# Azadirachtin use efficiency in commercial neem formulations

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**Biological efficacy of eight commercial neem formulations (CNFs) were investigated in two separate studies. The assays were conducted against second instar larvae of diamondback moth (DBM), *Plutella xylostella*, at different time intervals from the date of manufacture of formulations. The formulations represented a wide range of azadirachtin content and different formulation techniques. The studies demonstrated a possible loss of biological activity with increasing duration of storage of the CNFs. In both the studies, the $LC_{50}$ values of the formulations decreased with the concentration of azadirachtin in CNF and the observed decay functions were significant. However, since the decrease was not a linear function, the relationships were re-examined between the azadirachtin content in the CNFs and the $LC_{50}$ values expressed in ppm of azadirachtin. In both the studies it was observed that the relationships were strongly positive and followed power functions, suggesting lower use efficiency of azadirachtin with higher azadirachtin content in the CNFs. The observed lower efficiency may be due to two possible non-exclusive reasons. One, the concentration of azadirachtin results in the loss of other possibly synergistically active compounds found in neem, which are lost due to efforts of concentration of one chemical. Secondly, azadirachtin has several effects on the biology of insects. It is possible that the different activities may be more pronounced at specific concentrations and that less toxic antifeedant or repellent effects may be evident at higher concentrations that led to the observed results. Which of these two explanations is more plausible is an interesting question that needs to be pursued. Nevertheless the observed results call for a re-examination of efforts towards increasing the concentration of azadirachtin in CNFs.**

*ARISHTA*, the cure-all, is one of the Sanskrit names for neem, *Azadirachta indica* A. Juss (Meliaceae). The name largely stems from its multitudinal uses both in medicine and agriculture[1]. These properties are due to a large number of secondary plant metabolites found in various parts of the tree. However in the recent past, chemical constituents of neem seeds are being intensively explored since they have proved to be an excellent source of a wide variety of chemicals useful in the management of pestiferous insects and many plant diseases[1,2]. It has also been shown that a simple aqueous extract of neem seed kernels (NSKE) is an excellent, inexpensive home-made pesticide useful in tackling many serious pests of crops[3,4]. NSKE is widely recommended as a potent insecticide for the management of a large number of pests especially the Lepidoptera[4,5]. However, despite repeated demonstration of its excellent field performance, farmers rarely use NSKE largely due to the slightly cumbersome procedure of its preparation[6].

One way of promoting neem, therefore, as an alternative to synthetic pesticides, is to develop proprietary products. The knowledge of the high potency of azadirachtin (a constituent chemical found in relatively high concentrations in seed) against a wide variety of crop pests has now resulted in the development of many commercial neem formulations (CNFs)[7]. These formulations provide three great advantages. First, the fragile natural resource, azadirachtin, is highly unstable in the seeds and the potency of the seeds is lost exponentially upon storage[8,9]. CNFs provide an avenue to conserve this resource by reducing the rate of loss of azadirachtin manyfolds[9]. Secondly, they can be bought off-the-shelf and used with greater ease than the seeds. Another important advantage is their likely rapid action on the insects that might consequently reduce the rate of crop loss[10].

Since almost a decade, many CNFs, based on azadirachtin, are available in the Indian market for use by farmers. Earlier, products with azadirachtin concentrations of 300 and 1500 ppm were not found to be very useful under field conditions[7]. Therefore, intensive efforts were made to develop CNFs with higher concentrations of azadirachtin. As a result, during the last few years a series of new CNFs were launched by various firms with azadirachtin concentrations ranging up to 65,000 ppm. Now the question arises as to whether these formulations are superior to earlier formulations of lower azadirachtin content.

In order to address these issues, we conducted two laboratory studies using a range of CNFs. The first study involved a verification of three CNFs and the second involved a separate set of six CNFs. A series of bioassays were carried out using these CNFs against a lab culture of diamondback moth (DBM), *Plutella xylostella* (L.), collected from cabbage fields in Doddaballapura near Bangalore[8]. The culture was continuously maintained on mustard seedlings. At the beginning of the study, December 2000, the DBM culture was approximately 50 generations old and by July 2002, when the last set of bioassays were completed, the DBM culture was 75 generations old.

Bioassays were carried out using the standard leaf-dip method[8,11]. After an initial bracketing study, nine concentrations were set for each product and each assay. Successive dilutions were made on a semi-logarithmic scale using a stock solution of suitable concentration. Each treatment also contained 0.05% soap as the surfactant. Clean mustard leaves were cut to bits of approximately 10 sq. cm area with the stalk intact. Each leaf bit was

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**Table 1.** Details of the commercial neem formulations used in the study. Set I was used for the first study and set II was for the second study

| Commercial neem based products | Date of manufacture | Batch No. | Azadirachtin content (ppm) | Manufacturers |
|---|---|---|---|---|
| *Set I* | | | | |
| Nimbecidine® | October 2000 | 2 | 300 (0.03%) | T. Stanes and Company Ltd., Coimbatore |
| Econeem Plus® | October 2000 | 2310 | 10,000 (1%) | Margo Bio-control Pvt. Ltd., Bangalore |
| Soluneem® | August 2000 | 0800 | 65,000 (6.5%)* | Vittal Mallya Research Foundation, Bangalore |
| *Set II* | | | | |
| Limonool® | September 2001 | 28 | 300 (0.03%) | Bio Multi-tech (Pvt) Ltd., Bangalore |
| Neemgold®** | August 2000 | 10/2000–01 | 1500 (0.15%) | SPIC Biotechnology Division, Porur, Tamil Nadu |
| Econeem® | October 2001 | 10710 | 3000 (0.3%) | Margo Bio-control Pvt. Ltd., Bangalore |
| Econeem Plus® | October 2001 | 2210 | 10,000 (1%) | Margo Bio-control Pvt. Ltd., Bangalore |
| Fortune Aza® | September 2001 | No information (sample) | 30,000 (3%) | Fortune Bio tech Lab, Hyderabad, Andhra Pradesh |
| NeemAzal™-F | August 2001 | 0108 | 50,000 (5%) | EID Parry (India) Ltd., Chennai |

*Ascertained through oral enquiry; Soluneem is a water soluble powder and all other products are in liquid formulations; **Purchased from open market.

then dipped in the respective concentration of the product for 15 s and air-dried in shade. The stalk was then wrapped with fresh cotton soaked in clean water and transferred to a petri plate. Ten freshly-hatched second instar larvae of DBM were released onto these leaf bits. Three such replicates were maintained for each concentration of the CNFs. A 0.05% soap solution in distilled water served as the control. In all, 300 larvae were used for each assay. The petri plates were maintained in room conditions (23.5 ± 1.3°C). Observations were made once in 12 h post-treatment, but only the data of 96 h was used as these values were found to give minimum coefficient of variation. The mortality data were corrected using Abbott's formula and median lethal concentrations were computed through Probit analysis following Finney (1971)[12].

In the first study, three CNFs were used and the assays were repeated five times. In the subsequent study, six CNFs were used and three assays were conducted. Required CNFs were obtained either from the concerned manufacturer or purchased in sufficient quantities. The details of the products used in the study are indicated in Table 1. A new container was opened for each assay to avoid any possible degradation of the chemical due to exposure to atmospheric conditions. The $LC_{50}$s worked out were subjected to ANOVA to ascertain the differential performance of the CNFs. The $LC_{50}$ values for each CNF were then converted in terms of ppm of azadirachtin using the declared concentration of aza on the container label or product information leaflet.

Five bioassays carried out from December 2000 to August 2001 indicated two important features. First, the $LC_{50}$ values of the three CNFs decreased with the concentration of aza in the formulation. The mean $LC_{50}$ values for the three CNFs were 0.39 ± 0.09, 0.16 ± 0.02 and 0.03 ± 0.01 per cent respectively for Nimbecidine with 300 ppm, Econeem Plus with 10,000 ppm and Soluneem with 65,000 ppm of azadirachtin. ANOVA revealed the differences among the three CNFs to be significant ($F_{2,8}$ = 83.39; $P < 0.01$). Secondly, there was a monotonic increase

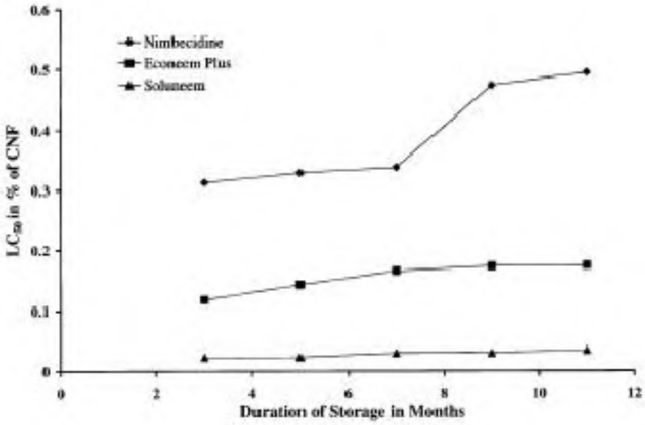


**Figure 1.** Trends in $LC_{50}$ values, expressed as per cent of formulations for the three neem formulations tested, reflecting the change in insecticidal property with time. The bioassays were conducted by leaf-dip method against the freshly-moulted second instar larvae of *Plutella xylostella*.

in the $LC_{50}$ values recorded for all the three CNFs with duration of storage (Figure 1). Nimbecidine registered an $LC_{50}$ value of 0.313 per cent concentration during December 2000 which increased to 0.494 per cent by August 2001. An initial $LC_{50}$ value of 0.118 per cent rose to 0.174 per cent by August 2001 for Econeem Plus. Similarly, an initial $LC_{50}$ value of 0.023 per cent for Soluneem increased to 0.035 per cent by the last assay. The differences between the months, however, were not significant ($F_{4,8}$ = 2.05; $P > 0.05$) suggesting possibility of greater stability of CNFs in comparison to neem seeds[8,9].

The second set of assays also revealed similar results for the six CNFs tested. Limonool with the lowest azadirachtin content of 300 ppm registered $LC_{50}$ values of 0.550 to 0.611 per cent, while the NeemAzal F with 50,000 ppm of azadirachtin registered $LC_{50}$ values of 0.057 to 0.069 per cent. ANOVA revealed the differences among the six CNFs ($F_{5,10}$ = 574.74; $P < 0.01$) to be significant. However, unlike in the earlier study, the differences between the months were found significant ($F_{2,10}$ = 17.74;

$P < 0.01$), thereby suggesting a loss of insecticidal property with time even in CNFs. This difference in result might be due to difference in the time gap between months of repetition of the bioassays which were large for the second study. But the rate of loss may be much lower than what it is in neem seeds[8,9].

Azadirachtin being the primary compound of insecticidal value in the CNFs, it is anticipated that the relationship between the $LC_{50}$s and the azadirachtin content in the CNFs is linear. Both the studies clearly indicated the $LC_{50}$ values to decrease with increasing azadirachtin concentration in CNFs. However, the expected linear relationships were not evident and the trends followed strong decay power functions (Figure 2 *a* and *b*). Both the relationships were significant ($P < 0.05$) and represented the best fit trends. Interestingly, in both the trend lines, the regression coefficients were similar and the observed differences in values were non-significant ($F_{1,29} = 0.081$; $P > 0.05$), although the intercepts differed ($F_{1,30} = 17.76$; $P < 0.05$). Thus the analysis of covariance (F test) convincingly demonstrates that the rate of change of $LC_{50}$ values with increasing azadirachtin content in the CNFs remains identical irrespective of the numbers and types of formulations used. Regression for the pooled data was also found to be significant with similar regression coefficient ($b = -0.453$). However, the observed difference in intercepts may be due to differing test conditions such as age of the insect culture, number of repetitions (five and three in the two studies), difference in the intervals of repetitions, etc.

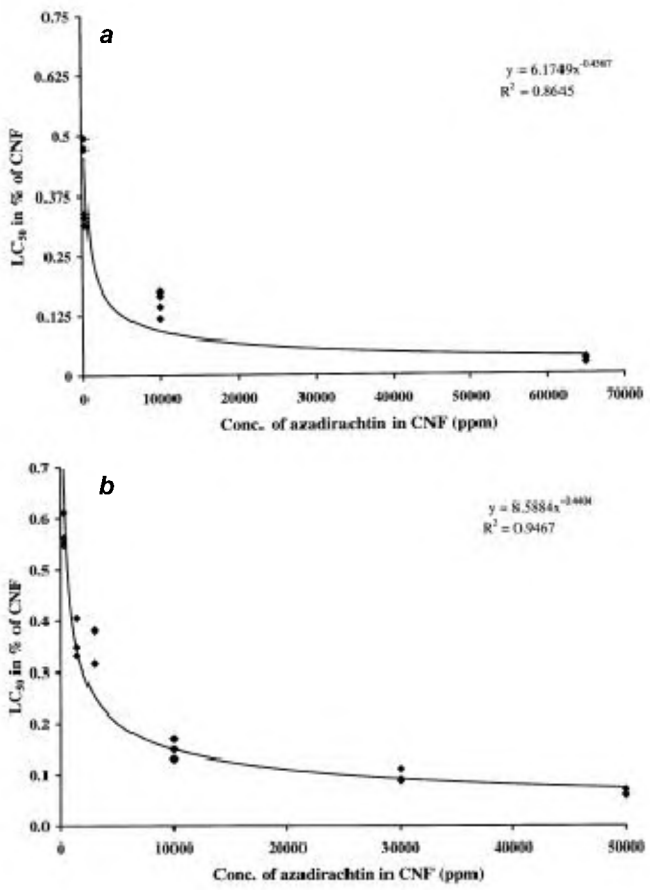


**Figure 2.** The trends in $LC_{50}$ values expressed as per cent of the formulation for the (***a***) three formulations in the first study and (***b***) six formulations in the second study when plotted against the azadirachtin content in the neem formulations. Both the relationships were significant and nonlinear. The lines represent the best fit functions. The $LC_{50}$s are based on bioassays conducted by leaf-dip method against the freshly moulted second instar larvae of *Plutella xylostella*.

These results therefore, suggest a possible nonlinear trend in the efficacy of CNFs with changing azadirachtin content. One way of checking this possibility is to evaluate the CNFs in terms of azadirachtin required for achieving median mortality of the test insect.

The $LC_{50}$s available in terms of per cent concentration of the CNFs were therefore converted into $LC_{50}$ values, in terms of ppm of azadirachtin. These values were plotted against the values of azadirachtin concentration available in the CNFs. We anticipated an invariant relationship, because, azadirachtin is expected to have the same biological activity against a test insect in any kind of formulation and irrespective of the level of concentration in different CNFs. However, the best fit regressions plotted for both the studies followed increasing power functions (Figure 3 *a* and *b*) and the analysis of covariance indicated a repetition of what was observed for the $LC_{50}$s expressed in terms of formulations. Clearly, the study indicated an increasing dose requirement of azadirachtin to achieve 50% kill of the test insect with the concentration of azadirachtin in the CNFs. Other studies have pointed to the possibility of lack of one-to-one correspondence in the association between the biological activity and the content of the major active ingredient, azadirachtin, in many commercial products[13,14] but the nature of the relationship was not evident. The present study strengthens evidence to this effect while providing an insight into the nature of trends in the activity of CNFs with the increasing azadirachtin content.

What are the implications of these results? As the azadirachtin content in the CNF increases, more and more quantity of the same is required to achieve the median mortality of the test insect. Therefore, one strong conclusion that can be drawn is the decreasing biological efficacy of azadirachtin with increasing concentration in the formulation. Another major implication is in conserving this resource. CNFs are expected to help conserve the natural resource, azadirachtin[9]. However, going by the above results, any attempt at developing formulations with high azadirachtin content will be counter productive due to reduced efficiency of the chemical for purposes of pest management.

It is possible that the azadirachtin content in the CNFs do not match exactly with the declared content due to various reasons. Such a variation can occur due to the variability in prevailing conditions at the time of manu-

facture and also due to degradation, with time, of the active ingredient[15]. Given this possibility, any variation in the azadirachtin content relative to the declared content can also result in the observed trend. In the absence of a chemical estimation data, we checked this aspect by working out the expected azadirachtin content in the three formulations of the first study, if the dose–response relationship is linear. This is possible by keeping the values of one of the formulations constant to serve as the standard (Table 2). The analysis would provide an opportunity to estimate the extent of deviation required to achieve the observed results.

It was observed that when 300 ppm was kept constant, for a linear response the 10,000 ppm and the 65,000 ppm formulations were expected to have just $3894 \pm 1109$ (mean $\pm$ s.d.) and $5007 \pm 1456$ ppm of azadirachtin respectively. Similarly, when 10,000 ppm was kept as the standard, the 300 and 65,000 ppm formulations were expected to have $800 \pm 233$ and $12,407 \pm 3367$ ppm of azadirachtin respectively. Alternatively, when 65,000 ppm values are kept constant and the corresponding values worked out for 300 and 10,000 ppm formulations, the expected values were $4369 \pm 1281$ and $52,728 \pm 14,043$ ppm of azadirachtin. This exercise amply demonstrates that the deviations expected to achieve a linear relationship among the three formulations need to vary enormously from the declared azadirachtin content. For example, the 300 ppm formulation needs to contain nearly three or 14 folds the declared azadirachtin value, if the 10,000 ppm or 65,000 ppm formulation is kept as the base value of response. Similarly, the 65,000 ppm formulation needs to have just 1/13 or less than 1/3 the declared azadirachtin content for a linear relationship to hold. Expectations of such an enormous deviation from declared content is unreasonable in any formulation. Therefore, marginal variation, if any, occurring in the azadirachtin content of the CNFs is unlikely to affect the results of the study. This conclusion can also be drawn by a careful examination of the Figures 3 *a* and 3 *b*.

In order to increase the concentration of azadirachtin in a formulation, exclusive formulation techniques may need to be followed. As a consequence, the differences in the formulation techniques might also contribute to the observed trend to indicate the lower efficiency of azadirachtin at higher concentrations. The first study represented three different kinds of formulations while the second represented two. But for one product, the two studies represented entirely different sets of products and manufacturers, possibly representing different manufacturing procedures. Despite these differences between the two studies, the slopes of the trend lines remained almost identical ($F_{1,29} = 0.081$; $P > 0.05$) suggesting the lack of impact of the type, number, kinds and other product differences of formulations in influencing the behaviour of the active ingredient, azadirachtin. Further, the congruence of the slopes indirectly suggests the declared azadirachtin content to be highly reliable at least in respect of the ratios between the products, if not in terms of the actual content. Therefore, the declared azadirachtin content is a sufficient indicator of the strength of the formulations used and does not constitute a limitation for the observed trend.

Neem has a bounty of limonoids that are of insecticidal value. A number of these chemicals found in the seed are expected to act synergistically to enhance the mutual biological activity. Azadirachtin among them, however, is considered the most biologically active and is also relatively abundant of such compounds. Therefore, the extraction and concentration of this particular chemical in the CNFs leads to the likely loss of other chemicals that are equally potent or synergistic in action, but are found in lower quantity in the seeds[16]. As a consequence, greater the concentration of azadirachtin lower will be the diversity of biologically active chemicals leading to the loss of potential benefits of other associated chemicals such as multiple actions and synergism. Eventually this leads to the decrease in effectiveness of the chemical at higher

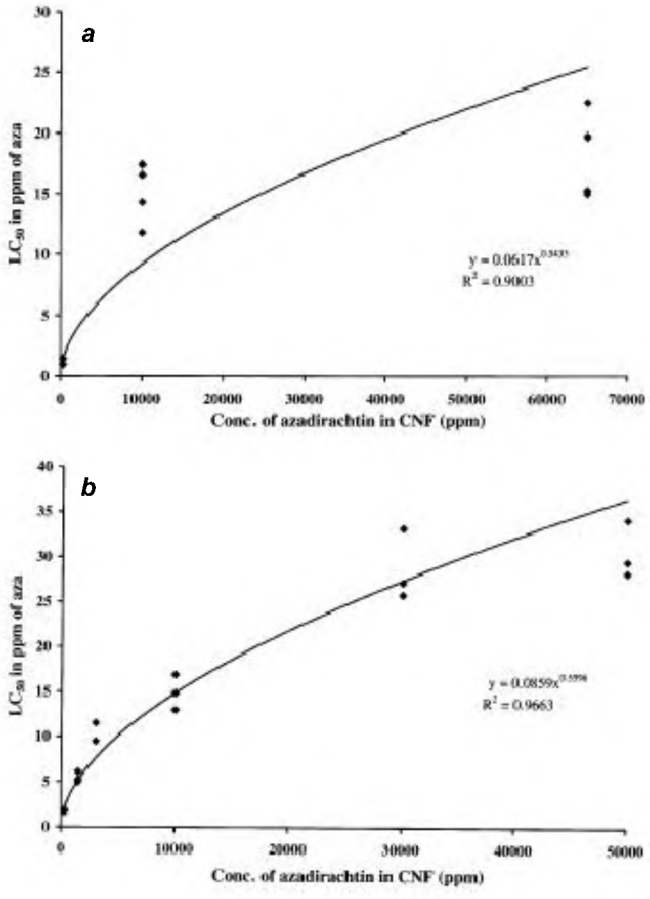


**Figure 3.** The trends in $LC_{50}$ values expressed in terms of concentration of azadirachtin for the (***a***) three formulations in the first study and (***b***) six formulations in the second study when plotted against the azadirachtin content in the neem formulations. Both the relationships were significant and nonlinear. The line represents the best fit power function. The $LC_{50}$s are based on bioassays conducted by leaf-dip method against the freshly moulted second instar larvae of *Plutella xylostella*.

**Table 2.** The expected concentration levels of azadirachtin in formulations with 300, 10000 and 65000 ppm considering one of the three as the base value and assuming a linear relationship for efficacy among the three formulations considered in the first of the two studies. (See text for more details)

| | Projected azadirachtin value (in ppm) in different formulations* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Declared azadirachtin content in the formulation (ppm) | | | | | | | | |
| | 300 | | | 10,000 | | | 65,000 | | |
| Base conc. in ppm | Min. | Max. | Mean ± s.d. | Min. | Max. | Mean ± s.d. | Min. | Max. | Mean ± s.d. |
| 300 | | | **300** | 2385 | 5571 | 3894 ± 1109 | 3039 | 7209 | 5007 ± 1456 |
| 10,000 | 540 | 1300 | 800 ± 233 | | | **10,000** | 8600 | 19,100 | 12,407 ± 3367 |
| 65,000 | 2730 | 6435 | 4369 ± 1281 | 33,930 | 75,400 | 52,728 ± 14,043 | | | **65,000** |

*The rows indicate the expected azadirachtin content in the other two formulations when one of the formulation is kept as the standard (in bold). Similarly, the columns indicate the expected concentration of azadirachtin in the respective formulations (normal fonts) when two other formulations are kept as standards (bold). The range, mean and the standard deviations were worked out considering the five $LC_{50}$ values as possible variants.

concentration thus making CNFs with higher azadirachtin content to be less potent than crude extracts. This possibility seems to be time and again substantiated by the fact that a well-prepared NSKE remains more effective than most CNFs with comparable or higher azadirachtin content[3,13]. Synergistic effects of plant allelochemicals is known previously in other systems[17]. In this context the observed results are not totally unexpected.

Further, neem and azadirachtin have multiple modes of action on insects. The biological effects like repellence[18], feeding deterrence, oviposition deterrence[19], mating disruption[20], insect growth regulating effect on metamorphosis[21], reduced fecundity and viability of eggs[22], egg sterility, ovicidal and insecticidal action[23] are known against a large variety of insects. In the present study, we have examined only the mortality of the larvae. Therefore it can be argued that other effects, if examined independently, might produce different results. However, these varied effects are age and stage dependent. We have used freshly-moulted second instar larvae. In such young larvae, these varied effects are expected to be subsumed in the death of the larvae and therefore, the results are unlikely to be different in the context of other independent effects.

A high concentration of azadirachtin in CNFs also poses another inherent danger. Synthetic insecticides have single biologically active compounds and their repeated use invariably results in build-up of resistance among the pestiferous target insects[4]. Great diversity of biologically active chemicals found in neem acts as an inbuilt resistance prevention mechanism among the target insects[24,25]. Any attempt at heavier reliance on a single chemical, azadirachtin in the present case, therefore, should lead CNFs only to the fates of such biological products as pyrethrum or *Bt* toxin[26,27].

Enriched extracts, however, are expected to provide many advantages besides reducing the content of unwanted chemicals[28]. The present study has clearly demonstrated the lower use efficiency of azadirachtin with its increasing concentration in the CNFs. Further, any difference in the rates of loss of bio-activity might potentially weigh against formulations with higher azadirachtin content. Thus, there is a need for a balanced approach in the development of CNFs of higher azadirachtin content. In this context, it would be of interest to explore whether there is an optimum concentration level for azadirachtin that can be used in neem-based formulations.


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# *Ropalidia marginata* – a primitively eusocial wasp society headed by behaviourally non-dominant queens

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***Ropalidia marginata* is a primitively eusocial polistine wasp widely distributed in peninsular India. As in most other primitively eusocial insects studied so far, colonies are headed by a single queen who monopolizes reproduction while the remaining female wasps in the colony function as sterile workers. Unlike in other species however, *R. marginata* queens are strikingly docile individuals who show little or no physical dominance. When such a behaviourally docile queen is removed from a colony, one of the remaining individuals becomes extremely aggressive, and is known from previous work, to go on to become the next queen if the original queen is not returned. When the original queen is returned after a day's absence, she re-establishes herself as the queen and she usually manages to do so with little or no aggression. We hypothesize that *R. marginata* queens use dominance behaviour to suppress worker reproduction in the beginning, and that they use pheromones to regulate worker reproduction once they establish themselves and start laying eggs. If this hypothesis is correct, *R. marginata* would be an ideal model system to study the possible evolutionary transition from physical inhibition to chemical regulation of worker reproduction, and the transition from primitively eusocial to highly eusocial in general.**

MANY insect species live in societies of varying degrees of complexity. To differentiate relatively loose social aggregations from true societies and to concentrate on the highest levels of social evolution, attention is usually focused on a subset of social species which are said to have achieved eusociality. Eusocial species are defined as those that exhibit overlap of generations, cooperative brood care and reproductive caste differentiation. Among these, primitively eusocial species are characterized by small colony sizes, absence of queen–worker dimorphism and physical inhibition of worker reproduction by the queens (e.g. many species of wasps and bees). On the other hand, highly or advanced eusocial species are characterized by large colony sizes, clear-cut queen–worker dimorphism and chemical (pheromonal) regulation of worker reproduction by queens (e.g. ants, termites and honeybees)[1].

*Ropalidia marginata* (Lep.) (Hymenoptera: Vespidae), widely distributed in Peninsular India has been considered a primitively eusocial species because of the absence of morphological differentiation between queens and workers and because many, if not all, female wasps can mate, develop their ovaries and function as solitary nest foundresses or as queens of multiple foundress nests[2]. There is growing evidence that *R. marginata* is different from other primitively eusocial species. In all other primitively eusocial species studied so far, queens are the most behaviourally dominant and active individuals and are thus expected to be capable of using physical aggression (dominance behaviour) to suppress worker reproduction[3,4]. In contrast, an *R. marginata* queen has been described as a behaviourally non-dominant, docile sitter, who cannot possibly inhibit worker reproduction by physical aggression and who probably uses pheromones to do so[2,5–7]. In

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