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Growth Regulating Effects of Natural Pesticide, Azadirachtin from Neem Tree (*Azadirachta indica* A. Juss) on Insects: A Review

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Abstract: Azadirachtin is a tetranortriterpenoid derived from the neem tree *Azadirachta indica* A. juss. Azadirachtin has attracted worldwide attention as promising feeding deterrent, insect growth disruptor and sterilent. Azadirachtin based pesticides become very much popular because of its biodegradability and least toxicity to non- target organisms. It has now been promoted as an alternative insecticide in integrated pest management programmes. Azadirachtin has an ability to modify or suppress haemolymph ecdysteroid and juvenile hormone titres through inhibition of the secretion of brain peptides prothoracicotropic hormone and allatotropic hormone, which are responsible for inducing the synthesis and release of ecdysone from the prothoracic gland and juvenile hormone from corpora allata, respectively. Blocking the synthesis and release of ecdysone and juvenile hormone leading to disruption of coordination of moulting process. Furthermore, this compound is known to cause degenerative changes in the cells of insect endocrine glands. This review focuses on growth regulating effects of natural pesticide, azadirachtin from neem tree (*Azadirachta indica* A. Juss) on insects.

Keywords: Azadirachtin, Biopesticide, Neem, Insect Growth Regulator, Integrated Pest Management

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Introduction

Annual crop losses caused by insects, weeds and diseases are estimated between 20 to 40 per cent, similar to those of 50 years ago due to the intensification of agriculture production together with effects of climate change (EAO, 2017). To safeguard and improve food security crop protection from pest is required and aimed to avoid or prevent crop losses or to reduce them to an economically acceptable level (Karuppuchamy and Venugopal, 2016).

Crop protection all over the world relies heavily on the use of synthetic pesticides. In the past, synthetic pesticides have played a major role in crop protection programes and have immensely benefited mankind. The discovery and use of DDT in 1940 and then BHC and subsequent development of the chlorinated cyclodienes marked a major advance in the field of crop protection. These chemicals have made great contribution to plant protection but have also raised a number of ecological and medical problems (Varma and Dubey, 1999). Nevertheless, their indiscriminate use has resulted in the development of resistance by pests (insects, weeds etc), resurgence and outbreak of new pests toxicity to non-target organism and hazardous effect on the environment endangering the sustainability of ecosystem (Jeyasankar and Jesudasan, 2005). It has been estimated that hardly 0.1% of the agro-chemicals used in crop protection reach the target pest, the remaining 99.9% enter the environment to cause hazards to non-target organism including human (Pimentel and Levitan, 1986). It has been described that more than 2.5 million tonnes of pesticides are used in agricultural crops protection for every year and the global damage caused by synthetic insecticides reaches more than $100 billion annually (USEPA, 2011). The reason behind this amount of cost is the high toxicity and residual properties of pesticides in soil, water, air and crops that affect human and domestic health (Carson, 1951). Hence, search for the eco-friendly, biodegradable pesticide for management of pest insects has been encouraged to be essential for last five decades.

After the publication of the silent spring by Rachel Carson (Carson, 1962), and to attenuate the negative impact of pesticides in the environment and public health, search for alternative control strategies and reduced risk of pesticides became a real challenge (Pimentel, 1997; Khater, 2012). Consequently, a drastic re-emergence of interest in the use of natural pesticide known as biopesticides was noted (Cantrell *et al*., 2012; Kumar 2015; Mishra *et al*., 2018; Haddi *et al*., 2020). The main advantages of biopesticides are that they are inherently less toxic than conventional pesticides by offering more targeted action against specific pests (Dhamalas and Koutroubas, 2018). Indeed conventional pesticides which exert their effect on the nervous system of insects often affect a broad spectrum of pests along with bird and mammalian species (Thakora, 2006). Furthermore, bio-pesticides often are effective in very small quantities and decompose quickly, resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides (FAO). When using as a component of integrated pest management (IPM) programs, biopesticides can supplement the conventional pesticides and greatly reduce their use and offer potentially higher crop yields (Thakora, 2006; Damalas and Koutroubas, 2018).

The ideal insecticide should control target pest adequately and should be target-specific (able to kill the pest insect but not other insects or animals), rapidly degradable and low in toxicity to humans and other mammals. Two classes of insecticides that exhibit some of these characteristics are the botanical insecticides and the insecticidal soaps. Botanical insecticides, sometimes referred to as “botanicals” are normally insecticides which have been derived from plants. Insecticidal soaps are soaps that have been selected and formulated for their insecticidal action (Weinzierl and Henn, 1991). Botanical insecticides have more advantage than synthetic one. The advantages of botanical pesticides mainly depending upon their quick degradation and lack of persistence and bioaccumulation in the ecosystem which have been key problem in chemical pesticide use. Several experiments with botanical pesticides showed that these pesticides are degraded in the environment in hours or days. Further it has clearly been shown that use of plant natural products provides low risk when compared with chemical insecticides. The availability and diversity of the secondary metabolites in botanical extract is renewable source. Also multiple analogs of one compound is known to increase the efficiency of phytochemical through synergism, reduce the rate of metabolism of the compounds and prevent the pest resurgence/pesticide resistance (Ascher, 1993; Senthil-Nathan and Kalaivani, 2005, 2006; Ntalli and Menkissoglu-Spiroudi, 2011). Plant community is the most efficient source for natural pesticide. It synthesizes numerous products, many of which have been shown to effect insect and other harmful organisms, including both vertebrates and invertebrates. But majority of plant derived compounds are affecting insects and are comparatively harmless to vertebrates. Such compounds are toxic causing mortality or reduced growth of pest insects. Phytochemical modes-of-action are more complicated. Most of them are affecting insect performance by repelling an insect and feeding deterrence or oviposition deterrence.

The Indian neem tree, *Azadirachta indica* A. Juss and its closely related China berry. *Melia azedarach* (Meliaceae) have been recognized since long for their unique properties. More than half century ago, it was discovered that leaves of the neem tree contain chemicals strongly inhibiting feeding by the locusts that are polyphagous in nature (Chopra, 1928; Volkonsky, 1937). Many studies have been performed on the pesticidal properties, especially after the demonstration of strong antifeedant properties of crude extract of the neem seed kernels to locusts by Pradhan *et al*. (1962, 1963), Gill (1972), Warthern (1979) and Schmutterer *et al.* (1981).

Growing attention has been given to neem tree, *Azadirachta indica* as the most prominent bipoesticide (Isman and Grieneisen, 2014; Aribi *et al*., 2020). In Asia, the neem tree is regarded as a wonder tree and has been used for centuries in Ayurvedic medicine as one of the oldest medical system in humanity (Biwas *et al*., 2002. Pasquato Stigliani *et al*., 2017). It acts as an antidiabetic, immunostimulant, anti-microbial, antiviral, cholesterol-lowering agents, contraceptive and anticancer remedy and it has long been recovered by ancient Indian people and is entitled "village drugstore'' (Tinghui *et al*., 2001; Hummel *et al*., 2016; Moga *et al*., 2018; Blum *et al*., 2019). Additionally, aqueous extracts of powedered neem kernels have been used as an insecticide in India for about 2,000 years for the control of insect pest (Schmutterer, 1995). In recent time and following the isolation of azadirachtin, the major active compound, that is mainly responsible for the insecticidal activity of neem, the use of neem-based insecticide has increased in the last 30 years (Chaudhary *et al*., 2017; Pasquoto-Stigliani *et al*., 2017). Currently azadirachtin is one of the prominent biopesticides in agricultural use worldwide (Isman and Griencisen, 2014; Chaudhary *et al*., 2017; Aribi *et al*., 2020). However, its mechanism of action is still unclear and remain to be clarified especially in relation to the neurophysiological and the possible long term activities. This review has been undertaken to describe the importance of natural pesticide azadirachtin and its effects on insects with special emphasis on its growth-regulating effects.

The Indian neem tree -- Azadirachta indica:

Neem is an evergreen fast-growing tree native to India and Burma. It grows in arid, semiarid, and tropical regions (Schmutterer, 2022). Today, the neem tree is widely distributed throughout tropical and subtropical Asia, Africa, Australia, and South America (Kumar *et al*., 2016). Neem products have been obtained from several species of neem trees belonging to the Meliaceae family. *Azadirachta indica* is the most important species of this group which is considered as a renewable resource of various useful domestic, medicinal and agricultural products (Kumar *et al*., 2016). All parts of the tree (leaf, flower, seed kennel, wood, bark, and twig) are a source of biologically active ingredients, and the maximum of activity is associated with the seed kernel (Kumar *et al*., 2016). More than 300 different phytochemicals have been reported from different parts of the neem tree (Gupta *et al*., 2017) and over 130 of these compounds belongs to limonoid-type titerpenoids that are endowed with potent medicinal and insecticidal properties (Chen *et al*., 2018). The most important neem limonoids include azadirachtin, nimbolide, salanin, nimbin, deacetylnimbin, mahmoodin, epoxy-azadiradione, deacetylgedunin, and gedunin (Nagini, 2014; Gupta *et al*., 2017). These compounds have been shown to possess many useful properties of which antifeedancy, insecticidal, and insect growth disruption are used in the management of pest (Schmtterer, 1995). Most of the triterpenoids of neem were found in very small quantities in various parts of the tree and accounts for the total bioactivity of the neem seed extract (Mordue *et al*., 2010). Azadirachtin A is the major active component and is responsible for 72 to 90% of the biological activity (Schmutterer, 1990; Mordue *et al*., 2010).

Azadirachtin has attracted worldwide attention not only as the most popular deterrent to insects but also as a promising growth regulator. The growing interest on the neem tree and azadirachtin in particular has led to detailed physiological and biochemical investigations on the action of this compound and development of pesticide formulations based on azadirachtin and neem oil (Subrahmanyam 1990).

Insecticidal properties of Azadirachtin:

Azadirachtin is a complex tetranortriterpenoid derived from the mevalonic acid pathway in the neem tree (Hansen *et al*., 1993; Arthy *et al*., 2018). It is a highly oxidized tetranorterpeniod natural product related to limonin, the bitter fruits are known as limoninds (Benuzzi and Ladumer, 2018). Azadirachtin-A is considered as the main constituent of azadirachtin's commercial formulations available on the world market for insect control in organic farming. It has a complex molecular structure and following the determination of its correct structure in 1985 (Kraus *et al*., 1985), the first total synthesis of this molecule was published two decade after discovery of the compound (Launch, 2008). Azadirachtin acts as a feeding deterrent, insect growth disrupter (IGD), sterilant and is used to control various agricultural pest species, including Coleoptera, Dipters, Orthoptera, and Isoptera (Morgan, 2009). The toxicity of azadirachtin varies among insect orders and is influenced by the different penetration rates and activities of detoxifying enzymes (Morakchi *et al*., 2021).

The chemical complexity of azadirachtin minimizes the potential risk of insect resistance (Mordure *et al*., 2010). Feng and Isman (1995) reported development of resistance to pure azadirachtin over 40 generation in the peach potato aphid *Myzus persicae* but no resistance was reported with neem seed extract. Bomford and Isman (1996) also showed habituation to pure azadirachtin in the tobacco cutworms with less sensitivity to the antifeedant properties of azadirachtin, but not to neem with the same absolute amount of azadirachtin. This might accounts for avoiding desensitization to commercial neem-based insecticides containing additional non-AZA-compounds (Bomford and Isman, 1996). Azadirachtin-A is very well-received by the root system, and subsequently, it is systematically distributed through the xylem into the green parts of plant tissues and stored in leaves in an unchanged form. In addition, a very low content of azadirachtin A in plant tissues may protect significantly plant damage against phytophagous pest larva (Pavela, 2016). In addition, azadirachtin has displayed remarkable selectivity, with low mammalian toxicity (Mordue *et al*., 2010). According to Raizada *et al*. (2001), azadirachtin has shown an LD_{50} value of more than 5,000 mg/kg which falls into class U (Unlikely to present an acute hazard) of the WHO (2009) toxicity rating.

Azadirachtin is registered in the United States as a general-use pesticide with a toxicological class Environmental Protection Agency (EPA) of IV (relatively non-toxic). Azadirachtin seems to be selective, non- mutagenic, and readily degradable and has also been reported as safer for non-target organisms and beneficial organisms (Medina *et al*., 2004; Cordeiro *et al*., 2010; Mordue *et al*., 2010; Celestino *et al*., 2014; Dai *et al*., 2019); however, the presumed safety of azadirachtin has been questioned, especially, in relation to natural enemies and pollinators (Barbosa *et al*., 2015; Lima *et al*., 2015; Xavier *et al*., 2015; Bernardes *et al*, 2017, 2018).

Nevertheless, semi-field and field studies may enable to reliably predict potential side effects of azadirachtin on non-target insects. However, azadirachtin is still considered as one of the best alternatives to conventional insecticides in IMP programs and considered as one of the most promising plant compounds for pest control organic agriculture (Tome *et al*., 2013; Bezzar-Bendjazia *et al*., 2017). Despite the progress on the physiological and biological activities and agricultural application of azadirachtin its exact mechanism of action, especially, at the molecular level is not yet fully understood (Lai *et al*., 2014; Dawkar *et al*., 2019).

Growth regulating effect of Azadirachtin:

Azadirachtin causes disorders in metamorphosis. It was Ruscoe (1972) who first demonstrated such an effect. Later these effects were reproduced in many insect species of several orders, on *Leptinotarsa decemlineata* (Coleoptera) by Steets (1976), on *Ephistia kuchniella* (Lepidopera) and *Apis melllifera* (Hymenoptera) by Remboid *et al*. (1987), on *Locusta migratoria* (Orthoptera) by Rembold and Sieber (1981) and Siber and Rembold (1983), on *Rhodnius prolixus* (Heteroptera) by Garcia *et al*. (1986). Typical disorders due to azadirachtin may be summarized by Subramaniyam (1990) are as under:

i) Induction of moult inhibition and mortally in a dose-dependent manner.

ii) Remarkable prolongation of instar duration accompanied by death or moult disruption. Locust fifth instar injected with a dose of 2 μg/g body weight may continue without adult moult for more than 60 days (normal intermoult period being 9 days), and *Rhodnius* bugs may survive beyond 5 month without moult.

iii) Treated larvae remain in pharate condition unable to shed their old cuticle successfully. Weak ecdysial movements that could last for several hour accompanied by incomplete shedding.

iv) Incomplete or depressed resorption of the exuvial fluid.

v) Incomplete selerotization and pigmentation of the new cuticle.

vi) Unplasticization of wing lobes leading to either wingless adult (in bugs) of adult with crippled wings (in moths).

vii) Severe deformities in head and thoracic appendages of the pupae of Holometabola.

viii) Disruption of oogenesis when injected into young adult (e.g. locusts) and inhibition of embryonic development when injected into adult at the end of vitellogeneis.

In insect, 20 hydroxyecdysone (20 E) and Juvenile hormone (JH) play a central role in the regulation of growth and development (Bensebaa *et al*., 2015) and the hormonal balance determines the outcome of each developmental transition (Dubrovsky, 2005). Therefore, any interface with hormonal homeostasis lead to interrupted development and is considered as potential specific target for pest control (Pener and Dhadialla, 2012). Azadirachtin is known as an antagonist of these two principal hormones, its major action was its ability to modify or suppress haemolyph ecdysteroid and JH titers through inhibition of the secretion of morphogenetic peptide hormone (PTTH) and allatotropins form the corpus cardiacum complex and this account for its well documented IGD effect defined mostly as reduced pupation, malformation or failure of adult emergence (Mordue and Blackwell, 1993; Bezzar-Bendjazia *et al*., 2017). In *Rhodnius prolixus* a single dose of azadirachtin reduced hemolymph ecdysteroid titers to level too low for induction of ecdysis (Garcia *et al*., 1990). This blockage of ecdysteroid production is not due to a direct action of azadirachtin on the prothoracic gland (Koolman *et al*., 1988) but is caused by two specific action of azadirachtin. These actions are in the cascade of events leading from brain activation and competency to molt to 20-hydro-xyecdysone (20E) production in the hemolymph and its effect on target tissue. First, the release of the morphogenetic brain peptide prothoracicotropic hormone (PTTH) responsible for inducing synthesis and release of ecdysone from the prothoracic gland is blocked in azadirachtin treated insect.

Azadirachtin directly affect the corpora cardiaca by inhibiting the release of hormones, perhaps by operating via effect on neurotransmitters such as acetylcholine, Y-aminobutyric acid (GABA), serotonin and octopamine (Bidmon *et al*., 1987; Kauser *et al*., 1987; Koolman *et al*., 1988; Banerjee and Rembold, 1992). Also through that axis, azadirachtin has been shown to slow down the rate of synthesis and transport of PTTH by the brain neurosecretary cells, for example in *Heliothis virescens* (Barnaby and Klocke, 1990). Furthermore, this compound is known to cause degenerative structural changes of the nuclei in all endocrine gland (prothoracic gland, corpus allatum, and corpus cardicum) responsible for controlling molting and ecdysis in insect which would contribute to a generalized disruption of neuroendocrine function (Mordue *et al*., 2010). Azadirachtin supplemented diet (74 ppm) affects the growth, suppresses ecdysis, and inhibits ecdysteroids synthesis in the larvae of *Ostrinia furnacafis* Guenée (Min-Li and Shin-Foon, 1987). In *Tenebrio molitor*, the injection of 1 μg of azadirachtin into freshly ecdysed pupae induced a significant depletion of level of immunoreactive ecdysteroids affecting 20-hydroxyecdysone levels and suppressing the ecdysteroid peak that normally appears at the middle of the instar (Marco *et al*., 1990). A drastic reduction of hemolymph ecdysteroid titers was also reported in *Rhodnius prolixus* after a unique dose of azadirachtin (Garcia *et al*., 1990).

In addition to its effects on morphogenetic PTTH, azadirachtin affect edysone 20-monooxygenase activity, the insect cytochrome P450-dependant hydroxylase responsible for the conversion of the steroid hormone ecdysone to its more active metabolite (Smith and Mitchell, 1988). Indeed, *in vitro* analysis of three insect species, homogeneates of wandering third instar larvae of *Drosphila melanogaster*, fat body or midgut from last instar larvae of *Manduca sexta* and abdomen from adult female *Aedes aegypti*, incubated with radiolabelled ecdysone and azadirachtin revealed inhibition of the ecdysone 20-monooxygenous with a dose-dependent relationship (Smith and Mitchell,1988). However, ingested or injected azadirachtin had no effect on ecdysone 20-monooxygenase activity in *Spodoptera frugiperda* (Yu, 2000).

Besides its negative effect on molting hormone, azadirachtin induced a delay or a reduction in JH titlers, primarily by hindering the release of the allatotropins and theraby blocking the synthetic and release processes of the JH (Mordue *et al*., 2010; Dhra *et al*., 2018). JH hemolymph titers are reduced or delayed which is demonstrated in a variety of ways. Azadirachtin inhibits JH stimulated supernumerary molts in chilled *Galleria mellonella* larvae (Malczewska *et al*., 1988) perhaps by the blockage of synthesis and release of JH. In the last instar larvae of *Manduca sexta*, azadirachtin causes supernumerary molts by delaying and extending the JH peak into the critical period for commitment to larval rather than pupal cuticle (Schlüter *et al*., 1985; Beckage *et al*., 1988).

The importance of timing of hemalymph JH level with ecdysteroid level is demonstrated by the selective destruction of larval crochets or hooked setae on the prologs in *Manduca sexta* (Reynolds and Wing, 1986; Beckage *et al*., 1988). The epidermal cells producing such structures require the presence of both ecdysteroids and JH in order to make cuticle and in the absence of JH (e.g. on the day 2 of the last larval instar) (Riddiford, 1981) the epidermis loses this ability. Other example of an imbalance of ecdysteroid and JH levels are seen in the occurrence of uneverted tanned pupal wing discs in *Epilachna varivestis* (Schlüter 1987), effects linked with high JH titers such as green hemolymph, brown and green cuticle, lack of black pigment and supernumerary moults in locusts, *Spodoptera littoralis* and *Galleria mellonella* (Schmutterer and Freres, 1990; Gelbic and Némec, 2001) and cuticular melanization resulting in black spots related to the absence of JH and low levels of ecdysteroids (Koul *et al*., 1987; Malczewska *et al*., 1988).

Azadirachtin is reported to impair the growth and moulting process of insect and induced robust development delays in the larva-to-pupa and the pupae-to-adult transition compromising their survival (Hasan and Ansari, 2011; Tome *et al*., 2013; Lai *et al*., 2014; Bezzar-Bendjazia *et al*., 2016). In addition, growth and nutrient intake are functionally linked processes in development and growth and body mass are directly affected by nutrient uptake principally governed by the insulin/insulin – like growth factor signaling (IIS) pathway (Tennessen and Thummel, 2011). Lai *et al*. (2014) reported that the inhibition of growth and development in *Drosophila melanogaster* after azadirachtin treatment was similar to those caused by disruption of the IIS pathway. In addition, azadirachtin can inhibit the excitatory cholinergic transmission and block partly the calcium channel (Qiao *et al*., 2014), and this might interfere with different endocrinological and physiological actions in insects (Morakchi *et al*., 2021).

Conclusion

Azadirachtin based insecticides have been promoted as an alternative, safe and non-hazardous pest control measures. Azadirachtin has now been used in integrated pest management programmes of insect control in different crops, but not yet been reached most of its potential utilization. Apart from Growth Regulating effects of azadirachtin in insects, several studies on the antifeedant effects and action of azadirachtin have been done. The effects of azadirachtin in blocking the cell division and effects on protein synthesis have been studied using insect cell lines and mammalian cell lines. Currently little information is available on the long term effects and field trials of azadirachtin on insects. A comprehensive information on this natural insecticide may improve its use in Integrated Pest Management programmes more effectively by determining the concentration, frequency of application and targeting the best time of its application.

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