Azadirachtin-based insecticides induce alteration in *Helicoverpa armigera* Hub. head polypeptides

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Azadirachtin obtained from seed kernel of Indian neem (*Azadirachta indica* A. Juss) is a plant-based insecticide for field-crop applications. *Helicoverpa armigera* Hub. (Lepidoptera: Noctuidae) is one of the most destructive and highly polyphagous insect pests of field crops and has developed resistance against most of the modern classes of synthetic insecticides worldwide. The ultimate instars of *H. armigera* larvae treated topically with 10 ppm of three azadirachtin-rich neem formulations, viz. Achook, Nimbicidine and Neem Jeevan, were found to significantly ($P < 0.05$) reduce total head protein after 0, 24, 48 and 72 h of treatment application over control. Achook in 24 h and Neem Jeevan in 48 and 72 h exhibited significantly ($P < 0.05$) reduced protein level compared to Nimbicidine and control. Azadirachtin applied by injection method in third instar larvae significantly ($P < 0.05$) reduced protein level at higher dose (5 µl). Similar trend was found in larvae of fourth instar. Diet mixed with different azadirachtin-based formulations and fed to fourth instar larvae of *H. armigera* also significantly ($P < 0.05$) reduced protein level. The experimental results also indicated that serial dilutions of both azadirachtin and Nimbicidine treatment resulted in significant ($P < 0.05$) negative response to various doses by injection as well diet-fed method respectively. SDS–PAGE analysis of proteins showed that azadirachtin suppressed 22, 26, 40 and 56 kDa head polypeptides in various treatments. However, haemolymph injection of azadirachtin (10 ppm) into third and fourth instar larvae of *H. armigera* reduced the total head protein level below that of control larvae and altered the polypeptide pattern. Alteration in SDS polypeptide pattern was also observed with different modes of application in a dose-dependent fashion.

Keywords: Azadirachtin, *Helicoverpa armigera*, insect, polypeptide alteration, total protein.

AZADIRACHTIN, a tetranortriterpenoid from Indian neem [*Azadirachta indica* A. Juss (Meliaceae)], is well known as a potent phytogenic insect growth inhibitor[1]. It inhibits feeding and growth of insects belonging to several orders like Lepidoptera[2], Diptera[3], Orthoptera[4,5], Hemiptera[6], Coleoptera[7,8], Hymenoptera[1], etc. through intervention in endocrine events[9–13].

Helicoverpa armigera (Hubner) (Lepidoptera: Noctuidae) is one of the most destructive pests of field crops worldwide. It is highly polyphagous and causes severe damage and loss to a wide range of food, fibre, oil, fodder, vegetable, horticultural, ornamental, aromatic and medicinal plants. Polyphagy, high mobility, high fecundity and facultative diapauses are its key physiological, behavioural and ecological characteristics that facilitate survival even in unstable habitats[14]. It has developed resistance against most of the modern classes of synthetic insecticides like DDT[15], organophosphate[16], pyrethroids[17], and carbamates[18]. Natural insecticides are ecologically and adaptogenically most suitable and sustainable tools of pest management. Azadirachtin acts by interference in food utilization and alteration of midgut catalytic digestion[19]. Azadirachtin-treated *H. armigera* exhibit a higher rate of firing of sensilla, lack of natural response pattern after simulation of receptors leading to complete inhibition of feeding[20]. Previously, we have demonstrated the effect of azadirachtin on head protein content of *H. armigera*[21]. Azadirachtin-based insecticidal formulations have attained higher commercial status worldwide. Hence it is important to generate further information on the biochemical effect of azadirachtin and its commercial insecticides. In this communication, we have investigated the influence of azadirachtin and neem-based insecticidal formulations on insect head polypeptide profile.

Azadirachtin was procured from Supelco (Bullefonte, PA, USA). Azadirachtin-rich neem formulations, Achook (0.15% azadirachtin), Nimbicidine (0.03% azadirachtin) and Neem Jeevan (0.03% azadirachtin) were obtained from Godrej Agrovet Ltd (Surat, India), T. Stanes & Co Ltd (Coimbatore, India) and Jegson Hi-Tech Industries (Gurgaon, India) respectively. BSA, vitamins, cholesterol, streptomycin, choline chloride, sorbic acid, sucrose, yeast extract and other chemicals were obtained from Hi-Media (Mumbai, India), SISCO Research Lab (Mumbai, India), and Sigma Chemicals Co. (USA).

The semi-synthetic diets consisted of host plant material, yeast powder, mixture of vitamins, anti-microbials, phagostimulators and fixing materials[22].

Larvae of field-collected test insect (*H. armigera* Hub.) were reared on the semi-synthetic diet at a photoperiod of 16 h light and 8 h dark cycle, 60–70% relative humidity and temperature 24 ± 2°C. Adult moths were reared in 10 l capacity jars for maturation, mating and egg-harvesting. Larvae were reared in culture tubes (2 × 5 × 6 cm) individually on semi-synthetic diet continuously over the year.

Azadirachtin (70% ethanol) was applied topically (2 µl/larva) at thoracic region of second and sixth instars larvae anesthetized with diethyl-ether, on dorsal side by Burkard Arnold Hand Microapplicator. In another experimental treatment, azadirachtin was injected in second abdominal proleg of third, fourth, and fifth anesthetized

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larvae using a 10 µl Hamilton syringe fitted with 26 S pointed needle. The doses (per larvae) were 1, 2, 4, 5, 6, 8 and 10 µl of 10 ppm solution.

Each larva was treated only once. Control larvae were applied with the corresponding carrier (5 µl/insect) alone[23]. Twenty larvae were used for each treatment and the treatments were replicated five times. Larvae were allowed to feed on normal diet for 0, 24, 48 and 72 h post-treatment. For comparative analysis of azadirachtin formulations Nimbicidine, Neem Jeevan and Achook were dissolved in distilled water (10 ppm active ingredients) and topically applied (2 µl/insect) at ultimate instar stage. Larvae were maintained on the normal diet for 0, 24, 48 and 72 h post-treatment. Controls were treated with distilled water (5 µl/larva) alone.

For feeding experiments, Achook, Nimbicidine and Neem Jeevan (0.01%, w/v) were mixed in freshly prepared semi-synthetic diet[24] and fed to fourth instars larvae as serially diluted concentrations of 50, 25, 6.25 and 3.12 ppm of Nimbicidine insecticide mixed in semi-synthetic diet and fed to third instars larvae for 6 days. Twenty larvae of each feeding set, replicated five times were sampled for analysis. Control larvae were allowed to feed on untreated diet.

Twenty larval heads were homogenized in 1 volume of an extraction medium (0.1 M Tris-Cl pH 7.5, 1% Triton-X 100 and 10% DMSO) by hand homogenization in a pestle and mortar. The homogenate was centrifuged at 15,000 *g* (15 min, 4°C) and the supernatant was precipitated with equal volume of trichloroacetic acid (12%). Precipitates were collected by centrifugation and dissolved in 0.1 N NaOH and protein was estimated by Lowry's method[25] using BSA as standard.

The polypeptide profiling of larval head proteins was performed by SDS–PAGE using a stacking gel (3%) and running gel (9%)[26] in a Mini Protean–II apparatus (Bio-Rad, USA). SDS samples were prepared by mixing with an equal volume of SDS sample buffer, keeping in boiling water bath (5 min) and cooling in liquid nitrogen. The samples were kept at –20°C until electrophoresed. Electrophoresis was carried out at constant voltage (120 V). The gels were fixed and stained for 3–4 h at 37°C with 0.2% Coomassie Brilliant Blue R 250 followed by de-staining by repeated slow rocking in 7% acetic acid containing 30% methanol on a platform rocker (Genei, Bangalore). Polypeptide molecular masses were computed using a calibration curve prepared on the basis of the migration of co-run mixture of polypeptides of known molecular weight.

Data were analysed by statistical analysis of known procedures used for least standard deviation at the 5% critical difference among the treatments over control, and standard deviation based on replicated treatments was also calculated[27].

The effects of azadirachtin and azadirachtin-based commercial formulations on insect head protein level and proteome (polypeptide profile) were investigated under three different modes of application – topical, injection, and diet-feeding.

In the case of topical application, the 2 µl dose of azadirachtin solution (10 ppm) applied topically resulted in no significant increase in protein level in head tissues of second instar *H. armigera* larvae during a prolonged period, i.e. 0 to 48 h of treatment application. Significantly ($P < 0.05$) higher amount of protein level (22.8 mg/g) was found in treated larvae of sixth instars in the initial period of 0 h compared to untreated control, where significantly higher amount of protein was observed (24.1 mg/g) in later age of larvae, i.e. 72 h period. The increase in mean total protein level in tissue of treated sixth instars larvae did not correspond to the period of topical application over control and second instars larvae (Table 1).

The protein content in tissues of ultimate instars larvae (full grown) of *H. armigera* in relation to azadirachtin-based commercial formulation was significantly higher (ranging from 23.1 to 24 mg/g) as observed during 0 to 72 h period in comparison to untreated control. However, protein levels in all the insecticidal formulation (Achook, Nimbicidine and Neem Jeevan) treatments were found at par statistically during the initial period of 0 h of treatment application. However, after 24 h of treatment application, a significant ($P < 0.05$) reduction in protein level was noted in larvae treated with Achook (18.07 mg/g tissue). Similarly, after 48 and 72 h of treatment application, significant ($P < 0.05$) reduction in protein level was found with Neem Jeevan, i.e. 19.1 and 19.2 mg/g tissue, respectively (Table 2).

An injection of azadirachtin solution (10 ppm, 5 µl) in third instars larva was found to result in significantly ($P < 0.05$) lower amount of head protein level (10.75 mg/g) compared to a 2 µl dose, during 72 h of treatment application. Similarly, lower protein level (8.92 mg/g) was recorded in fourth instar larvae at 5 µl over the 2 µl dose (11.2 mg/g) application during 72 h (Table 3). Azadirachtin (10 ppm) injections in fifth instar larvae of *H. armigera* at different doses also showed significantly ($P < 0.05$) lower protein level (13.73 mg/g) at 10 ppm doses compared to lower doses (1–8 µl) during 48 h of treatment application (Table 4).

Feeding azadirachtin-rich commercial insecticides (0.01%), Achook, Nimbicidine and Neem Jeevan, to the fourth instar larvae of *H. armigera* mixed with semi-synthetic diet was also found to reduce head protein level over control significantly ($P < 0.05$) during 48 h of treatment application. Amongst the insecticides, significantly ($P < 0.05$) lower amount of protein level was found by feeding Nimbicidine (Table 5). Further, third instars larvae fed with Nimbicidine (50 ppm) mixed diet had significantly ($P < 0.05$) lower protein level (9.7 mg/g tissue) compared to a lower dose (3.12 ppm) as well as controls fed with neem-free diet (Table 6).

A comparison of electrophoretic pattern of head polypeptides of ultimate instar 48 h after treatment with 10 ppm

Table 1. Protein level in second and sixth instar larvae of *Helicoverpa armigera* influenced by topical application of azadirachtin

	Mean* protein level (mg/g tissue) as applied topically on					
	Second instars		Sixth instars			
	Treated		Untreated		Treated	
Period (h)	Observation	SD (±)	Observation	SD (±)	Observation	SD (±)
0	0.752[c]	0.060	22.794[d]	0.174	22.798[a]	0.009
24	1.504[b]	0.042	23.062[c]	0.105	17.838[d]	0.057
48	1.586[a]	0.015	23.984[b]	0.044	19.652[c]	0.005
72	1.566[a]	0.071	24.110[a]	0.077	22.702[b]	0.007
SEM	0.0224		0.0524		0.0125	
LSD at 5%	0.0779		0.1819		0.0434	

*Means of five replication.

Table 2. Comparative protein content of ultimate instars larvae of *H. armigera* influenced by topical application of azadirachtin and different azadirachtin-based formulations

	Mean* protein level (mg/g tissue) in larvae due to application of different industrial formulations of azadirachtin									
	Achook		Nimbicidine		Neem Jeevan		Control (untreated)		ANOVA	
Period (h)	Observed	SD (±)	Observed	SD (±)	Observed	SD (±)	Observed	SD (±)	SEM	LSD at 5%
0	22.808[b]	0.038	22.806[b]	0.027	22.796[b]	0.011	23.912[a]	0.542	0.1220	0.4233
24	18.072[d]	0.068	18.884[c]	0.021	19.424[b]	0.009	23.122[a]	0.077	0.0231	0.0802
48	19.890[b]	0.023	19.366[c]	0.029	19.104[d]	0.011	23.960[a]	0.019	0.0080	0.0277
72	20.048[c]	0.048	23.060[b]	0.059	19.220[d]	0.027	24.148[a]	0.048	0.0191	0.0661

*Means of five replication.

Table 3. Protein level of third and fourth instars of *H. armigera* influenced by injection of azadirachtin (10 ppm) after 72 h of treatment application

	Mean* protein level (mg/g tissue) at different instars of larvae			
	Third		Fourth	
Treatment dose (µl)	Treated	SD (±)	Treated	SD (±)
2	13.384[b]	0.021	11.226	0.015
Control	13.976[a]	0.018	–	–
5	10.750[c]	0.025	08.926	0.043
Control	13.954[a]	0.030	14.108	0.039
SEM	0.0121		–	
LSD at 5%	0.0420		–	

*Means of five replication.

azadirachtin topically and control, loss of 40, 56, 26, 22 kDa head polypeptides was noted. 10 ppm Achook also resulted in loss of some of these polypeptides (22, 26 and 40 kDa). Similarly, 10 ppm Nimbicidine resulted into loss of two of the above polypeptides (22 and 26 kDa) after 48 h of treatment. Whereas azadirachtin treated head samples lacked 12 and 22 kDa polypeptide, up to 72 h compared to control. Interestingly, some additional low molecular weight polypeptides of up to 11 kDa could be also visualized (Figure 1).

Diet-mixed feeding of 50 and 25 ppm Nimbicidine resulted into characteristic loss of 25, 15 and 25 kDa polypeptide, in treated third instar larvae after 6 days of treatment. However, no polypeptide pattern alteration in the fourth instar (treated up to 48 h) was noted, similar to 48 h after injection of an azadirachtin.

The head protein level was about twenty-two times higher, although in sixth instar (full grown) larvae compared to second instars larvae. Azadirachtin-treated larvae were found to have significantly ($P < 0.05$) suppressed protein level compared to control although the period of observation. Among azadirachtin-based commercial formulations, topically applied on sixth (ultimate) instars significantly ($P < 0.05$) reduced head protein level with Achook in 24 h and Neem Jeevan in 48 and 72 h. A significantly higher reduction of protein was shown by application of azadirachtin on third, fourth, and sixth instars by topical as well as injection method. Simi-

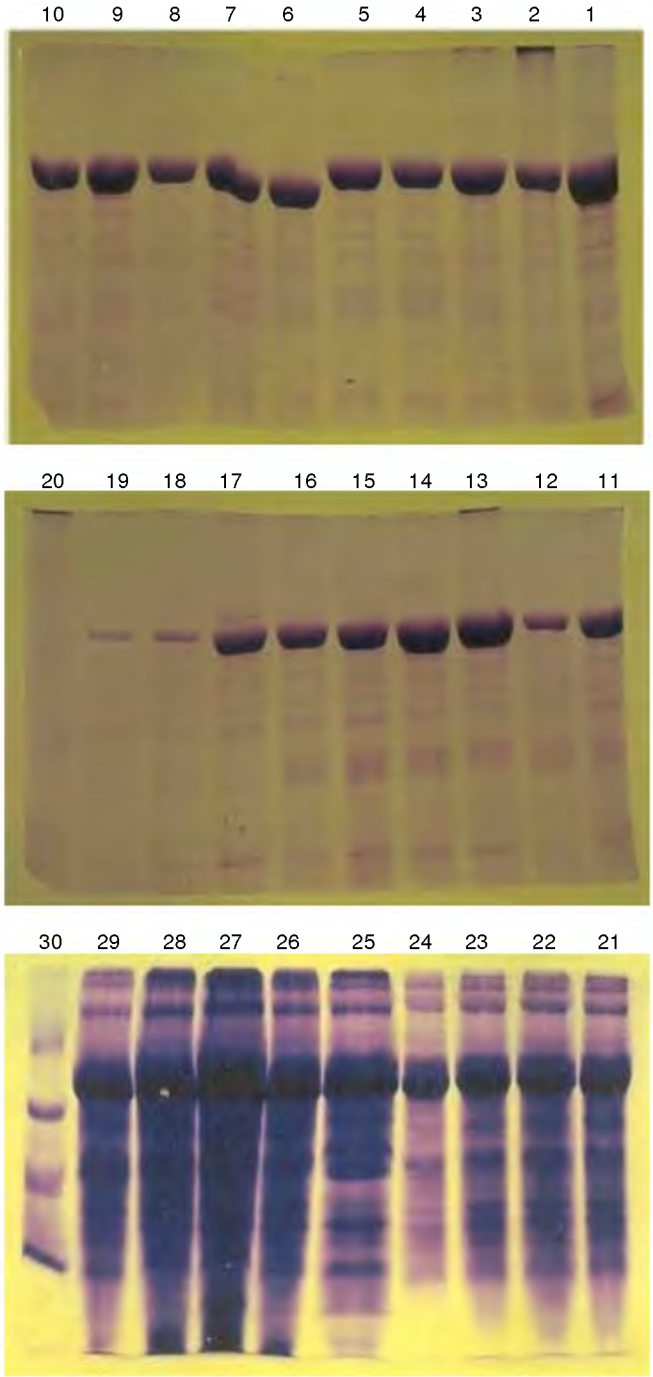

Figure 1. Lanes 1–4, Achook, Nimbicidine, Neem Jeevan (0.01% each) and control fed to fourth instar larvae for 48 h; lanes 5–10, Fourth instar larvae injected with 10, 8, 6, 4, 2 and 1 µl azadirachtin (10 ppm), respectively for 48 h; lanes 13, 14, Fourth instar injected with 5 and 2 µl azadirachtin (10 ppm) for 72 h; lanes 15, 16, Third instar injected with 5 and 2 µl azadirachtin for 72 h; Lanes 11, 12, Control of fourth and third instar larvae; lanes 18, 19, 25 and 50 ppm nimbicidine-treated diet fed to third instar larvae for 6 days; lane 17, Control; lanes 21–29, Third instar larvae topically treated with Neem Jeevan, Nimbicidine, Achook, azdirachtin (10 ppm), control for 72 and 24 h, respectively; lanes 20, 30, Molecular weight marker.

Table 4. Protein level in fifth instar larvae of *H. armigera* as influenced by injection of azadirachtin (10 ppm) after 48 h of treatment application

	Mean* protein level (mg/g tissue)	
Treatment dose (µl)	Observed data	Standard deviation (±)
1	20.672[b]	0.008
2	16.958[c]	0.013
4	15.360[d]	0.055
6	14.942[e]	0.008
8	14.114[f]	0.013
10	13.736[g]	0.013
Control	21.020[a]	0.013
SEM	0.00488	–
LSD at 5%	0.01420	–

*Means of five replication.

Table 5. Head protein level in fourth instar larvae of *H. armigera* as influenced by diet mixed with different azadirachtin-based formulation after 48 h of treatment application

	Mean* head protein level (mg/g tissue)	
Treatment (0.01%)	Observed value	Standard deviation (±)
Achook	11.416[c]	0.021
Nimbicidine	17.378[b]	0.023
Neemjeevan	11.266[d]	0.018
Control	18.292[a]	0.084
SEM	0.0076	
LSA at 5%	0.0266	

*Means of five replication.

Table 6. Protein level in third instar larvae of *H. armigera* as influenced by diet mixed with Nimbicidine after 6 days treatment application

	Mean* protein level (mg/g tissue)	
Treatment dose (ppm)	Observed data	Standard deviation (±)
3.12	14.612[b]	0.026
6.26	14.414[c]	0.013
12.5	13.604[d]	0.444
25.0	11.060[e]	0.039
50.0	9.7120[f]	0.013
Control	16.248[a]	0.008
SEM	0.0363	
LSD at 5%	0.1069	

*Means of five replication.

lar but most drastic trend was exhibited in case of serially diluted dose application of Nimbicidine on third instar larvae.

The observed drop in head protein concentration in treated second instar larvae of *H. armigera* of azadirachtin treatment was qualitatively similar to observation on effect of azadirachtin treatments in *Spodoptera litura*[28]. Also azadirachtin is known to reduce haemolymph protein concentration in *Periplanata americana* and *S. gregaria*[29,30]. The process sets in after about 6 h of treatment and continued till 72 h. Contrarily, cyclic changes in concentration have been observed in *Achaea janata*[31], *Schistocerca gregaria*[4], *Bombyx mori*, *Hyalophora cecropia* and *Manduca sexta*[32]. Fluctuations in protein concentration in an insect are net balance of its synthesis, breakdown, and water movement between tissue and haemolymph. Haemolymph volume changes under insecticide stress resulting in alteration in protein concentration[33]. Azadirachtin may either change the hormonal level in haemolymph to signal protein synthesis or degradation, or inhibit the proteins or the neurosecretory cells which control the endocrine glands in *Helicoverpa*. It may also alter the feeding behaviour of the insect toward food avoidance and maintain body metabolism at the expenses of storage or cellular proteins, however, avoided dietary practice may be resumed adaptively.

Azadirachtin is absorbed by *H. armigera* larvae and passed into the haemolymph, from where it is gradually metabolized or accumulated (mainly in the gut) after a delay in case of 72 h. Thereby, significant changes in head protein level might have been observed after 72 h of topical application of both azadirachtin and its commercial formulations. These findings are similar to *Peridroma saucia*, where ingested azadirachtin in larvae was largely recovered from frass[34]. Significant accumulation occurs in the digestive tract, integument and other tissues like head, gonad and malpighian tubules within 72 h, gradually eliminate ingested azadirachtin. The situation is however, at variance with *Rhodinus*[35], where even after six days of treatment haemolymph concentration of azadirachtin remains the same as at 24 h after oral feeding.

The effect of azadirachtin in neuroendocrine system does not appear to exist up to 72 h as topically administrated azadirachtin is eliminated[36] from the haemolymph by 24 h, by excretion in frass where it increases up to 48 h and retention in the body tissue was up to 72 h and the depot of retention has been shown to be integument > gut > malpighian tubules[32]. Among the phytogenic insecticides identified so far, azadirachtin-based formulations have been reported to successfully control *H. armigera*[37] and are considered to hold promise of perpetual utility and enhancement of potency. Advancement in this direction necessitates monitoring facets of their biochemical modulation vis-à-vis insect morphogenesis.

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Diagenetic talc of Jhiroli, Kumaun Himalaya

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Talc/soapstone deposit of Kumaun Lesser Himalaya is mainly associated with magnesite. It occurs in small, irregular patches and pockets within the magnesite and sometimes with dolomite. Talc in the interstitial spaces of magnesite occurs as fillings of stylolitic veins, as well as in patches heavily corroding the magnesite grains lying in contact. Tremolite, chlorite and other low grade metamorphic minerals are absent while chalcedony is sometimes present in intimate association with talc in the stylolitic veins and in the patches. Talc is extremely fine-grained, sometimes scaly and at times fibrous. These features indicate that talc is neither hydrothermal in origin nor is produced by stresses acting on the carbonate rock. Presence of pyrite grains in talc and host magnesite is indicative of a reducing environment. X-ray diffraction and infrared studies reveal that there is no other metamorphic mineral present. Fluid-inclusion study of host magnesite suggests that the minimum temperature of homogenization (*Th*) ranges between 220 and 300°C. The antipathic relationship between silica and talc suggests that the latter is a product of diagenetic processes, being formed by reaction of magnesite and silica at temperature less than 300°C.

Keywords: Diagenetic processes, Kumaun Lesser Himalaya, metamorphic minerals, talc.

PROTEROZOIC talc/soapstone deposit of Deoban Formation in the Jhiroli area is a part of Kumaun Lesser Himalaya. It is situated between lat. 29°45′30″ and 29°47′30″ N, and long. 79°44′ and 79°46′ E. Talc is a hydrothermal or metamorphic mineral, characteristic of green schist facies and occurs in shear zones where it is considered as an alteration product. The genesis of talc/soapstone which revolves around the metamorphic/hydrothermal process, is still debated. Some workers suggest that talc is a product of hydrothermal replacement[1,2], while others contend that it represents a low-grade metamorphic reaction product of magnesite and silica[3–6].

The Deoban Formation consists of a thick pile of calcareous and siliceous metasedimentary rocks belonging to Kumaun Lesser Himalaya Upper Middle Riphean age[7]. It occurs in and around the study area, surrounded by three villages, i.e. Chaugaon Chhinna, Bilori and Kathpuria. The basement of the Deoban Formation is the Rautgara Formation that consists of predominantly pink, grey and

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