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Effect of ethanolic plant extractives on cephalic neuroendocrine system of BmNPV inoculated 5th instar larvae of *Bombyx mori* L. (Lepidoptera, Bombycidae)

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ABSTRACT: In this research 3000, 5000 and 8000ppm concentration of ethanolic extractives of leaves of *Eupatorium odaratum*, *Hyptis suaveolens* and fruits of *Aegle marmelos* were tested on mulberry silkworm fifth instar larvae of PMxCSR2 inoculated with *Bombyx mori* Nuclear Polyhedrosis Virus (BmNPV). Extractives of *A. marmelos*, *H. suaveolens* and *E. odaratum* showed promising results against BmNPV of silkworm larvae @8000 ppm. Total improvement occurs in the function of neurosecretory cells (NSC), A1 and A2 cells of median neurosecretory cells (MNC) group. Also found that, NCC I and II, corpus cardiacum (CC) lobes and fine branching of NCA I over corpus allatum (CA) showed strongly stained PF positive granules of NSM due to the subsequent treatment of given ethanolic plant extractives which reduced BmNPV infection about (30 - 40%). © 2024 Association for Advancement of Entomology

KEYWORDS: PMxCSR2, silkworm larvae, *Eupatorium odaratum, Hyptis suaveolens, Aegle marmelos,* neurosecretory cells

INTRODUCTION

Sericulture is agro-cottage based industry. *Bombyx mori* L. (Lepidoptera, Bombycidae), silkworm is susceptible to various diseases causing by viruses and bacteria. Grasserie is one of the important diseases of silkworm due to infection by *Bombyx mori* Nuclear Polyhedrosis Virus (BmNPV) which causes more than 35-50 per cent loss (Chitra *et al.,* 1975; Samson, 1992; Ranjith kumar *et al,* 2022). BmNPV infected larvae become restless, crawl on the edges of the rearing trays and exuding white body fluid through skin wounds (Aruga, 1994). The endocrine system regulates the physiological, developmental, reproductive and a behavioural activity in insect has been explored to a great extent (Wigglesworth, 1965; Engelmann, 1970; Gilbert and Kings, 1973; Tembhare, 1988; Bauah and Chinmoyee Kalita, 2020). Fukuda (1940) elucidated the role of prothoracic gland and showed that when both the corpus allatum and prothoracic gland are active, larval ecdysis takes place. When corpus allatum loses its activity, prothoracic gland alone induced active pupation. Fukuda (1944) reported that the brain controls the secretion of sub oesophageal ganglion in the pupal stage, so brain oesophageal ganglion system played an important role in regulating diapause behaviour of eggs in *B. mori*. It is clear that the brains, corpus cardiacum, corpus allatum, sub-oesophageal ganglion are

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important cephalic endocrine organ of the insect. The groups of neurosecretory cells are present in the medial and lateral region of the brain. In *B. mori* they are located in the groups namely two median and two lateral (Kobaayshi, 1957; Tembhare and Barsagade, 2000). As plants contain active secondary metabolites which act as insect growth regulators, phagostimulant, JH analogues, pesticides and antimicrobial agents, the ethanolic extractives of leaves of *Eupatorium odaratum*, *Hyptis suaveolens* and fruits of *Aegle marmelos* were tested on mulberry silkworm fifth instar inoculated with BmNPV.

MATERIALS AND METHODS

Disease free laying of the silkworm $PMxCSR_2$ was incubated and the larvae were reared as per the recommended regimen of Krishnaswami *et al.* (1978, 1979). Appropriate modifications in the rearing techniques were made to suit the local conditions.

Isolation, Purification and Inoculation of BmNPV: During the regular rearing of cross breed (PMxCSR2) race of silkworm, natural infection of grasserie diseases were occurred. The pathogen BmNPV infection was in fourth and fifth instars. The larvae with typical symptoms of grasserie infection [intersegment swelling and look like a 'bamboo like appearance', inactiveness, shiny and translucent skin, bursting body wall, oozing white turbid haemolymph and worms crawling on the edges of the rearing trays], were isolated and individually checked for virus infection by preparing smear for microscope observation and turbidity test. The histological preparation of midgut and salivary glands were stained with Azan stain (Mallory Hiedenhains, 1938) to check the infection of the viral bodies in these tissues. The BmNPV infected fifth instar larvae were collected and Polyhedron Inclusion Bodies (PIBs) obtained directly from the haemolymph of infected larvae to get stock of BmNPV in sterilized double distilled water. The isolated PIBs were purified by repeatedly centrifugation process. The isolated PIBs re-suspended in the sterile distilled water and centrifuged at 5000 rpm for 10 minutes. The centrifugation process was repeated for three times so as to obtained whitish residues of PIBs at the bottom of centrifugation tubes. Then the PIBs were washed in sterilized double distilled water and stored in refrigerator at 4^oC until their use.

Inoculation and determination of LC_{50}: Just after completion of fourth moult, about 500 larvae were starved for 6 hours and divided into 10 groups containing 50 larvae each. Each larva from all groups was fed with the piece of mulberry ($1cm^2$) smeared with 10 ìl of each dilution of PIBs suspension. A group of larvae was kept as normal control in which the larvae were fed with mulberry leaves smeared with distilled water and air-dried. Then all the larvae from all the groups were fed on fresh mulberry leaves for a period of six days. The larvae from each group were observed daily and mortality due to BmNPV infection was recorded. The LC_{50} dose was calculated from the observed mortality due to BmNPV infection [$LC_{50} =$ log dilution above 50% + PD x dilution factor]. The observed LC_{50} value 1.413 PIBs/ larva at 10^5 concentrations was used for inoculating the larvae in the further experiments.

Preparation of ethanolic extractives: Leaves of *E. odaratum* and *H. suaveolens* and fruits *of A. marmelos* were collected dried at room temperature and prepared in fine powder. Fine powder of 50g each was macerated separately with 500ml of ethanol. Extracts were evaporated by using speed vacuum evaporator to obtain a thick paste like extracts. These extracts were collected by spatula in glass bottle. The crude extracts (condensed product) were weighed and kept at 4^oC prior to test. For the preliminary testing for antimicrobial activity, the stock extracts of these plant parts were dissolved in warm sterile distilled water (40^oC) to make a concentration of 3000, 5000 and 8000 ppm. Newly moulted 500 - 600 fifth instar larvae of $PMxCSR_2$ were starved six hours and divided in to 11 groups, each group containing 50 larvae. Except normal groups the larvae of remaining groups were fed with the piece of mulberry leaf ($1cm^2$) coated smeared with 10 µl of $1x10^5$/ml dilution of PIBs suspension of BmNPV. After inoculation, all the groups were fed with

mulberry leaves, which were dipped in 3000, 5000 and 8000ppm concentration of the ethanolic extractives. One group was kept as inoculated control in which the larvae were fed with mulberry leaves smeared with distilled water instead of plant extractives, another control reared on fresh mulberry leaves without any application and it is served as normal control. The treatments were given before morning feeding (9.00am) once in a day. For the studying the effect of BmNPV and the subsequent application of ethanolic plant extractives on the neuroendocrine system of silkworm race multivoltine cross breed PMxCSR2 were utilized. After following the usual mode of inoculation and subsequent application of plant extractives, the larvae from the untreated control (normal control), inoculated control, and the treated groups were used for the preparation of their cephalic neuroendocrine complexes. At least five larvae from each group were dissected for obtaining their neuroendocrine complexes under stereoscopic binocular microscope in the cold insect ringer solution, then the neuroendocrine complexes fixed in a aqueous Bouin's fixative for 24 hours, then they were transferred in 70 per cent alcohol for the removal of yellow colour of Bouin's fluid by giving 4-5 changes of 70 per cent alcohol even after this, if the yellow colour of picric acid persist in the tissue was removed by giving treatment of lithium carbonate in 70 per cent alcohol. After removal of yellow colour, tissues were hydrated by passing through graded series of alcohol, which were utilized in whole mount preparation by using aldehyde fuschin stain (PF) as per the methodology described by Dogra and Tondon (1994). The stained whole mounts observed under the microscope. Observation on the neurosecretory 'A1' and 'A2' cells of MNC groups regarding their staining intensity, size was scored according to the following criteria - unstained stain, slightly stained, moderately stained cell and strongly stained cells. The secretary activity of 'A1' and 'A2' cells and nuclear diameter of these cells and the presence of variable quality of PF positive granules in them (Highnam *et al.,* 1969). The size of the CA was also measured for determining their activity in all the groups during study.

RESULTS AND DISCUSSION

Anatomy of cephalic neuroendocrine complex of 5th instar larva in both the races of *B. mori* showed typically lepidopteran type which comprises of neurosecretory cells of brain, paired corpora cardiaca, paired corpora allata (CA) and aorta (Plate I, Figs. 1, 2). In the 5th instar silkworms, the brain is well distinct bilobed structure situated middorsally in the head capsule, which was connected to the sub oesophageal ganglion by a pair of circum oesophageal connectives. The corpora cardiaca (CC) are the paired elongated structure connected posteriorly to the brain by a pair of nerve corporis cardiaci I (NCC-I) and II and the CA are also paired elongated oval structure which are joined to the lower end of the CC of their respective side by single nervi corporis allati. Thus, corpora cardiaca-allata complex is situated laterally to the oesophagus. Aorta with its cephalic ends terminates behind the brain, which is mid dorsal to the oesophagus. In whole mount by using aldehyde fuschin, staining techniques only PF + ve A type of neurosecretory cells (NSC) are stained and they are seen in the 4 distinct neurosecretory cell groups. All these groups are in paired the A-cells can be further classified into subtypes namingly larger A_1 type and small A_2. The four pairs of NSC groups are named according to their position in the brain tissue (Plate I, Figs. 3):

1. Paired median neurosecretory cells (MNC) (Plate I, Figs. 1, 2)
2. Paired lateral neurosecretory cells (LNC) (Plate I, Figs.1, 2)
3. Paired anterior neurosecretory cells (PANC).
4. Paired posterior NSC groups (PNC).

MNC occupies antero-middorsal region of pars intercerebralis of therotocerebrum of either side of the medial line in each hemisphere of brain. Each group consists of four large A_1 cells measuring $14.12 \pm 1.07\mu m$ in PM x CSR2 with centrally placed nucleus measuring $7.3 \pm 0.75\mu m$ in PM x CSR2. Each MNC group also contains 5 to 6 A_2

cells, which are smaller containing PF + ve granules in their pericaria. These cells measure 11.00 ± 0.98ìm in PM x CSR2 with centrally placed nucleus measuring 5.97 ± 0.45 in PM x CSR2 (Plate I, Figs. 1, 2; Plate II, Figs. 9, 11). The lateral neurosecretory cell group also known as cerebropleural NSC groups located anterior lateral region of protocerebrum lying between the pars intercerebralis and corpora pedunculata. Each lateral neurosecretory cells (LNC) group consists of 5 to 6 A_2 type of cells only and possible number PF –ve (B type) cells (Plate I, Fig. 2, Plate II, Figs. 2, 8). Each anterior group consists of two A_2 types of cells and the posterior group has 3 to 4 A_2 type of cells. PF –ve B cells could not be observed in the whole mount preparation (Plate I, Fig. 3). The axonic pathways of MNC group cross over each other in the centre of the brain and they joined to the axonic pathway of LNC group of the opposite side which emerges from posterior as NCC 1. Axonic pathway of posterior group emerges laterally as NCC-II. The axonic pathway for anterior group could not observe in the present preparation (Plate I, Fig. 3; Plate II, Figs. 5, 7.). NCC I and II enters into to the corpora cardiaca of their sides which do not acts as neurohaemal organ. Axon of the NCC I and II from central axonic core in the corpora cardiaca and emerging from the posterior end as nervi corporis allata- I which forms the fine branching over the anterior surface of the corpus allatum through which NSM is seen to be released in the form of neurosecretory granules in the surrounding haemolymph. (Plate I, Fig.1, Plate II, Figs. 2, 5, 7, 10). Corpora cardiaca paired elongated structure situated on each side of oesophagus covered externally with connective tissue sheath. The centre of each CC lobe is occupied by central axonic core made from NCC I and II peripherally intrinsic and extrinsic secretary cells are present which cannot be stained in whole mount preparation. In the lumen corpora cardiaca NSM in the form of granules is observed (Plate II, Figs. 5, 7, 14). CA is paired elongated oval structure made up from 18 to 26 oval secretary cells, which shows cyclic secretary activity. Externally each CA lobe is covered with connective tissue peritoneal sheath and it is connected to the lower end of which fine axonic barbarization is seen in which granule neurosecretory material is observed (Plate I, Fig. 1; Plate II, Fig. 5 and 7). Somewhat similar findings were reported by Tembhare and Barsagade (2000).

Effect of BmNPV and subsequent treatment of the ethanolic extractives on 5th instar: The infection of BmNPV on the 5th instar produced the pathogenic condition causing severe damage to each and every internal tissue including body wall. The effects infection noted after 3-4 days. Free matured polyhedra were seen in the central nervous system which probably came from the innervating trachea to the central nervous system. Hence tracheal innervation was the possible source for the infection of CNS by BmNPV. Similar observations were reported by Torquaito *et al.* (2006) and Blissard (1996). Through tracheal innervations the BmNPV disseminated into the central system of 5th instar and penetrated in to glial nerve and perineurium cells. The NSC (modified motor nerve cells) must also get infected with the BmNPV due to which their normal function of neurosecretion were altered (Plate II, Fig. 3). Hence the effect of BmNPV infection on neurosecretory cells A1 and A2 type of MNC group was under taken.

Changes in neurosecretory A_1 and A_2 cells of MNC groups: After four days inoculation of BmNPV to 5th instar of PM x CSR_2 race, the inoculated control NSC A_1 and A_2 cell and their nuclei, showed slightly increased size as compared to the normal control (Plate II, Fig. 1), indicating the hypertrophy A1 and A2 cells and their nuclei of infected cell. These cells of inoculated control were practically devoid of PF positive NSM and the same was not observed in NCC I and II as well as in the CC and also in the fine branching NCA I over the CA, indicating that the normal functions of NSC A1 and A2 was disturbed, because BmNPV infected virogenic stroma and polyhedron to the central nervous system. These findings are correlated with the findings of Torquaito *et al.* (2006) and Bauah and Chinmoyee Kalita (2020). The neurosecretory cells probably got infected with BmNPV. As this virus multiply in the nuclei of the infected cells and probably getting into the nuclei of A1 and A2 cells and as the secretion of these cells was in the form of electron dense granules

PLATE - I

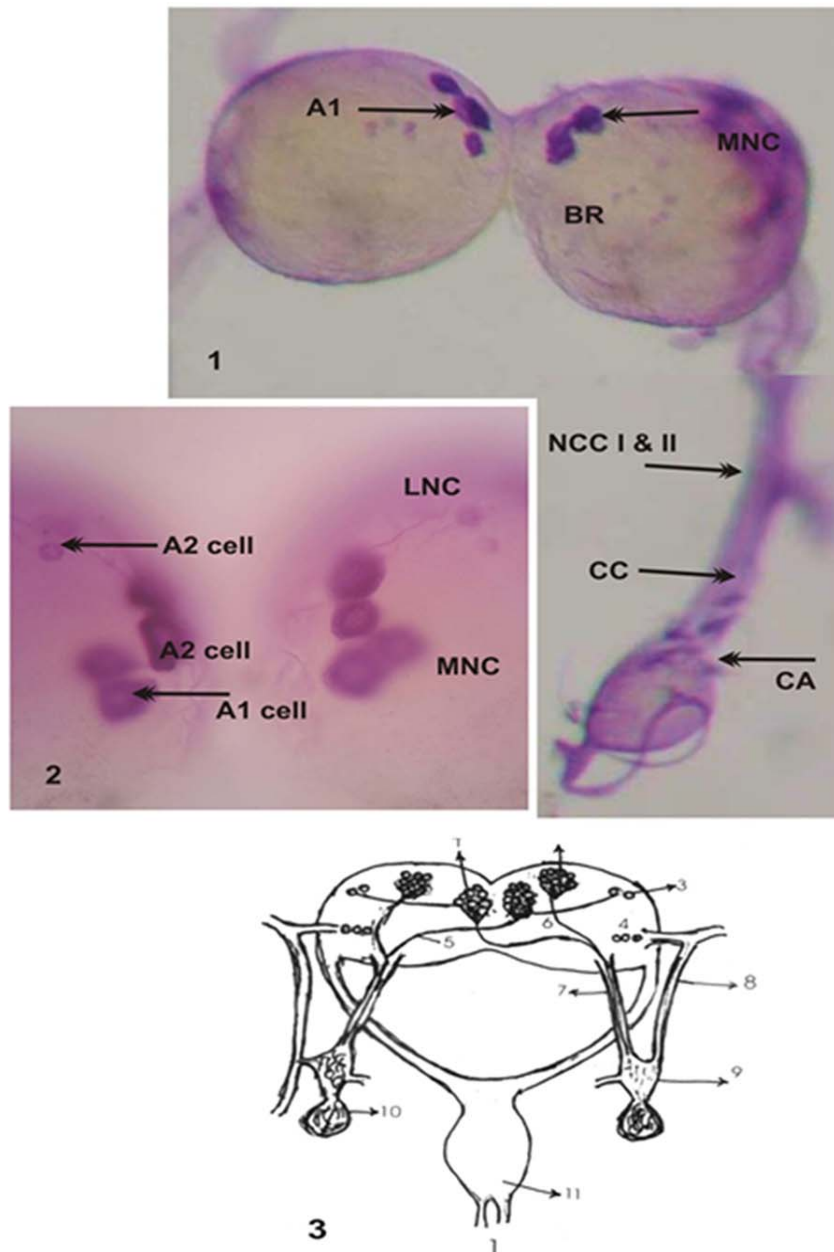

Fig. 1 Whole mount preparation of the brain and CC-CA complex of cross breeds PMxCSR2 showing 4 A1 cells in each MNC group, NCCI and NCCII.

Fig. 2 Whole mount preparation of brain MNC with A1 cells and LNC with A2 cells.

Fig. 3 Diagrammatic representation of cephalic neuroendocrine complex of 5th instar of *B. mori* PMxCSR2 showing- 1. Median Neurosecretory Cell group (MNC), 2. Lateral Neurosecretory Cells (LNC), 3. Anterior Neurosecretory Pathway (ANC), 4. Posterior Neurosecretory Cells (PNC), 5. Median Neurosecretory Pathway (MNSP), 6. Lateral Neurosecretory Pathway (LNSP), 7. Nervi Corporis Cardiaci (NCC I), 8. Nervi Corporis Cardiaci (NCC II), 9. Corpus Cardiacum (CC), 10. Corpus Allatum (CA), 11. Prothoracic Gland (PG), 12. Sub-Oesophageal Gland (SOG)

PLATE - II

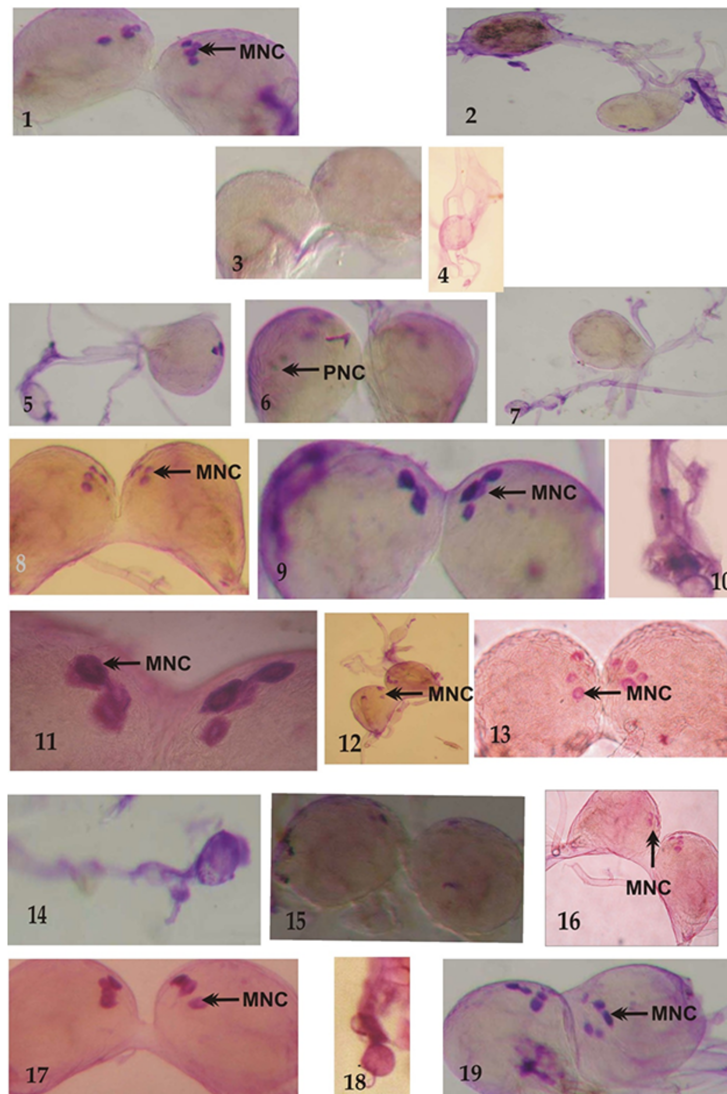

PLATE-II

Fig.1 & 2: Whole mount preparation of endocrine complex of control larvae of PM x CSR2 race showing MNC groups with Al cells containing moderate amount of PG +ve granules (Fig. 1) and NCC I & II and CC showed granular moderate amount of NSM. Fig. 3 & 4: Whole mount preparation of endocrine complex of BmNPV control showing MNC devoid of NSM (Fig. 3) and CC-CA complex with NCC- I and II also devoid of NSM group. Fig. 5 & 6: Whole mount preparation of 3000 ppm concentration of *Aegle marmelos* treated larvae showing slight amount of NSM in Al cells of MNC and in NCCI and II and CC -CA complex. Fig. 7 & 8: Whole mount preparation of 5000 ppm concentration of *Aegle marmelos* treated larvae showing moderate amount of NSM in A1 cells of MNC and in NCC I and II and CC -CA complex. Fig 9, 10 & 11: Whole mount preparation of 8000 ppm concentration of *Aegle marmelos* larvae showing strongly stained similar to the control group of Al cells of MNC group (Fig. 9-11) and increased amount of NSM also observed in NCC I and II and CC -CA complex (Fig. 10). Fig. 12: Whole mount preparation 3000 ppm concentration *Hyptis suaveolens* treated larvae showing slight amount of NSM in Al cells of MNC and in NCCI and II and CC- CA complex. Fig. 13 & 14: Whole mount preparation of 8000 ppm concentration of H. suaveolens *Hyptis suaveolens* treated larvae showing strongly stained similar to the control group of A cells of MNC group (Fig. 13) ad increased amount of NSM also observed in NCC I and II and CC- CA complex (Fig. 14). Fig. 15: The whole mount preparation 3000 ppm concentration of *Eupatorium odaratum* treated larvae showing slight amount of NSM in Al cells of MNC. Fig. 16: Whole mount preparation of 5000 ppm concentration of *Eupatorium odaratum* treated larvae showing moderate amount of NSM in Al cells of MNC Fig. 17, 18 & 19: The whole mount preparation of 8000 ppm concentration of *Eupatorium odaratum* treated larvae showing strongly stained similar to the control group of Al cells of MNC group (Fig. 17 & 19) and increased amount of NSM also observed in NCC I and II and CC -CA complex (Fig. 18).

over 1000A^0 diameter and protein nature which requiring the involvement of nuclei in their synthesis. These cells were devoid of PF positive. NSM indicating that these cells inoculated control were infected by that these cells inoculated control were infected by viral stroma and polyhedra interfering and hampering the synthesis of proteinaceous granular NSM in A1 and A2 cells of MNC groups (Plate II, Fig. 3). These cells had the source of prothroacotrophic, allotropic and allostatatic hormones, hence total process of the metamorphosis getting disturbed. Bauah and Chinmoyee Kalita (2020) reported similar observation. Physiological process leading to the disease condition and finally death of infected larvae occurred in the last two days of 5th instar. However, the NSC $A_1$and A_2 cells of MNC group of plant extract treated larvae showed gradual recovery from the infections. The plant extracts treated group of 3000ppm *A. marmelos*, *H. suaveolens* and *E. odaratum* where in the $A_1$and A_2 NSC of MNC group contain slightly stained granules showed PF positive NSM in three days. The PF positive NSM was quite evident in the NCC II and I CC lobes and in the fine branching of NCA1 over CA indicating the NSC A1 and A2 performing normal functions (Plate II, Figs. 5, 6, 11, 12). The improvement in the functioning of the NSC A_1 and A_2 cells of MNC groups of three days plant extracts treated group of 5000 ppm (where in $A\text{-}_1$ & $A_{\text{-}2}$ cells, NCC I and II and fine branching of NCA I over CA moderately stained granules of positive materials immediately this treatment) had protective effect and did not allow virus to infect the CNS the size of A_1 and A_2 cells with very negligible hypertrophy (Plate II, Figs. 7, 8, 16). There was total improvement in the function of NSC A1 and A2 cells of MNC group of 3 days plant extractives treated group with 8000ppm concentration, where in A1 and A2 cells of MNC groups, NCCI and II, CC lobes and fine branching of NCA I over CA showed strongly stained PF positive granules of NSM (Plate II, Figs. 9, 10, 11, 13, 14, 17, 19). Thie was more or less similar with normal control groups indicating this treatment had offered total protection to the components of cephalic neuroendocrine system from the infection of BmNPV and these larvae of this groups showed nearly normal feeding behaviour and their body weight were slightly less than the untreated group larvae. The silkworms of PMxCSR2 treated with the ethanolic plant extractives @3000, 5000 and 8000ppm along with the mulberry leaves showed normal behaviour after the 5th day of BmNPV inoculations and were having the more or less similar body weight compared to untreated control groups. Their feeding also was normal and hence they were recovered from the BmNPV infection. Subsequently their feeding rate and the weight gain showed that some factors in plant extractives either growth promoters and phagostimulant or the juvenomimic (terpenoids) compounds which had the effect on the growth of the larvae either due to phagostimulant or by elevating the JH levels in the haemolymph by which, NSC A1 and A2 cells secreting the hormone and activate corpora allata to secrete juvenile hormone. Ranjith Kumar *et al.* (2022) reported similar observation. Bhaisare (2023) reported that the additional consumption of food and the additional growth in larva leading to the improvement in the cocoon characters after the treatment of ethanolic plant extractives of BmNPV inoculated larvae. Plants are having potential of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found to have antimicrobial properties (Manimegalai *et al.*, 2006; Chanda, 2011, Sithi Jameela Muthu Mohammed *et al.*, 2023). Recently the efforts have been made to promote the use of plant products against the infectious diseases of silkworm as an alternative for chemical control. The present research work, the ethanolic plant extractives showed the significant positive result against viral disease caused by BmNPV of *B. mori*. These are the preliminary observations in search of the bioactive compound having antimicrobial properties and the results obtained were encouraging.

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