Characterization and testing of fine powder formulation of whole neem fruits

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Azadirachtin (Aza) is a key ingredient of neem-based pesticides. However, use of neem pesticides is limited due to storage instability of Aza. In this work, free-flowing fine powder of whole dry neem fruits (powder neem formulation, PNF) is developed without separately extracting Aza. The optimal particle size was found to be −44 + 60 mesh. PNF is characterized by Fourier transform infrared, X-ray diffraction, Brunauer–Emmett–Teller surface area, particle-size distribution and scanning electron microscope. Stability of Aza was found to be improved and it was assessed by studying the effect of particle size, temperature, UV light exposure and release study in buffered and natural water samples.

Keywords: Azadirachtin, characterization, free-flowing powder, particle-size, stability.

BIOPESTICIDES are obtained from plants and microorganisms and have gained significant attention in recent times due to their harmful effects of chemicals. Besides, these compounds are more selective and biodegradable. The most commonly used biopesticides are neem-based. Since ancient times, different parts of *Azadirachta indica* (neem) tree such as leaves, bark, flowers, fruits and seeds are used to control pests and for various other medicinal applications. Azadirachtin (Aza) is a key ingredient of neem-based biopesticide responsible for inhibition of pests. It is a powerful insect antifeedant and growth-regulating substance; besides controlling pests, neem extracts are also found to inhibit nitrification and retard nematode growth[1,2]. However, use of neem pesticides is limited owing to Aza storage instability.

The three major factors that influence instability of Aza are degradation in water, pH sensitivity and photodegradation. Szeto *et al.*[3] studied hydrolysis of Aza in natural water and aqueous buffered solution, and found that these active materials are non-persistent in aquatic environment. Andrew *et al.*[4] suggested that Aza is highly stable in mildly acidic (pH 4 and 6) aqueous solutions at room temperature, but it is unstable in mildly alkaline and strongly acidic solutions. Durege *et al.*[5] noted that Aza is highly photolabile. The (E)-2-methylbut-2-enoate ester group in Aza isomerizes into Z-2-methylbut-2-enoate ester when irradiated as a thin film under UV light at 254 nm, due to the presence of π-electrons. Kumar *et al.*[6] studied the stability of Aza in different solid carriers and found that degradation is least in attapulgite.

In this study, whole neem fruits are used in their original form without extracting Aza separately. This method ensures better Aza stability as well as effective utilization of other limonoids in neem fruits. According to Verkerk and Write[7], presence of other limonoids in neem seeds increases the biological activity of pure azadirachtin. Whole neem fruits are dried and ground with dolomite into fine powder using a special technique developed by them. This method reduces particle size and creates new surface area, which in turn increases the availability of Aza on the surface of the particles. Dolomite acts as an inert material and does not have any effect on plant growth except increasing the leaf size[8,9]. Normally, oil seeds cannot be converted into fine powder because they contain oil, and therefore the neem fruit powder is limited to coarser size, which results in a comparatively small surface area. This problem is overcome by grinding dry neem fruits with dolomite, which absorb or adsorb released oil and make PNF free flowing. PNF produces a sustained release effect since the ingredients are gradually released, thus ensuring long-term protection from insects. PNF could also be directly dusted near the root of crops or may be sprayed directly mixed with water, on the leaves. Thus, the insecticidal effect could be much stronger and long lasting.

Dry neem fruit and dolomite powder were purchased from a local market. Aza (95% purity) was purchased from Sigma Chemical Co. Methanol, water and acetonitrile (all HPLC grade) were purchased from Fisher Scientific India.

Pre-treatment of whole dry neem fruit included washing, cleaning and drying. Cleaned dry fruits were then pulverized with dolomite powder (85 mesh size) with 1 : 1 ratio by weight in a hammer mill (RPM-9000; HP-2 Shubh Micro Baby Pulverizer, India). After pulverization, PNF was sieved and its moisture content was determined according to the Bureau of Indian Standards (IS 3579-1966). Moisture contents in all samples were 6% ± 0.5% (wt/wt). Figure 1 shows dried whole neem fruit, dolomite powder and PNF.

Each study was conducted by analysing the concentration of Aza in PNF, which was determined by HPLC (Water Make Model no. 515) method[10–12]. The chromatographic determination of Aza from different PNF samples was achieved using a C-18 analytical column with a water–acetonitrile mixture (65 : 35) as the mobile phase (flow rate 1 ml/min; UV detector at 214 nm). A 10 μl sample was then directly injected into the column. The retention time of Aza was 18.49 min (ref. 12). A stock solution (40 ppm) of standard Aza (95% purity) was prepared in methanol and stored in a volumetric flask.

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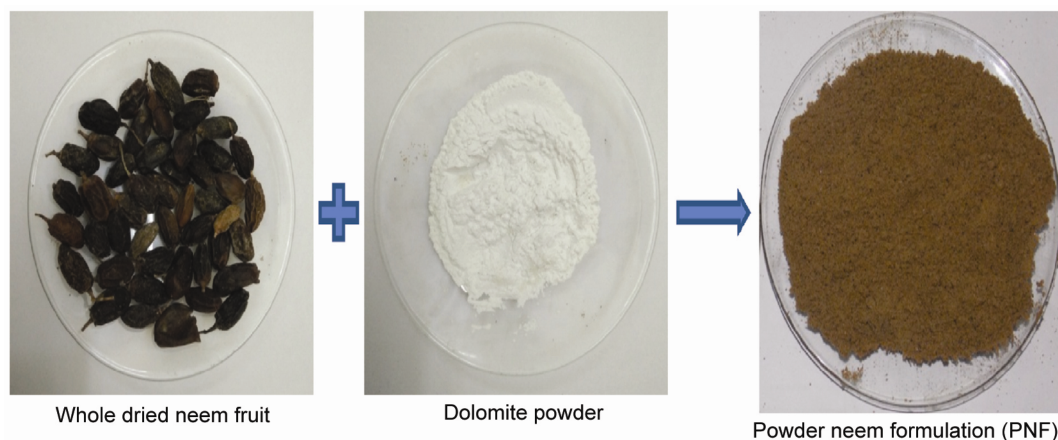

Figure 1. Free-flowing fine powder of whole dry neem fruits.

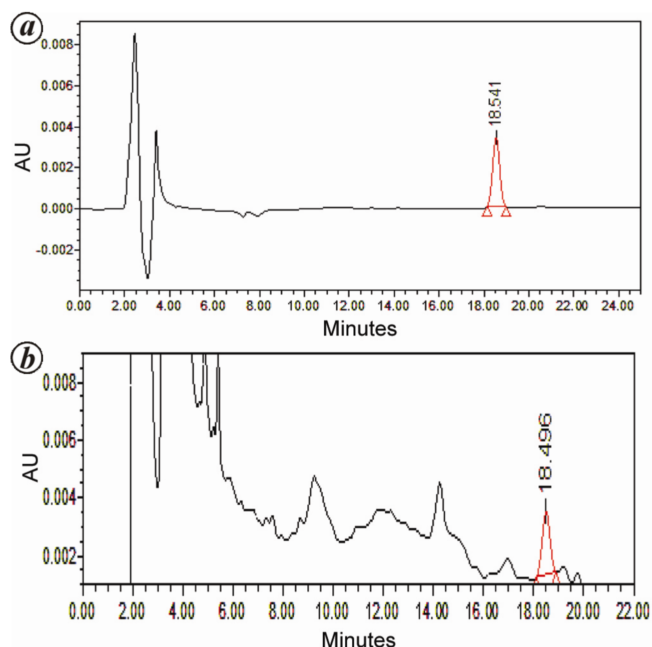

Figure 2. HPLC peak of Aza with retention time of 18.496; ***a***, Standard Aza sample in methanol; ***b***, Crude extract of PNF sample in methanol.

The flask was then kept inside a refrigerator (below 0°C). Using this stock solution, Aza solutions of different concentrations (5, 10, 15 and 25 ppm) were prepared. These solutions were analysed by HPLC. Retention time of pure Aza (i.e. 18.49 min) and the peak area of different solutions were determined. A calibration plot was used to correlate the concentration of unknown sample. Aza content in PNF was determined by ultrasonic assisted extraction of the sample in methanol using a water bath sonication process (Ultrasonics Pvt Ltd, Mumbai, India). About 10 g of the PNF sample was mixed with 100 ml of methanol (HPLC grade) and sonicated for 30 min. This sample was then centrifuged and the extract phase containing Aza was separated. The extract was then concentrated to 10 ml by rotary evaporation (Jain Scientific Glass Work). The concentrated sample was passed through a fluorescein column to remove colour impurities and then passed through a syringe filter with a pore diameter of 0.456 μm (Millipore, USA), diluted, and finally loaded onto the HPLC column[13]. Before conducting the experiment, the initial concentration of Aza in PNF was determined each time, in mg per 10 g of PNF sample. HPLC peaks of standard Aza (95% pure) in methanol and the extract of PNF in methanol are shown in Figure 2 *a* and *b* respectively.

PNF was characterized by different techniques. Particle-size distribution was analysed using Fritsch particle sizer (ANALYSETTE 22) to obtain size fractions of PNF. FTIR of dry neem fruit, dolomite and PNF were recorded using a Perkin Elmer spectrometer. The spectral data were used to compare the functional groups of raw materials and PNF. The XRD patterns were recorded on a Philips X'Pert Pro PANalytical PW3040/60 diffractometer to obtain information about crystallographic structures. SEM analysis (JEOL 6380A/JFC 1600) was carried out to study the surface morphology of PNF. BET surface area of dry neem fruit, dolomite and PNF was determined using a surface area analyser (Smart Sorb 92/93).

PNF contains Aza in its natural form as well as other limonoids. Effect of various parameters, such as particle-size, temperature and UV exposure, on the stability of PNF was studied. In addition, we also studied the release of PNF in buffered and natural water.

Five fractions of different sizes of PNF were taken for this study (Table 1). Each fraction was quantified by Aza available on the particle surface and Aza present inside the PNF particles. Aza content on the surface was determined by sonication-assisted extraction at ambient temperature for 30 min. Aza present inside the particles was quantified by Soxhlet extraction in methanol. In brief, this involved the following steps: 10 g of PNF wrapped in

Table 1. Surface and total extractable Aza in PNF samples with different particle sizes

Sieve mesh no. (as per BSS)	Mean size (μm)	Surface azadirachtin (mg/10 g of sample)* (*a*)	Total extractable azadirachtin (mg/10 g of sample)* (*b*)	% Aza available on surface (*a*/*b*) × 100
−16 + 25	957	10.7	22.3	48
−25 + 44	533	13.6	24.3	56
−44 + 60	303	20.9	26.9	77.7
−60 + 85	215	21.8	27.1	80.5
−85 + 100	165	23.1	28.6	80.8

*Mean of three replicates determined in five samples. B.S.S – British Stander Sieves BSS 410-2000.

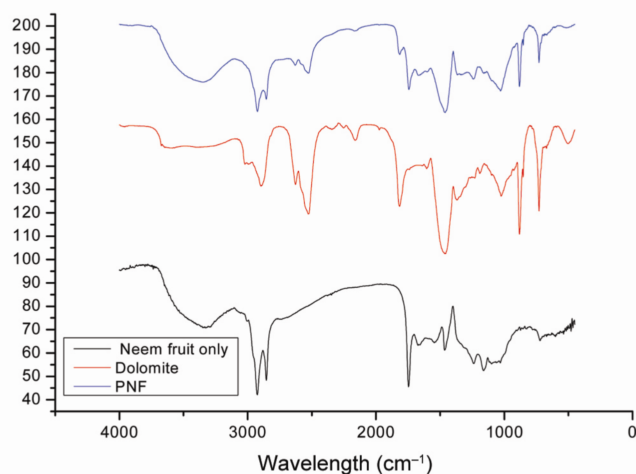

Figure 3. FTIR of dry neem fruit, dolomite and PNF.

Table 2. Release study in different aqueous solutions

Concentration (ppm)	% Aza remaining in PNF cake	% Aza release in filtrate
Buffered solution of pH 9.2	NF	NF
Buffered solution of pH 4.0	100	NF
NMC water sample pH 8.02	82.9	17.02
Well water sample pH 7.57	87.8	12.1

*The study was conducted for total period of 48 h in a dark compartment with an average temperature of 23°C. NMC, Nagpur Municipal Corporation; NF, Not found (no peak appears at retention time of 18.46 min).

a filter paper was placed in a Soxhlet apparatus. About 150 ml of methanol (HPLC grade) was added to carry out the extraction at 64.7°C for 4 h (ref. 12). The solvent was recovered and the PNF extract in methanol was concentrated to 10 ml. The concentrated sample was passed through a fluorescein bed and then through a syringe filter. The clear sample was diluted and loaded onto the HPLC column.

An optimal size fraction of PNF was taken to study the effect of temperature on its stability. Experiments were conducted at 50°C and 100°C respectively. Initially, 100 g of PNF sample was taken in a petri dish and kept in an oven at 50°C. The sample was withdrawn at regular intervals of 1, 3, 7 and 14 days. The concentration of Aza in each sample was determined by HPLC. The same experimental procedure was followed to study the effect of temperature at 100°C.

To study the effect of UV radiation on PNF, 100 g of powder was taken in a petri dish and kept in an UV chamber (Biotechnic India-32, model no. 19222) for 14 days. The UV lamp was placed at a distance of around 18 cm from the PNF sample and a uniform temperature of 50°C was maintained inside the chamber. The sample was withdrawn at regular intervals of 1, 3, 7 and 14 days. Aza concentration in each sample was determined by HPLC.

The release of PNF was studied using the following four aqueous samples: buffer solutions of pH 9.2 and 4.2, laboratory tap water (pH 7.57), and local municipal corporation water supply (pH 8.02) (Table 2). About 10 g of PNF was mixed with 50 ml of each aqueous solution. After 48 h, the solution was filtered, and the cake and filtrate were collected separately. The cake was dried and Aza content determined. The filtrate was completely evaporated at 60°C and the Aza content of the residue was determined.

It was observed that as the particle-size of PNF decreases, the percentage of Aza available on particle surface increases (Table 1). This is because, as the particle-size decreases, a new surface of PNF is created, which increases the availability of Aza on the surface. Approximately 48% of Aza is available for −16 + 25-mesh size fraction of PNF and 80.8% is available for −85 + 100 fractions. In other words, finer the PNF particle, larger will be the Aza molecules available on the surface. Table 1 shows that a size fraction of −44 + 60 mesh gives 77.7% surface Aza. By further decreasing the size to −85 + 100 mesh, Aza on the surface increased by only 3.1%. Size reduction beyond 100 mesh is energy-intensive and moreover it is observed that below 100 mesh size, there is no substantial increase in surface Aza. When energy requirement for size reduction below 100 mesh is compared with available Aza, it is found inefficient. Similar observations were recorded by Jadeja *et al.*[12]. For better surface Aza and proper energy for size reduction, PNF powder of mesh size −44 + 60 was considered optimal.

FTIR spectra of pure neem fruit, dolomite and PNF are shown in Figure 3. FTIR spectra of neem fruit showed the

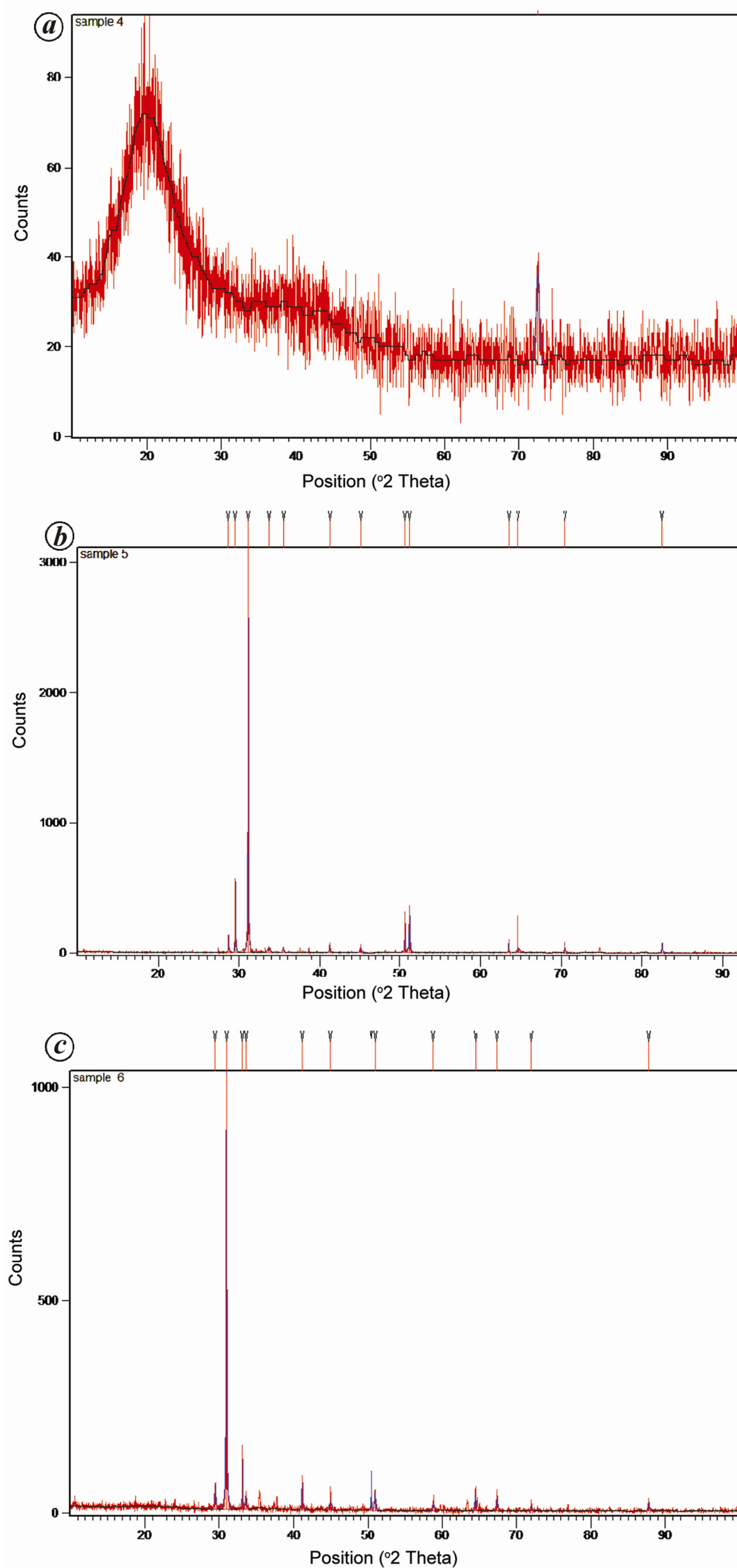

Figure 4. XRD of (***a***) dry neem fruit, (***b***) dolomite and (***c***) PNF.

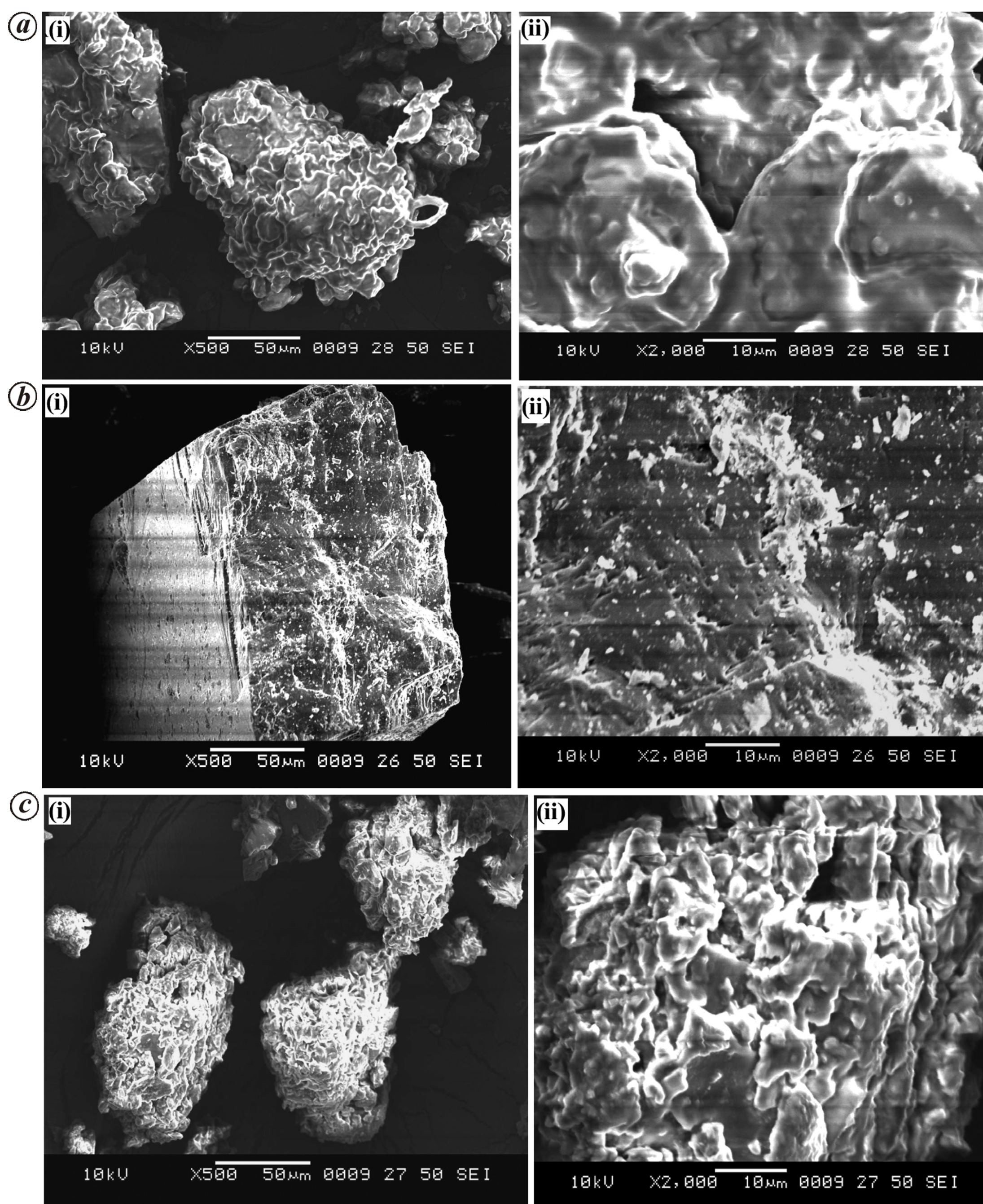

Figure 5. SEM (i) 500× and (ii) 2000×: ***a***, dry neem fruit seed; ***b***, dolomite; ***c***, PNF.

following bonds and corresponding functional groups: 3326-hydroxyl O–H stretch (hydrogen bonded) in alcohol/phenol, 3306-C=H stretch in alkene, 2924-2854-C–H stretch in alkene, 1746-C=O stretch in ester/saturated aliphatic, 1702-1672-C=O stretch in α–β unsaturated aldehyde and keton, 1465-C–H stretch in the aromatic ring, 1238-C–H stretch in alcohol carboxylic acid ester ether, 1161–1128, 1097-C=O stretch in alcohol carboxylic acid ester ether, 840-C–Cl stretch in alkyl halides, and 720-C–H rock in alkanes. Similar bonds were also observed in a study on neem leaves powder by Baljit Singh and Sharma[14]. In the FTIR spectrum of pure dolomite, several bands characteristic of carbonate group were observed. The bands at 1464 and 1191 cm^{-1} corresponded to stretching vibrations of C–O; those at 881 and 729 cm^{-1} were attributed to the out-of-plane and in-plane bending mode of $(CO_3)_2$ ion; and combinations of internal mode frequencies with a lattice mode were observed at 2538 and 2627 (1/cm) respectively[15]. In the FTIR spectrum of PNF, all the aforementioned peaks (neem and dolomite) were

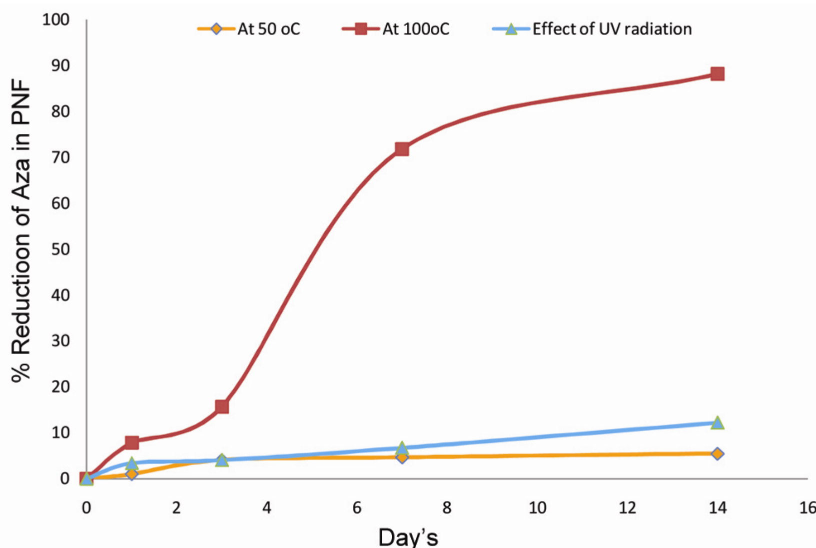

Figure 6. Effect of temperature (50°C and 100°C) and UV radiation on PNF.

observed (Figure 3). This proved that there was no chemical reaction or thermal degradation of chemicals in neem due to size reduction. Neem was present in PNF in its very natural form.

XRD spectra of neem fruit (Figure 4 *a*) showed a hump, which indicated the amorphous nature of neem powder. The *d*-spacing value of dolomite XRD (Figure 4 *b*) was compared with the reported *d*-spacing value[16] and these were found to match. A sharp peak confirmed the crystalline structure. XRD of PNF showed both crystalline and amorphous nature due to the presence of neem powder and dolomite.

SEM images provide insight into surface morphology. SEM images of pure dry neem fruit, dolomite and PNF are shown in Figure 5. The micrographs (500 × magnification) showed the assembly of fine particles that were not in regular shape and size. At 2000 × magnification, the SEM image of pure neem fruit powder appeared bulky/cloudy. This is because when the dry neem fruits are crushed, oil is released and lumps are formed, which causes cake formation. A rigid crystalline structure of dolomite was observed at 2000 × magnification. The image of PNF at 2000 × magnification indicated that the surface was free from oil. This is because the oil that oozes out during crushing is immediately adsorbed/absorbed by dolomite and a homogeneous free-flowing powder is obtained.

BET surface area of PNF was found to be 4.5 m^2/g. Normally oil seeds cannot be converted into free-flowing fine powder because of oil content. Neem fruit powder was limited to coarser size, which produced a comparatively small surface area. This problem was overcome by grinding dry neem fruit with dolomite, which removed oil and made the product free flowing with improved surface area.

Effect of temperature (50°C and 100°C) on PNF was studied (Figure 6). A minor reduction of Aza was noticed when PNF was treated at 50°C, compared to that at 100°C. These results confirmed an earlier study by Andrew *et al.*[4]. In general, if the on-field temperature does not exceed 50°C, then the PNF shows good stability. However, PNF should be applied on crops during morning hours. At 100°C, it was observed that degradation of Aza was slow in the initial period (up to 3 days) after which, it increased suddenly. The half-life period of Aza at 100°C was 5 days.

Effect of UV radiation on PNF showed that it was quite stable and its half-life period was more than 14 days. Standard Aza has a reported half-life of 3.9 days when exposed to UV light[11]. Thus, it can be concluded that Aza in its natural form in PNF is more stable under UV radiation than the pure isolated Aza.

Release study of Aza in PNF in buffer solutions (pH 9.2) showed that Aza was quite unstable and underwent complete degradation in 48 h. Thus, its half-life is less than 48 h. In a pH 4.0 buffer solution, the amount of Aza in the filtrate was negligible. This confirms that Aza was completely present in cake and has a half-life of more than 48 h. In Municipal Corporation water pH 8.02 sample, around 17% of Aza was present, whereas in laboratory tap water (pH 7.58), 12% of Aza was released from PNF. Thus, it can be concluded that the stability of Aza decreased with pH. These results demonstrate that at pH 7.58, release of Aza was slow compared with that at pH 8.02, and these also agreed with the study by Andrew *et al.*[4].

The present study has been directed towards the use of whole dry neem fruits in their natural form and making it more effective in terms of Aza content. Separation or isolation of Aza can decrease their stability. This will also add the need for unit operations such as depulping, and extraction with solvent, which unnecessarily increase the manufacturing cost. Developing a simple technique for manufacturing PNF could improve its effectiveness as

well as reduce the overall cost of manufacturing. Our results show that PNF is economical, stable, and can have a long-lasting effect on crops.

1. Mohanty, S., Patra, A. K. and Chhonkar, P. K., Neem (*Azadirachta indica*) seed kernel powder retards urease and nitrification activities in different soils at contrasting moisture and temperature regimes. *Bioresour. Technol.*, 2008, **99**, 894–899.
2. Akhtar, M., Plant growth and nematode dynamics in response to soil amendments with neem products, urea and compost. *Bioresour. Technol.*, 1999, **69**, 181–183.
3. Szeto, S. Y. and Wan, M. T., Hydrolysis of Azadirachtin in buffered and natural water. *J. Agric. Food Chem.*, 1996, **44**, 1160–1163.
4. Andrew, P., Jarvis, S., Johnson, E. and David, M., Stability of the natural insecticide Azadirachtin in aqueous and organic solvents. *Pestic. Sci.*, 1998, **53**, 217–222.
5. Durege, P. and Johnson, S., Photodegradation of azadirachtin-A: neem-based pesticide. *Curr. Sci.*, 2000, **79**, 1700–1703.
6. Kumar, J. and Parmar, B. S., Stabilization of Azadirachtin A in neem formulations: effect of some solid carriers, neem oil and stabilizers. *J. Agric. Food Chem.*, 1999, **47**, 1735–1739.
7. Verkerk, R. H. J. and Write, D. J., Biological activity of neem seed kernel extract and synthetic azadirachtin against larvae of *Plutella xylostellia* L. *Pestic. Sci.*, 1993, **37**, 83–91.
8. Benjawan Chutichudet, P., Chutichudet, S. and Kasewsit, Effect of dolomite application on plant growth international. *J. Agric. Res.*, 2010, **5**(9), 690–707.
9. Chen, G. C., He, Z. L., Stoffella, P. J., Yang, X. E., Yu, S. and Calvert, D., Use of dolomite phosphate rock (DPR) fertilizers to reduce phosphorus leaching from sandy soil. *Environ. Pollut.*, 2006, **139**, 176–182.
10. Kanth, M. S. and Sudaram, Azadirachtin biopesticide: a review of studies conducted on its analytical chemistry, environmental behaviour and biological effects. *J. Environ. Sci. Heal. B*, 1996, **31**, 913–930.
11. Dai, J., Yaylayan, V., Vijaya, A., Raghavan, G. S., Jocelyn, R. and Pare, Extraction and colorimetric determination of azadirachtin-related limonoid in neem seed. *J. Agric. Food Chem.*, 1999, **47**, 3738–3742.
12. Jadeja, G. C., Maheshwari, R. C. and Naik, S. N., Extraction of natural insecticide Azadirachtin from seed kernels using pressurized hot solvent. *J. Supercrit. Fluid*, 2011, **56**, 253–258.
13. Babic, S., Petrovic, M., Kastelan, M and Macan, Ultrasonic extraction of pesticides from soil. *J. Chrom. A*, 1998, **823**, 3–9.
14. Baljit Singh and Sharma, D. K., Development of a new controlled pesticide delivery system based on neem leaf powder. *J. Hazard. Mater.*, 2010, **177**, 290–299.
15. Gunasekaran, S. and Anbalagan, G., Thermal analysis of natural dolomite. *Bull. Mater. Sci.*, 2007, **30**, 339–344.
16. Samtani, M., Skrzypczak-Janktun, E., Dollimore, D. and Alexander, K., Thermal analysis of ground dolomite confirmation of results using an X-ray powder diffraction methodology. *Thermochim. Acta*, 2001, **367–368**, 297–309.

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Distribution and conservation status of the western tragopan *Tragopan melanocephalus* in Jammu and Kashmir, India

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In India, western tragopan is reported from Jammu and Kashmir (J&K), Himachal Pradesh and Uttarakhand. We documented the current status and distribution of western tragopan in J&K. We also predicted its potential distribution in the state. We used literature, field surveys and semi-structured questionnaire surveys to ascertain the distribution and conservation status of the pheasant species in J&K. Between 2007 and 2011, we conducted counts of western tragopan in five areas: Tattakuti Wildlife Sanctuary, Khara Galli Conservation Reserve (CR), Limber Wildlife Sanctuary (WLS), Lacchipora WLS and Kazinag National Park (NP) to assess its current status. We estimated 113 callers of western tragopan from Kazinag NP, Limber WLS, Lacchipora WLS, Tattakuti WLS and Khara Galli CR. We also discovered four new sites – Tattakuti WLS and Khara Galli CR (through direct surveys), Noorpur Galli and Narian-Ratannard (through secondary surveys) – of this species. We have confirmed the presence of western tragopan in Lacchipora WLS and re-confirmed its presence in Padder, Bhadarwa and Sudh Mahadev. Our habitat model predicted potential distribution of western tragopan, adding few additional potential sites. There is an urgent need to plan long-term monitoring and initiate appropriate measures to conserve the species.

Keywords: Conservation status, distribution, hunting, *Tragopan melanocephalus*.

WESTERN tragopan *Tragopan melanocephalus*, a threatened pheasant species[1], is endemic to the Western Himalaya[2]. Its distribution ranges from northwestern Pakistan[3] through Kashmir[4] into Himachal Pradesh (HP)[5] and possibly the western parts of Uttarakhand[6]. The current global population of western tragopan is reported to be between 2500 and 3500 individuals[7], although earlier studies estimated it to be around 5000 individuals[2]. The major reasons for its global decline are habitat degradation and

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