wirkstoffe des Neem-Baumes aufmerksam werden lassen.

Herr. Kleeberg hat uns verschiedene NeemAzal Formulierungen seiner Firma zur Verfügung gestellt.


Dazu wurden 9 mittelgroße Hunde mit ca. 100 Flöhen / Tier der Art Ctenocephalides felis besetzt. 48 h später wurden 6 dieser Hunde, nachdem die Untersuchung einen starken Flohbefall zeigte, mit je 50 ml Neem-Azal Shampoo gewaschen.

2 Hunde blieben als Kontrolle unbehandelt.

1 Hund wurde nur mit der Shampoo-Grundlage, ohne Inhaltsstoffe, gewaschen.

Bereits am darauffolgenden Tag waren auf den Neem-Azal behandelten Tieren deutlich weniger Flöhe zu sehen.

3 Tage später haben wir die 7 Hunde nochmals gewaschen, einen davon nur mit der Shampoo-Grundlage die anderen 6 w.o. mit je 50ml Neem-Azal.

Am darauffolgenden Tag war eines dieser 6 behandelten Tiere flohfrei, die anderen 5 zeigten im Gegensatz zu den drei Kontrollen einen sehr geringen Flohbefall.

Am 4. Tag hatten 3 der 6 behandelten Hunde keine Flöhe mehr.


Zum Abschluß behandelten wir noch die beiden Kontrollhunde w. o. jedoch mit der doppelten Menge Shampoo d.h. mit 100 ml / Tier.

2 Tage später hatten beide Hunde keine Flöhe mehr.
Durch diese Behandlung hatten übrigens alle Hunde ein wunderschönes glänzendes Fell bekommen. Um eine möglichst schnelle Parasitenfreiheit auf dem Tier zu erreichen, scheint eine entsprechend hohe Wirkstoffmenge notwendig zu sein.


Wie aus dem Entwicklungszyklus des Flohs ersichtlich ist der Einfluß auf die Entwicklungsstadien in der Umgebung von besonderem Interesse. Um nun die Wirkung des Neem-Azals auf die Reproduktion der Flöhe zu untersuchen haben wir uns entschlossen dies mit einer sogenannten spot-on Formulierung an Gerbils auszuprobieren.


Proben der Einstreu wurden täglich im Stereomikroskop kontrolliert. Nach einigen Tagen waren in der Einstreu der beiden unbehandelten Kontrolltiere bereits Flohlarven zu sehen. In den 4 anderen Proben der behandelten Gerbils wurden auch nach intensiver Durchsicht nur in 2 Schalen je eine Larve entdeckt, die sich jedoch nicht weiter entwickelt.

In den Kontrollschalen zeigte sich eine Weiterentwicklung bis zu adulten Flöhen.

Durch diese Ergebnisse ermutigt planen wir den Aufbau einer standardisierten Versuchsreihe.

Wir haben 6x ca. 200 Flöhe Xenopsylla Cheopis mit Hilfe einer Volumenmessung ausgezählt. 6 zylindrische Glasbehälter wurden mit je einem abgewogenen Gemisch von Sägespänen, gemahlenem Körnerfutter und Larvenährmedium bestückt. In jedes Glas wurde ein männlicher Gerbil von ca. 100 g gesetzt und mit den jeweils 200 ausgezählten Flöhen infestiert. Die Gläser wurden in den Inkubator bei ca. 27 °C und 80% Luftfeuchtigkeit gestellt. Am nächsten Tag wurden 4 der Tiere mit der Neem-BD Formulierung behandelt, d.h. sie bekamen diese ölige Zubereitung im Nackenbereich aufgeträufelt.


In den Schalen der beiden unbehandelten Kontrolltiere waren massenhaft Larven vorhanden.

In den vier anderen waren nur ganz vereinzelt mal eine Larve zu sehen. 24 Tage nach Versuchsbeginn schlüpften die ersten Flöhe in den Schalen der Kontrolltiere. Ich beobachtete das Schlüpfen der Flöhe über einen Zeitraum von 27 Tagen.


NeemAzal in der Anwendung bei Pferd und Esel

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in der praktischen Anwendung des Mittels Neem-Azal machten wir folgende Erfahrung:


Wir versuchten unseren Pferden durch Waschungen mit Alugan zu helfen, das uns vom Tierarzt empfohlen worden war. Es half auch zeitweise. Allerdings hatten wir immer ein ziemlich ungutes Gefühl bei der Anwendung. Pferde treiben intensive Körperpflege, indem sie sich gegenseitig das Fell kraulen. Um sich auch an den Körperstellen jucken zu können, die sie selbst nicht erreichen können, bitten sie sozusagen ihre Herdengenossen, ihnen zu helfen. Dabei kommen die Pferde natürlich in Kontakt mit den Giften, die gegen Schädlinge ins Fell eingerieben sind.


So machten wir einen Versuch mit dem "neuen Mittel". Wir stellten fest, daß so ohne weiteres das Fell der Pferde nicht benetzt werden konnte. Neem Azal drang nicht bis auf die Haut durch. Pferdefell ist stark fetthaltig, besonders bei im Offenstall gehaltenen Tieren, baut sich zum Schutz der Haut gegen Nässe ein Fettfilm im Haar auf. Esel hingegen haben keinen Fettfilm, da sie aus warmen Ländern kommen, wo sie keinen Schutz gegen Kälte und Regen benötigen.

So wuschen wir zuerst mit einem Shampoo die Pferde an den Stellen,(Mähne, Schweifansatz, Brust) an denen die Haarlinge besonders stark auftreten, um das Fell zu entfetten. Danach trugen wir mit einem Schwamm das vorschriftsmäßig verdünnte Neem-azal tupfend in das Fell des Pferdes und des Esels auf. Wir ließen es eine Stunde einwirken, spülen die Tiere dann mit dem Wasserstrahl ab.

Schon am nächsten Tag, konnten wir feststellen, daß die Prozedur offensichtlich gewirkt hatte, da die Pferde sich nicht mehr juckten.
ANTI BACTERIAL ACTIVITY OF NEEM EXTRACT: DETERMINATION OF MINIMUM ACTIVE CONCENTRATION AGAINST DERMATOLOGICAL BACTERIA (STAPHYLOCOCCUS AND PSEUDOMONAS SP.).

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ABSTRACT: The ethanolic extract of Neem seeds cake was made according to Schroeder and Nakanishi method (1987), and used for the study of biological action of neem products on human dermatological bacteria. Staphylococcus and Pseudomonas Species isolated from infested people of the national hospital have been used for test. After 18 hours incubation on Mueller Hinton culture medium with 2.5mg/ml of ethanolic neem extract, we obtain growth inhibition of bacteria. The bactericidal tests in the same conditions give a good result. Chemical analysis of neem extract shows a high concentration of azadirachtin in the solution.

KEY WORDS: Neem extract, anti bacterial effect, human dermatosis, azadirachtin.

INTRODUCTION

Neem extract is commonly used in medicine by populations living in the tropical region. Neem products are known for many centuries to have properties in medicinal applications (Shaktin Upadhyay, Charu Kaushic and G.P. Talwar, 1990, Sami A. Khalid et al 1989), virus and bacteria infections treatment (R.B.S. Sangar, and M.K. Dwingra, 1982), (V.K. Ganesalingam, 1986; A.V.B Sankaram et al, 1986), Insects and nematodes fighting (H. Schmutterer, 1983; M. Jacobson et al (1983); By A.V.B Sankaram et al, (1986); W. Kraus et al, (1986). Recently, some molecules have been isolated from neem components and characterized as particularly active. Azadirachtin is the most important of these molecules which have been purified and identified by many authors as a powerful insecticide (R. Bryan Yamasaki et al.). Neem products are widely used in Asia. But in Africa, neem has been introduced beyond the 1973th year to fight drought and desert. The decoction of leaves is widely used as anti malarial product. In Africa, some NGO working on public health field reported that neem oil is also used against human skin pathology. The most of infections are caused by bacteria like Staphylococcus and Streptococcus sp. We report in this paper, the results of a case study in which neem seed extract can be used as curative medication for human dermatologic infections.

MATERIALS AND METHODS

NEEM EXTRACT

Neem components were extracted from seed cake after the oil extraction by hand press, according to method describe by Schroeder and Nakanishi (1987).

50g of Neem cake containing up to 20% of residual oil were grounded and poored to 200ml of 95% ethanol. The suspension was stirred at room temperature for three hours following by decantation during 24 hours at 4°C. The mixture is then filtrated.
The supernatent was evaporated under vacuum to dryness. The residues were dissolved in 100 ml methanol-water 90/10. This solution is washed three times with 50 ml n-hexane. the Methanolic phase was separated and evaporated to dryness. Residues were dissolved in 100 ml ethyl-acetate following by three times washing with 50 ml water. This solution is evaporated again to dryness and the residue dissolved in 10 ml of ethanol to obtain the final solution for essays. The concentration of Azadirachtin in final solution was determined by HPLC to be about 0.2 mg / ml. This solution is concentrated in vacuo to give up to 5.0 mg / ml of Azadirachtin.

**BACTERIAL STRAINS**

The tests were carried out with strains of Staphylococcus aureus and speudomonas sp. isolated from sick people (post-chirurgical sickness, skin infested) in national hospital of Ouagadougou. These wild strains are further identified using the purified strains from "Institut Pasteur.

**MEDIUM AND GROWTH CONDITION**

We used for all the tests Mueller Hinton (MH) liquid and solid medium.

1) *growth in submerged culture*

2 ml of neem extract containing 0.2 mg / ml of Azadirachtin were filtered through 45μ under vacuum, and mixed with 18 ml of MH culture broth in test tubes. 10 μl of inoculum with 10⁶ bacteria /ml are added. The tubes are placed at 37°C for test.

2) *growth on solid medium*

10 μl of neem extract are used to inoculate petri dishes filled with MH agar medium.

3) *The control medium* was made of 20% ethanol solution in water. The temperature of growth is 37°C.

**INOCULUM**

Two Bacterial suspensions were made by 10⁶ and 10⁴ bacteria /ml. I₁ = 10⁶bact. /ml, I₂ = 10⁴ bact. /ml.

**AZADIRACHTIN ESTIMATION IN NEEM EXTRACT**

Azadirachtin concentration was determined by HPLC in the following conditions, using standard provided kindly by Trifolio : Analytical HPLC: PH 1050 MDW Colonne Supelco LC 18, 5μm, 25cm.

Solvents: ACN/water 60/40

Elution isocratic, 1.5 ml/mn, 10μl sample.

**CRUDE AZADIRACHTIN SOLUTION FOR TESTING**

The final solution from neem extract is diluted to have 0.1 mg / ml of Azadiractin.
RESULTS AND DISCUSSION

EFFECT OF INOCULUM CONCENTRATION

Table 1. Effect of concentration of bacteria in inoculum during incubation with neem extract

<table>
<thead>
<tr>
<th>Test conditions</th>
<th>number of Bacteria after 18 hours incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ml of Neem Extract + 10 μl inoculum (10⁶ bact./ml)</td>
<td>7000</td>
</tr>
<tr>
<td>10ml of Neem Extract + 10 μl inoculum (10⁴ bact./ml)</td>
<td>0</td>
</tr>
<tr>
<td>10 ml control medium + 10 μl (20% alcohol)</td>
<td>2 000 000</td>
</tr>
</tbody>
</table>

After 18 hours incubation, the viability of bacteria was reduced to 0.35% for 10⁶ bact. / ml and 0% for 10⁴ bact. / ml. 20% ethanol without neem extract does not affect the growth. 18 hours was taken as experimental time and 10⁴ is the bacterial concentration in inoculum. The effectiveness of neem extract depends of bacterial concentration in inoculum. 10⁶ bact. / ml inoculum is more active than 10⁴ bact. / ml.

EFFECT OF NEEM EXTRACT ON STAPHYLOCOCCUS AUREUS IN LIQUID MEDIUM

Two trials were performed to find the effect of neem extract in liquid medium. Table 2 gives the results of essays on Staphylococcus aureus.

Table 2. Effect of neem extract on bacterial growth: Staphylococcus aureus strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of bacteria after 0h incubation</th>
<th>Number of bacteria after 18h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains 1 (Post-chirurgical sick)</td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>Strain 2 (skin decease)</td>
<td>400</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Effect of neem extract on bacterial growth: Peudomonas strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of bacteria after 0h incubation</th>
<th>Number of bacteria after 18h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains 1 (Post-chirurgical sick)</td>
<td>380</td>
<td>0</td>
</tr>
<tr>
<td>Strain 2 (skin decease)</td>
<td>300</td>
<td>0</td>
</tr>
</tbody>
</table>

Using samples with a defined number of bacteria, we get the neem extract action in the conditions of medication. The two tables here above describe the efficacy of neem ethanolic extract to stop pathogenic bacterial growth.
EFFECT OF NEEM EXTRACT ON STAPHYLOCOCCUS IN SOLID CULTURE

In the trials on petri dishes, 18 ml of MH solid medium are added to 2 ml of neem extract. 10 μl of 106 bact./ ml are used to inoculate.

An important bacterial growth occurs after 18 hours for the two strains. This result shows that neem extract does not affect microorganisms growth. This is probably due to the reaction of neem active molecules with the solid medium.

ASSESSMENT OF MINIMAL ACTIVE CONCENTRATION OF NEEM EXTRACT

The objective of this test is to make up the minimal concentration of neem extract for bacterial growth inhibition or bactericidal effect.

Testing method
The minimum active concentration tests were carried out in the same conditions as previously, with a diluted sample of neem extracts. For the control, Azadirachtin from Trifolio was used. The contact time was kept at 18 hours. The following tables give the main results of these tests.

Table 4. Determination of active concentration of neem extract: Test with strain N°1 of Pseudomonas sp (post-chirurgical host)

<table>
<thead>
<tr>
<th>neem extract mg/ml</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2.5</th>
<th>2</th>
<th>1.5</th>
<th>1.0</th>
<th>0.5</th>
<th>0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of bacteria after 18h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15000</td>
<td>2000</td>
<td>45000</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Azadirachtin (from Trifolio)</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2.5</th>
<th>2.0</th>
<th>1.5</th>
<th>1.0</th>
<th>0.5</th>
<th>0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of bacteria after 18h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5. Determination of active concentration of neem extract: Test with strain N°2 of Pseudomonas sp. (skin decease host)

<table>
<thead>
<tr>
<th>neem extract mg in sol.</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2.5</th>
<th>2</th>
<th>1.5</th>
<th>1.0</th>
<th>0.5</th>
<th>0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of bacteria after 18h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>1000</td>
<td>18000</td>
<td>20000</td>
<td>nc</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Purified neem extract (Trifolio)</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2.5</th>
<th>2</th>
<th>1.5</th>
<th>1.0</th>
<th>0.5</th>
<th>0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of bacteria after 18h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

nc= number of bacteria non determined.

For the Pseudomonas strains, the minimum concentration of neem extract was found 2.5 mg / ml. It is ten folds less concentrated (0.25 mg/ml) when the Trifolio purified extract was used.
Table 6. Determination of active concentration of neem extract: Test on Staphylococcus aureus Strain 1 (post-chirurgical host).

<table>
<thead>
<tr>
<th>neem extract mg in sol.</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2.5</th>
<th>2</th>
<th>1.5</th>
<th>1</th>
<th>0.5</th>
<th>0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of bacteria after 18h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>800</td>
<td>1400</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td>Purified neem extract (Trifolio)</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2.5</td>
<td>2</td>
<td>1.5</td>
<td>1</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>number of bacteria after 18h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
</tr>
</tbody>
</table>

Table 7. Determination of active concentration of neem extract (Test with Staphylococcus aureus Strain 2).

<table>
<thead>
<tr>
<th>neem extract mg in sol.</th>
<th>5</th>
<th>4</th>
<th>3</th>
<th>2.5</th>
<th>2</th>
<th>1.5</th>
<th>1</th>
<th>0.5</th>
<th>0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of bacteria after 18h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>300</td>
<td>700</td>
<td>nc</td>
<td>nc</td>
</tr>
<tr>
<td>Purified neem extract (Trifolio)</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2.5</td>
<td>2</td>
<td>1.5</td>
<td>1</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>number of bacteria after 18h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

The results list above show the effect of neem extract on pathogenic bacteria. The efficacy of neem extract increase when the purification lead to higher level of Azadirachtin in the solution.

**BACTERICIDAL AND INHIBITION EFFECT OF NEEM EXTRACTS**

According to our previous results, neem extract can act by two pathways: The first one may be inhibitory effect by which the bacterial growth is inhibited but bacteria remain alive. The second case occurs when the neem compounds cause the bacteria irreversible decay. To check these effects, fresh growth medium is used to inoculate samples of inactive populations from previous culture broth. In a positive case of bactericidal effect, no growth again can be observed. In case of inhibition, growth of bacteria starts again. The results are summed up on the following table.

Table 8: Test for inhibition control

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number bact. after 18h incubation</th>
<th>Number bact. after 72h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neem crude extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus 1</td>
<td>0</td>
<td>1600</td>
</tr>
<tr>
<td>Staphylococcus 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas 1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
These results indicate that the neem extract has bactericidal effect. Only one sample shows an inhibitory effect (Staphylococcus Strain 1). The neem extract can be considered at these concentrations to have anti bacterial effect on Staphilococcus and Pseudomonas species.

CONCLUSION

The neem compounds have anti bacterial and inhibition effect against some pathogenic bacteria, especially staphylococcus sp. and Pseudomonas Sp. The minimum active concentration of equivalent Azadirachtin in neem extract is 2.5 mg / ml in a crude extract. The anti bacterial effect is higher with a purified extract containing 0.25mg / ml of Azadirachtin (solution from Trifolio). Ours results are different of those published later by A.V.B. Sakaram et al who did not find activity against Staphylococcus et Pseudomonas species. That may be due to the difference of anti bacterial molecules concentrations in the solutions used by the authors. (They used 62.5 μg / ml, when we use about 2500μg / ml in our tests). The Azadirachtin seems to be the main responsible of anti bacterial effect of neem crude ethanol extract. In the economical point of view, the purification and concentration of neem active compounds are difficult and expensive particularly for developing countries. So, it will be better to use crude extract. For african people, this is the best way to reduce the importation of manufactured antibiotics and to promote a low cost health. However, more research must be done to explain the role of Azadirachtin (or its synergistic action with other compounds) as active and anti bacterial compound in the neem extract. It is also necessary to elaborate new products with neem extract (emulsion, lotion . . .) more suitable for African use in health and cosmetics scope. That could be the best way to stimulate the protection of neem trees and to make its by products more available in African tropical regions.

ACKNOWLEDGMENTS

We thank DR H. Kleeberg (Trifolio-M-GmbH, Germany) who gently provides us a purified sample with high azadirachtin contain. We are particularly grateful to Dr R. SOUDRE, the head of faculty of medicine, University of Ouagadougou, Dr SANGARE Senior Research, dermatose department, National Hospital of Ouagadougou for their continuous and valuable assistance during our tests. This study was supported by a grant awarded by IDRC (International Development Research Centre, Canada) and France Cooperation Ministry.

REFERENCES


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| Pseudomonas 2 | 0 | 0 |
| Sample from Trifolio |
| Staphylococcus (1 & 2) | 0 | 0 |
| Pseudomonas (1 & 2) | 0 | 0 |


Effect of Azadirachta indica (Neem) extract on Paracetamol induced hepatotoxicity

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Amira M. El-Noweihi, Adel A. Gomaa and I.M. Kelany
Departments of Forensic Medicine and Toxicology, Biochemistry, Histology and Pharmacology, Faculty of Medicine, Assiut University, Assiut, Egypt
and Faculty of Agriculture, Zagazig University, Zagazig, Egypt

ABSTRACT

Azadirachta indica, popularly known as “neem” is an important plant commonly used in agriculture and in indigenous medicine. It is used mainly for controlling insect pests. Recently, there are arguments about the effect of this plant on the liver. In the present work, we investigated the effect of the water soluble extract in rats given hepatotoxic dose of paracetamol on histopathological and biochemical basis.

The animals were divided into 4 groups, each was 12 rats. The first group received neem extract and paracetamol, the second group was given neem extract and saline, the third group was given paracetamol. The last group received saline and served as controls. The liver of each animal was examined histopathologically and part of it homogenized and the homogenate was used to determine the liver enzymes, GOT, GPT and albumin. Also the homogenate was used to determine the degree of oxidant
stress by determination of total thiols and catalase. The histopathological results showed that the hepatotoxic effects of paracetamol were greatly ameliorated by plant extract. Biochemically, this was shown by significant decrement of liver enzymes GOT and GPT and degree of oxidant stress manifested by increased levels of total thiols and catalase. These data, thus, support the hepatoprotective effect of the plant on paracetamol induced toxicity.

INTRODUCTION

Azadirachta indica (Syn. Melia Zadirachta Linn., Meliaceae) popularly known as "neem" is an indigenous plant commonly grown in India, Sri Lanka, Burma and Malaya. Due to its manifold uses, not only does it grow in the wild, but it is also cultivated by the people in these countries (Chopra et al., 1956 and Chattopadhyay et al., 1992). Nowadays the plant is found in Asia and in East and West Africa. In the Middle East it has been introduced in Yemen and Saudi Arabia (Van der Nat et al., 1991) and recently in Egypt. The plant is rich in active compounds that belong to the structure classes of limonoids, flavonoids, macromolecular substances together with sulphurous compounds in smaller concentrations. All parts of the neem tree contain active compounds, however most of the active ingredients are concentrated in the seeds followed by the leaves, bark and twigs. The effects of the various active ingredients are quite different (Hla Tin Oo., 1987 and Van der Nat, 1991).
Azadirachta indica has been used for a long time in many fields, where it proved useful for agricultural and industrial application. In agriculture, neem extracts of the seeds, kernels and cake have a potential role in controlling insect pests. They are a natural resources which are effective against a broad spectrum of insect pests. They act as a repellent, phago-deterrent, antifeedant and as growth-regulators (Jacobson, 1986; Hla Tin Oo., 1987 and Saxena, 1989).

The medicinal properties of the tree have drawn attention. It is used as antiseptic, has a role in wound healing and curing skin diseases, anti-ulcer activity, anti-inflammatory and anti-rheumatic, anti-pyretic effects. Furthermore, anthelmintic, emetic, stimulatory as well as anti-malarial and anti-diabetic effects are attributed to neem preparations (Van der Nat et al., 1991).

Recently, hepatoprotective activity of the plant leaves has been reported (Chattopadhyay et al., 1992). They related the hepatoprotective activity of the plant to the flavonol-o-glycosides present in the leaf extract. In contrast, hepatotoxic effect of neem leaf extract was also reported by other investigators (Obaseki et al., 1985).

The present work was designed to evaluate the effect of the neem seed kernels on the structure and function of the liver to detect whether it has a protective or toxic effect. The hepatoprotective effect of the neem extract was investigated in rats by using paracetamol, as it is well known by its hepatotoxic activity (Jollow et al., 1973; Potter et al., 1973; Strubelt and Younes, 1992).
MATERIALS AND METHODS

Materials:

Paracetamol (acetaminophen) was obtained from EIPCO, Cairo. Watery neem extract was prepared from the powder of neem seed kernels [(NEEMAZAL-W), a product of Trifolio-M GmbH Company, Lahnau, Germany] by dissolving 4 g of powder in 100 ml distilled water.

Animals:

Forty eight young male albino rats were used in this study. The average of their weight was 100-120 gm. The animals were fed normal purina cow and water ad libitum.

Animals were divided into four groups of 12 rats each:

- The first group of animals (A) was injected daily (I.P.) by 20 mg/kg of neem extract, for three weeks. Then the animals were injected with paracetamol 0.8 mg/kg (I.P.) (as 0.25 M supersaturated solution at 40°C).

- The second group of animals (B) was given 20 mg/kg of neem extract (I.P.) daily for 3 weeks, then the animals were injected by saline.

- The third group (C) was injected by saline for 3 weeks then the animals were injected by paracetamol 0.8 mg/kg (I.P.).
The fourth group was given saline and served as a control group.

All animals were scarified after 12 hr. from the last injection.

**Biochemical and histopathological studies:**

After decapitation of rats, liver samples were obtained for biochemical and histopathological investigations.

**Biochemical analysis:**

Part of the liver of each animal was homogenized in saline and the homogenate was used to determine the 2 transaminases GPT and GOT according to the method of Reitman and Frankel (1957), albumin by the dye binding technique using the method of Doumas and Biggs (1972). Meanwhile, the concentration of total thiols was performed as described by Ellman (1959) while catalase determination was done according to Beutler (1975). Total protein of the homogenates was determined by the method of Lowry et al. (1951).

**Histopathological study:**

Small specimens of the liver of each animal were taken and fixed in Bouin’s solution. The specimens were paraffin embedded and sectioned at a thickness of 7 μm. The sections were stained with hematoxylin and eosin to study the general histological structure according to Drury and Wallington (1980). Other small pieces were fixed in glutaraldehyd and processed as for electron microscopic examination. Semithin sections were cut at 2 μ and stained with 0.5% toluidine blue. Ultrathin sections were
contrasted with uranyl acetate and lead citrate. Electron micrographs were taken with Jeol TEM.

The data obtained were calculated and statistically analyzed using Student’s t-test.

**RESULTS**

**Biochemical results:**

Table 1 presents the results of GOT, GPT, albumin, total thiols and catalase measurements in the group of animals given paracetamol and the control group given saline only. GOT, GPT and catalase levels were significantly increased in the paracetamol group compared with the control group. Total thiol level was significantly decreased in the paracetamol group compared with the control while the levels of albumin were insignificantly decreased in the paracetamol group in comparison with controls.

Table (2) shows the mean levels of the various indices in the group given neem with paracetamol compared with the control. No changes were observed in these indices except for albumin which show significant decrease in their levels.

On the other hand, the levels of GOP, GPT, albumin and catalase in the group received this mixture (neem and paracetamol) were significantly decreased compared with the group received paracetamol alone. Contrarily, the level of the total thiols was
increased in the first group compared with the paracetamol group. However, this increase was insignificant (Table 3).

Table (4) clarifies that the levels of all the different indices were not significantly changed by the administration of neem given alone to a group of animals compared with the same indices of the control group.

The individual values of GOT, GPT, albumin, total thiols and catalase in the different groups of animals are depicted in Figs 1, 2, 3, 4, 5 respectively, where the differences between the various groups are further clarified.

**Histopathological results:**

The liver of control animals displayed features similar to those characteristic of normal tissue. The cells are arranged in cords or plates separated by blood sinusoids lined by flat endothelial cells. The liver cells are polyhedral in shape. The cytoplasm is eosinophilic with uniform granulation. Each liver cell has one or two central, rounded vesicular nuclei with one or two prominent nucleoli (Fig. 6). The examination of semithin sections stained with toluidine blue showed the large polyhedral cells with large vesicular rounded nuclei. The cytoplasm shows uniformly distributed cellular organelles which appear as dark areas. The cells are separated by blood sinusoids (Fig. 7). Electron microscopic examination showed that the surface of the hepatocytes that faces the space of Disse bears many microvilli protruding into that space. The liver cells has rounded nucleus with one or two typical nucleoli and fine dispersed
chromatin. The cytoplasm has abundant endoplasmic reticulum of smooth and rough varieties. The liver cells have numerous elongated and rounded mitochondria usually in association with rough endoplasmic reticulum, lysosomes and Golgi bodies. The cytoplasm frequently contain glycogen in the form of coarse electron-dense granules (Fig. 8).

In animals treated with paracetamol only, there was focal distortion of the tissue architecture, the liver cells showed evidence of cellular degeneration. The nuclei became small, irregular and dense. The cytoplasm showed loss of cellular architecture. Some cells appeared smaller than normal with dense nuclei and dark granulated cytoplasm having coarse granules. These cells were separated by irregular more dilated sinusoids compared with that of the normal liver. An accumulation of inflammatory cells was also observed (Fig. 9). In semithin sections, the periportal areas were the most affected parts of the hepatic lobules. Some cells showed loss of cytoplasmic architecture. These cells undergo frank degeneration with dark cytoplasm and small dense irregular nuclei. Other cells showed complete distortion of the cytoplasm with small or large vacuoles. Deposition of fibers was also observed between the cells (Fig. 10). By electron microscopic examination, there is frank degeneration of the hepatocytes with complete distortion of the cells. The nuclei became pychnotic and there was accumulation of filamentous structures and intracytoplasmic vacuoles which were variable in size, shape and contained materials (Fig. 11).
Table (1): GOT, GPT, albumin, total thiols and catalase in animals given paracetamol compared with controls. Data are expressed as mean±S.E.

<table>
<thead>
<tr>
<th>Biochemical indices</th>
<th>Control (D) (n=12)</th>
<th>Paracetamol (C) (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOT (mU/mg protein)</td>
<td>8.01±0.34</td>
<td>10.4±1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>GPT (mU/mg protein)</td>
<td>31.2±4.9</td>
<td>45.8±4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Albumin (gm/ml extract)</td>
<td>0.83±0.09</td>
<td>0.650±0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>Total thiols (mmol/mg prot.)</td>
<td>0.12±0.01</td>
<td>0.09±0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Catalase (K/gm protein)</td>
<td>0.620±0.03</td>
<td>1.0±0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Table (2): GOT, GPT, albumin, total thiols and catalase in animals given paracetamol and neem compared with controls. Data are expressed as mean±S.E.

<table>
<thead>
<tr>
<th>Biochemical indices</th>
<th>Control (D) (n=12)</th>
<th>Paracetamol + neem (A) (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOT (mU/mg protein)</td>
<td>8.01±0.34</td>
<td>7.8±0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>GPT (mU/mg protein)</td>
<td>31.2±4.9</td>
<td>28.3±1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>Albumin (gm/ml extract)</td>
<td>0.83±0.09</td>
<td>0.32±0.040</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Total thiols (mmol/mg prot.)</td>
<td>0.12±0.010</td>
<td>0.14±0.030</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>Catalase (K/gm protein)</td>
<td>0.62±0.03</td>
<td>0.7±0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
</tbody>
</table>
Table (3): GOT, GPT, albumin, total thiols and catalase in animals given paracetamol and neem compared with group given paracetamol. Data are expressed as mean±S.E.

<table>
<thead>
<tr>
<th>Biochemical indices</th>
<th>Paracetamol+neem (A) (n=12)</th>
<th>Paracetamol (C) (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOT (mU/mg protein)</td>
<td>7.8±0.4</td>
<td>10.4±1.2</td>
</tr>
<tr>
<td>GPT (mU/mg protein)</td>
<td>28.3±1.7</td>
<td>45.8±4.5</td>
</tr>
<tr>
<td>Albumin (gm/ml extract)</td>
<td>0.32±0.035</td>
<td>0.650±0.06</td>
</tr>
<tr>
<td>Total thiols (mmol/mg prot.)</td>
<td>0.14±0.03</td>
<td>0.09±0.009</td>
</tr>
<tr>
<td>Catalase (K/gm protein)</td>
<td>0.70±0.05</td>
<td>1.0±0.10</td>
</tr>
</tbody>
</table>

Table (4): GOT, GPT, albumin, total thiols and catalase in animals given neem compared with controls. Data are expressed as mean±S.E.

<table>
<thead>
<tr>
<th>Biochemical indices</th>
<th>Control (D) (n=12)</th>
<th>Neem (B) (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOT (mU/mg protein)</td>
<td>8.01±0.34</td>
<td>9.03±0.7</td>
</tr>
<tr>
<td>GPT (mU/mg protein)</td>
<td>31.2±4.9</td>
<td>37.9±3.2</td>
</tr>
<tr>
<td>Albumin (gm/ml extract)</td>
<td>0.83±0.09</td>
<td>0.51±0.05</td>
</tr>
<tr>
<td>Total thiols (mmol/mg prot.)</td>
<td>0.12±0.01</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>Catalase (K/gm protein)</td>
<td>0.72±0.03</td>
<td>0.70±0.08</td>
</tr>
</tbody>
</table>
In animals treated with both paracetamol and neem, most of the liver cells appeared normal apart from the presence of mildly dilated blood sinusoids. Some cells were slightly smaller with coarse granulated cytoplasm. The tissue also showed few cellular infiltration (Fig. 12). In semithin sections, most of the liver cells possessed one or two large, rounded vesicular nuclei. The cell organelles appeared as dark areas besides the intracytoplasmic vacuolations. The vacuoles were variable in size and shape and contained granulated materials (Fig. 13). By electron microscopic examination, the liver cells exhibit more or less normal architecture with nearly normal distribution of cellular organelles (Fig. 14).

Although, there was slight affection of the hepatocytes in animals treated with neem only, dilated vasculature that include both the sinusoids and the central veins were observed. Some cells were smaller in size and have dark granulated cytoplasm. Other cells showed vacuolated cytoplasm. Few infiltrated cells were also observed (Fig. 15). In the semithin section, some cells appeared dark with dark nuclei, others appeared light with large vesicular nuclei. The cytoplasm of both types of cells showed numerous vacuoles that were varied in sizes and shapes (Fig. 16). Electron microscopic examination, there were intracytoplasmic vacuolation. These vacuoles were membrane bound and irregular in shapes, some of these vacuoles were swollen mitochondria with loss of cristae and loss of density of ground substance (Fig. 17).
Fig. (1): Individual values of GOT in the 4 studied groups

Fig. (2): Individual values of GPT in the 4 studied groups
Fig. (3): Individual values of albumin in the 4 studied groups

Fig. (4): Individual values of total thiols in the 4 studied groups

Fig. (5): Individual values of catalase in the 4 studied groups
DISCUSSION

In recent years, Azadirachta indica (neem) was introduced in Egypt for agricultural and medicinal purposes. Among its agricultural uses are its potential value in controlling insect pests where it affects more than 195 species of insects. It is a promising source for natural sound pesticides (Saxena, 1989). Besides, the plant has wide medicinal values. Of special interest is its role as a hepatoprotectant. Paracetamol is a drug known to induce hepatic damage (Potter et al., 1973). In the present study the hepatotoxic effects of paracetamol was further confirmed (Table 1). The toxic effect was manifested by significant increase in GPT and GOT levels in liver homogenates. Such increment could be due to increased synthesis of the enzymes in order to correct for metabolic derangements induced by paracetamol in the form of mitochondrial dysfunction, where the compound or its metabolites inhibit oxygen consumption of rat hepatocytes and to deplete mitochondrial ATP (Burcham and Harman, 1991 and Strubelt and Younes, 1992). The increase in transaminases could represent a trial to increase carbohydrate intermediates. Nevertheless, the significantly decreased hepatic thiols in the present study, could be due to depletion of glutathione by binding to it and then binds extensively to the sulfhydryl groups of cellular proteins (Hoffman et al., 1985). Besides, hepatocyte protein thiols are also depleted through oxidation. In addition, paracetamol leads to the conversion of the enzyme xanthine dehydrogenase to xanthine oxidase. This conversion, in conjunction with decrease in the activity of enzymes that affect cellular thiol homeostasis and the degradation of adenine nucleotides, may lead to transient production of activated oxygen species and
promote the oxidation of protein thiols (Trimenstein and Nelson, 1990). In a trial to overcome such stress, catalase activity is enhanced as evident in Table (1).

The hepatotoxic effect of paracetamol was histologically evident in the form of focal distortion of the tissue architecture. The cells have dense nuclei and dark granulated cytoplasm. Hepatic necrosis induced by paracetamol was also reported by Dixon et al. (1971) after administration of a single dose of the drug. The main events were hydropic vacuolation, centrilobular necrosis and macrophage infiltration. French et al. (1988) reported that there are multiple focal inflammation that included macrophage mononuclears and multifocal centrilobular spotty necrosis were seen. There is also increased collagen deposition among the cells. Recent studies proposed that paracetamol hepatotoxicity result from the formation of reactive metabolites of paracetamol during its biotransformation. This action is through induction of a microsomal drug metabolizing enzymes which has been shown to increase the susceptibility to hepatotoxicity of paracetamol (Nassar et al., 1993).

When animals were supplied with paracetamol and neem seed kernel extract, the liver transaminases showed significant decrease in comparison with group supplied by paracetamol while catalase activity exhibited significant decrease accompanied by insignificant increment of total thiols. This is an indication of hepatoprotective effect of neem on cellular level against hepatotoxic effect of paracetamol. In a previous study Chattopadhyay et al. (1992) reported that administration of neem leaf extract and paracetamol resulted in significant decrease of serum SGOT and SGPT, which was
accompanied by little deviation from the histopathological picture of normal rats. According to these investigators, the increased levels of serum enzymes in the rats treated with paracetamol alone were due to induction of hepatic necrosis by drug. The extract of neem leaves seem to afford protection from such effect. They attributed such protective effect to the active ingredients in the leaves of the plant. Among these are the flavonol-o-glycosides which are known to be responsible for blood sugar lowering activity against streptozotocin induced diabetes in rats (Chakraborty et al., 1989). From our study at the hepatocellular level, it could be suggested that many of the active compounds present in neem seed kernel have hepatoprotective effect against paracetamol. These compounds could inhibit the effect of paracetamol on oxygen consumption on rat hepatocytes and its role on depletion of ATP. Flavonol-o-glycosides which stimulate glucose oxidation and lower blood glucose could partly mediate this action. Also, neem is rich in components which could act as antioxidants and thus result in increase in thiols and decrease in catalase. However, extensive detailed studies are required to exactly understand such hepatoprotection. The histopathological study in this work was also support the hepatoprotective activity of the neem seed extract against paracetamol.

When the rats were supplied with neem seed kernel extract, the livers of the animals were not affected except the decrease of albumin concentration (Table 4). This decrease seem to reflect a reaction to the injection of the drug. However, slight affection of hepatocytes after neem administration were observed histopathologically, there was some vacuolation of the cytoplasm. By electron microscopic examination,
some of these vacuoles appeared as swollen mitochondria with loss of their cristae. Sato et al. (1981) described the vacuolated mitochondria in the hepatocytes with administration of acetaminophen after chronic ethanol consumption in the rats.

Thus, besides its hepatoprotective effect against paracetamol, the neem plant is without marked deleterious side effects. Awareness of the undesirable side effects of synthetic pesticides is growing rapidly in our community during the last years. Therefore, due to its pesticidal properties, neem seem to be a promising natural pesticide that would replace the synthetic pesticides.

ACKNOWLEDGMENT

The authors wish to thank Dr. Hubertus Kleeberg, Lahnau, Germany for kindly providing the NEEMAZAL-W formulation used in the present investigation.
Fig. (6): A photomicrograph of a paraffin section of control liver showing; cords of cells with vesicular nuclei and granular cytoplasm. Note the blood sinusoids separating the cells (†).

(H, E x 30)

Fig. (7): A photomicrograph of a semithin section of control liver showing; the hepatocytes with uniformly distributed cytoplasmic organelles appear as dark areas and large vesicular nuclei.

(Toluidine blue x 1250)
Fig. (8): An electron micrograph of a control liver cell showing: numerous mitochondria (m), RER, lipid granules (L), lysosomes (Ly); glycogen particles (†), microvilli (mv) and bile canaliculi (bc).

(x 5,000)
Fig. (9): A photomicrograph of a paraffin section of liver of rats treated with paracetamol showing: loss of architecture of tissue, dense nuclei and dense cytoplasm (↑). Note the inflammatory cellular infiltration (Cl).

(H, E x 320)

Fig. (10): A photomicrograph of a semithin section of liver of rats treated with paracetamol showing: loss of cellular architecture, dense nuclei and dark cytoplasm. Note the deposition of collagen fibers (cf)

(Toluidine blue x 1250)
Fig. (11): An electron micrograph of liver cells of rats treated with paracetamol showing; dense nuclei (N), the cytoplasm is largely devoid of structure (↑), large vacuoles (V).

(x 5,000)
Fig. (12): A photomicrograph of a paraffin section of liver of rats treated with paracetamol and neem showing hepatocytes return its normal structure. Note slight dilatation of blood sinusoids (↑).

(H, E x 320)

Fig. (13): A photomicrograph of a semithin section of liver of rats treated with paracetamol and neem showing, normal hepatocytes. Note the intracytoplasmic vacuoles (V).

(Toluidine blue x 1250)
Fig. (14): An electron micrograph of a liver cell of rats treated with paracetamol and neem showing; the cytoplasm contain numerous mitochondria (m) and strands of RER. (x 5,000)
Fig. (15): A photomicrograph of a paraffin section of liver of neem treated rats showing dilatation of blood sinusoids and shrinkage of hepatocytes (↑).
(H, E x 320)

Fig. (16): A photomicrograph of a semithin section of liver of rats treated with neem showing; some cells appear dark with dark nuclei, others appear light with vesicular nuclei. Note the vacuoles in the cytoplasm inside both types of cells (V)
(Toluidine blue x 1250)
Fig. (17): An electron micrograph of a liver cell of neem treated rats showing; intracytoplasmic vacuoles (V), vacuolated mitochondria with loss of cristae and loss of density of ground substance (Vm).

(x 5,000)

REFERENCES


