INVESTIGATION OF THE EFFECTS OF BORIC ACID

ON HEMOCYTE MEDIATED IMMUNE SYSTEM

IN *GALLERIA MELLONELLA* L. (LEPIDOPTERA: PYRALIDAE)

LARVAE

MASTER'S DEGREE THESIS

Rehemah GWOKYALYA

May, 2019

Eskişehir

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MASTER'S THESIS

Department of Biology

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May, 2019

FINAL APPROVAL FOR THESIS

This thesis titled "**Investigation of The Effects of Boric Acid on Hemocyte Mediated Immune System in *Galleria mellonella* L. (Lepidoptera: Pyralidae) Larvae**" has been prepared and submitted by **Rehemah GWOKYALYA** in partial fullfillment of the requirements in "Anadolu University Directive on Graduate Education and Examination" for the Degree of Master of Science in Biology Department has been examined and approved on **27/05/2019**.

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF BORIC ACID ON THE HEMOCYTE MEDIATED IMMUNE SYSTEM IN *GALLERIA MELLONELLA* L. (LEPIDOPTERA: PYRALIDAE) LARVAE

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Anadolu University, Graduate School of Sciences, May, 2019

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Boric acid (BA) is used as an insecticide, fungicide and in various industrial processes and thus different populations are exposed to it. The aim of this study was to analyze the effects of BA on the hemocyte mediated immune mechanism and its insecticidal potential on the model organism *Galleria mellonella* L. (Pyralidae: Lepidoptera). Different BA concentrations (78.125- 10,000 ppm) were administrated to the larvae using force-feeding method. Concentration-dependent mortality was observed in all experimental groups. According to the data obtained from the probit analysis, the LC_{30}, LC_{50} and LC_{70} values of BA were determined as 112.438, 320.133 and 911.484 ppm respectively. These lethal concentrations were used in all biological and immunological assays. In BA treated groups, larval developmental time increased while pupal and adult weight, pupal developmental time and adult longevity reduced. Post BA treatment, significant decreases in the total hemocyte counts, prohemocyte, plasmatocyte and oenocyte ratios and increments in granulocyte and sperulocyte counts were observed. Hemocyte viability assays showed a decrease in the numbers of live cells and an increase in necrotic and apoptotic ratios. However, no significant change was observed in the mitotic indices of larval hemocytes. Using Sephadex DEAX beads as encapsulation and melanisation targets, results showed that encapsulation and melanisation decreased with increasing BA concentrations. Nonetheless, the nodulation ability of the larvae exposed to BA significantly reduced post-laminarin injection. This investigation is the first of its kind to demonstrate the potential immunotoxicity of BA in insects, and our results suggest *G. mellonella*'s immune system is suppressed at high BA concentrations.

Key Words: Boric acid, *Galleria mellonella*, Hemocyte, Immunity, Model insect.

ÖZET

BORIK ASIT'İN *GALLERIA MELLONELLA* L. (LEPIDOPTERA: PYRALIDAE) LARVALARINDA HEMOSİT ARACILI İMMÜN SİSTEM ÜZERİNE ETKİLERİNİN İNCELENMESİ

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Anadolu Üniversitesi, Fen bilimleri Enstitüsü, Mayıs, 2019

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Borik asit (BA) insektisit, fungisit ve çeşitli endüstriyel işlemlerde kullanılmaktadır. Böylece birçok farklı populasyon bu kimyasala maruz kalmaktadır. Bu çalışma'da, BA'nın model organizma Galleria mellonella L. (Pyralidae: Lepidoptera) türü üzerindeki insektisidal ve hemosit aracılı immün savunma üzerindeki etkilerinin analiz edilmesi amaçlanmıştır. Zorla besleme yöntemi ile larvalara faklı BA konsantrasyonları (78,125-10.000 ppm) uygulandı. BA'ya maruz kalan deneysel gruplarda larval mortalitenin uygulanan konsantrasyona bağlı olarak arttığı gözlendi. Probit analizine göre, BA'nın LC_{30}, LC_{50} ve LC_{70} değerlerinin sırasıyla 112,438, 320,133 ve 911,484 ppm olduğu belirlenerek, bu değerler immünolojik deneylerde kullanıldı. BA konsantrasyonlarına maruz kalan gruplarda kontrol grubuna kıyasla, larva gelişim zamanında bir artış, pupa ve ergin ağırlığında, pupal gelişim zamanında ve ergin ömründe azalma olduğu belirlendi. BA uygulama sonrasında, toplam hemosit sayısında, prohemosit, plazmatosit ve oenosit oranlarında önemli bir azalma, granülosit ve sferülosit sayısında ise artışlar gözlendi. Canlı hemosit oranında ise konsantrasyona baglı azalma, nekrotik ve apoptotik oranlarda artış belirlendi. Fakat, mitotik indeks, BA uygulanan gruplarda değişiklik göstermedi. Hemositlerde belirlenen enkapsülasyon ve melanizasyon oranları ise BA konsantrasyonun artmasıyla azaldı. Bununla birlikte, laminarin enjeksiyonundan sonra BA'ya maruz kalan larvalardaki nodülasyon kabiliyetinin düştüğü belirlendi. Bu çalışma, BA'nın böceklerdeki immünotok potansiyelini gösteren ilk çalışmadır ve sonuçlarımız G. mellonella'nın bağışıklık sisteminin BA'nın yüksek konsantrasyonlarında baskılandığını göstermektedir.

Anahtar Sözcükler: Borik asit, *Galleria mellonella,* Hemosit, İmmunite, Model organizma

ACKNOWLEDGEMENTS

This thesis wouldn't be a success if it weren't for the support of various people to whom I am very grateful. Firstly, I give glory to God who has brought me this far and with Whom everything is possible.

I specially thank my supervisor, Assoc. Prof. Dr. Hülya ALTUNTAŞ, whose dedication and inspiration are invaluable. You not only nurtured this project from its initial state, but you have co-piloted this work till its final stages. Your discipline and work ethics let alone your dedication and availability for consultation every step of the way are commendable and have added great value to my career.

Special thanks go to the Turkish Scholarship Committee for the financial support rendered to me throughout my master's education.

I am grateful to my lab mates Nur BAYRAM, Aybüke İREMSAN and Selin ÇIM for their support in all laboratory procedures. I also thank my dear friends, Donald KALE, Velsa VELIU, Sara ZEQIRI and Nergis KARADUMAN for their endless support in every aspect of life during my stay in Turkey and for providing a happy distraction to rest my mind outside my research.

Last but never least, very special thanks go to my family to whom I always come first, especially my life coach and eternal cheer leader Mr. Lutaaya Muhammad, I owe it all to you Dad. To my loving mama Mrs. Prossy Kiwanuka who always reminds me how incredibly gifted I am and what I am capable of doing in times when I lose track and courage, you bring out the best of me. And to my brothers, sister, nieces and nephews, thank you for the endless calls, emotional and moral support. I missed home but was never home-sick. That's all thanks to you.

Rehemah GWOKYALYA

27/05/2019

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby truthfully declare that this thesis is an original work prepared by me; that I have behaved in accordance with the scientific ethical principles and rules throughout the stages of preparation, data collection, analysis and presentation of my work; that I have cited the sources of all the data and information that could be obtained within the scope of this study, and included these sources in the references section; and that this study has been scanned for plagiarism with "scientific plagiarism detection program" used by Anadolu University, and that "it does not have any plagiarism" whatsoever. I also declare that, if a case contrary to my declaration is detected in my work at any time, I hereby express my consent to all the ethical and legal consequences that are involved.

Rehemah GWOKYALYA

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ABBREVIATIONS

BA	:Boric acid
USDA	:United States Department of Agriculture
IPM	:Integrated Pest Management
EU	:European Union
USA	:United States of America
USCG	:United States Coast Guard
USDA	:United States Department of Agriculture
ATSDR	:Agency for Toxic Substances and Disease Registry
EPA	:Environmental Protection Agency
CID	:Compund Identification Database
U.S EPA	:United States Environmental Protection Agency
WHO	:World Health Organisation
TEM	:Transmission Electron Microscopy
DNA	:Deoxyribonucleic acid
ANOVA	:Analysis of Variance
e.g	:For example

1. INTRODUCTION

Today, various chemicals are used to improve product quality in sustainable agriculture, obtain more product from a limited arable area and to ensure that the products are more resistant to pests and diseases. The most important and largest portion of these chemicals is undoubtedly pesticides.

However, the;

- ✓ Excessive and uncontrolled use of pesticides,
- ✓ High cost of pesticides,
- ✓ The disappearance of useful species,
- ✓ Cancerous, teratogenic and mutagenic effects on mammals,
- ✓ Incomplete elimination of pests due to resistance gained with time necessitating increments in the doses or usage of other pesticides
- ✓ Direct and indirect effects of the chemicals not only on target species but also non-target organisms have raised concerns about chemical usage and the possible ecotoxic risks they pose today.

Many under-developed and developing countries profoundly use chemical control methods to combat pests in agricultural systems. The use of chemicals such as arsenic, organic chloride and organic phosphate containing compounds causes resistance in pests and also negatively affects non-target organisms. Because these chemicals are persistent, they enter the food chain in different ways and accumulate in the fat bodies and also induce carcinogenic, teratogenic and mutagenic effects in non-target organisms (Isman,2006; Senthil-Nathan, 2013). Due to the side effects imposed on the local ecosystems by these chemicals, their use in agricultural practices is a huge debate (Klatt et al., 2016) and most countries have imposed restrictions and bans on the use of these chemical compounds (e.g. DDT) (Schumann, 1991). For this reason, chemicals such as pyrethroids (e.g. deltamethrin) and carbamates that are rapidly degraded from the environment, less harmful and are least transmitted up the food chain are prefered for pest control (Jeschke and Nauen, 2008; Jeschke et al., 2010; Casida and Durkin, 2013). However, with time, pests develop resistance to these chemicals (Simon-Delso et al., 2015; Brevik et al., 2018), and this, is the

most pressing issue currently requiring immediate intervention. Additionally, secondary environmental effects of traditional insecticides have led to further research about species-specific substances (Casida and Quistad, 1998) and/or the use of biological insecticides (Kaakeh et al.,1997). Even though a variety of biological and biochemical pest control approaches are available, insecticides continue to be the main control method. Economically, the consumption of insecticides is 31%, 26% herbicides and 20% fungicides by value (Delen et al., 2004). The world pesticide production is around 3 million tons per year and the annual sales amount has reached 30 billion Euros on average. Turkey's share of this amount is only about 0.6% (Ozturk, 1997).

Because chemical control of insect pests in agricultural systems is common due to the above-mentioned reasons (Cox, 1996; Lee et al., 1996; Andow et al., 1997; Ahmad et al., 1997; Soderlund and Knipple, 1999; Montagna et al., 1999; Ribeiro et al., 2003), research about new control methods where very little or no chemicals are used has gained importance in recent years. In developed countries in particular, a method called "Intergrated Pest Management (IPM)" is being used for pest control in agricultural systems (Hillocks, 1995; Elad and Shtienberg, 1995; Sierpiñska, 1998; Edge et al., 2001). In IPM, population levels are protected by taking into consideration the environment, diseases, pests and weeds belonging to the cultivated plants and products. This method involves multiple chemical, biological and physical pest control methods which complement each other and the principles on which it is based are the protection of human and environmental health, prevention of losses in quantity and quality of products and the economic success of all initiatives (Özkan, 2012). The aim of IPM is to minimize the use of pesticides, investigate all pest control strategies and best utilize the natural enemies of pests (Hillocks, 1995; Hill and Foster, 2000; Wells et al., 2001; Simmonds et al., 2002; Tomberlin et al., 2002; Schneider et al., 2003). For this reason, recent studies have focused on the use of environmentally friendly chemicals as alternative methods of combating pests while protecting non-target beneficial insects, humans, and other living things.

It is also emphasized that the sustainability of sustainable agricultural practices depends on the development of environment friendly techniques and reducing the usage of chemical pesticides (Azizoğlu et al., 2011). For this purpose, it is necessary to come up

with insecticides which have a relatively low cost, a wide spectrum, the least or no negative effect on human population and the environment and can be used in greenhouse and field conditions (Loomans and Van-Lenteren, 1995; Rabasse and van Steenis, 1999). In this regard, some pesticides have been described as low risk or environmentally friendly by the Environmental Protection Agency (EPA). In the United States of America (USA) and many European Union (EU) countries, the registration of environmentally friendly pesticides has been encouraged and some incentives have been introduced for their use. In Turkey, similar measures have been taken since the adoption of the Ministry of Food, Agriculture and Horticulture 2013-2014 strategic plan. These measures include the use of pesticides that will ensure plant and animal health and welfare and also pose the least harm to the environment in IPM programs.

Among these environmentally friendly insecticides boron-based insecticides have gained importance. Boron, which was identified for the first time as a mineral in the year 1808 is a very important micronutrient for animals (Fort et al., 1998, Rowe et al., 1998, Park et al., 2004) and plants (Warington, 1923). In the environment, it mainly exists as borax, boric acid (BA) and also as borates which are not degraded any further to any compound (ATSDR, 2010). Easily soluble in water, BA exists as an odorless, slightly bitter, non-volatile, white crystalline powder or granules, stable cassolite mineral (Lang and Trecee, 1972, Ebeling, 1995). The melting point of BA is 171°C, it is miscible with and submersible in water. For this reason, it may leak through the soil. Its persistence in soil depends on the minerals and pH of the soil (USDA report, 2006). It occurs naturally at concentrations of 1-5 ppm in both fresh and marine waters. It also exists in the earth's crust at various concentrations for example, 300 mg/kg of soil (Eckel and Langley, 1988).

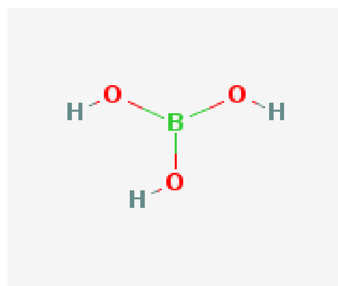

Figure 1. 1. *Chemical structure of boric acid.*

(National Center for Biotechnology Information; Pubchem Compound Database; CID=7628, https://pubchem.ncbi.nlm.nih.gov/compound/7628 (accessed May 13, 2018)

BA is known to be one of the oldest insecticides used to control termites, cockroaches and fungi. It has been marketed under many commercial names and can be supplied in an emulsifiable, reconstitutable and soluble powder form or ready to use in a microencapsulated form. Some insecticides containing BA and its derivatives e.g. Perma-Dust PT240 (Boric acid), Bora-care and Timbor (Sodium borate/disodium octaborate tetrahydrate) are available in American and European markets. These BA based insecticides are excellent alternatives to the organophospate based chemicals used to control agricultural pests (Yang et al., 2000).

Studies conducted in both animals and humans reported that BA is rapidly absorbed following oral exposure (81-95% BA in approximately 96 hours post ingestion) (ATSDR, 2007) and in most cases, majority of boron containing compounds are absorbed as undissociated BA (Pahl et al., 2001). Additionally, the absorption of borax, disodium octaborate tetrahydrate and BA via undamaged skin is relatively low; however, if the skin is damaged for example due to eczema, psoriasis or urticaria, the absorption rate of these compounds is usually high (U.S EPA, 2006). In another study where talcum powder (composed of 5% boric acid) was administered to 1-10 months old infants dermally for a period of one month, it was reported that only trace/negligible amounts of BA could penetrate the babies' skins (WHO, 1998).

Fatal doses of BA are recorded as 2,000–3,000 mg, 5,000–6,000 mg and 15,000–20,000 mg for infants, children and adults respectively (WHO, 1998; U.S EPA, 2006). A report by U.S. EPA, (2006) suggested that BA possesses a relatively low acute toxicity reporting 3450mg/kg and 4080mg/kg oral LD_{50} doses for male and female rats respectively. Similar deductions were made for borax reporting a LD_{50} doses of 4550mg/kg and 4980mg/kg for male and female rats respectively (U.S EPA, 2006). Additionally, fetal mortality in pregnant females and testicular atrophy in males are reportedly the most sensitive toxicological symptoms of both BA and borax. Considerably low doses of both compounds led to reduced fetal weight and abnormalities in the skeleton of off springs in

rats (U.S EPA, 2006). However, studies conducted in dogs reported significantly lower LD_{50} values (631mg/kg body weight for BA and 974mg/kg body weight for borax) following oral exposure suggesting that dogs are more sensitive to these two boron compounds compared to rats (U.S EPA, 2006).

While BA has been declared to have low toxicity on some birds, fish and aquatic invertebrates (Weir and fisher, 1972), a report by U.S EPA, (2006) stated that BA exhibits moderate acute toxicity symptoms and is thus regarded as a toxicity category III compound for majority of the acute effects. Additionally, it has been classified as a group E carcinogen since it does not show symptoms of carcinogenicity in rats and mice (Fishel, 2005). At the same time, because of its low toxicity to honey bees, BA is regarded as one of the least toxic insecticides in beekeeping (Harper et al., 2012). Due to this, BA is evaluated among the environmentally friendly chemicals in the struggle against insect pests, which directly or indirectly threaten human and animal health in enclosed areas, especially cockroaches, ants, termites and fleas (Lang and Trecee, 1972; Ebeling, 1995).

In Turkey, pesticides such as BA are traditionally used among the people in the struggle against pests, however, inorganic commercial insecticide formulations of such chemicals are not available and as such not used in a professional sense (Senthil-Nathan, 2013). Besides pest control, BA is an important ingredient of fire-retardant glass, wood preservatives, bulb and porcelain or ceramic glaze production; also, during coal burning and electricity generation, it oscillates around the environment. In the medical field, it is used as medicine in the treatment of fungal infections (Harper et al., 2012). For these reasons, the possibility of exposure of target and non-target organisms to BA in the ecosystem is increasing. The mode of action of BA is not fully known but it has been shown in various studies to affect the biological functions of various animals including insects (Rowe et al., 1998; Park et al., 2004; Büyükgüzel et al., 2013). Studies have also showed that BA and other borate salts are abrasive to insect exoskeletons (U.S EPA, 2006), act as stomach poisons (Cochran, 1995) and neurotoxins (Habes et al., 2006), and also inhibit growth (U.S EPA, 1996).

Several studies have shown that, when mixed with other substances BA has a profound effect on various organisms. Doanne and Wallis, (1964) reported that a combination of BA and *Bacillus thuringiensis* (Berliner) increases the mortality rate of the *Prothetria dispar*. In another study investigating the pathogenesis of *B. thuringiensis* against some termite species, a mixture of 1% boric acid and *B. thuringiensis* increased the mortality rate of the termites (Khan, 2006). Similar results were obtained from mixtures of 0.5% and 1% boric acid prepared with nucleopolyhedroviruses (NPV) on *Lymantria dispar* (Linnaeus) and *Spodoptera litura* (Fabricius) (Shapiro and Bell, 1982; Chaudhari, 1992). Xue and Barnard, (2003) also reported that a 1% BA and 10% sucrose mixture solution reduced the population of *Aedes albopictus* by 98% and that high BA concentrations inhibited nutrient absorption and usage of various nutrients like carbohydrates, nucleotides and vitamins. However, unlike these studies, Cisneros et al., (2002) investigated the lethal effect of nükleopolihedrovirus (sfmnpv) virus on *Spodoptera frugiperda* using BA as a phagostimulant or viral enhancer and results revealed that concentrations of 0.5% and 1% of BA alone had no significant effect on the mortality of *S. frugiperda.* Likewise, corn flour granules containing 1% and 4% BA in the laboratory environment were not toxic to *Dorus taeniatum* (Cisneros et al., 2002).

In a study conducted on *Blattella germanica*, dietary BA has been reported to affect the cuticle structure of the insects (Kilani-Morakchi et al., 2005), reduce the size and number of oocytes produced per paired ovaries (Kilani-Morakchi et al., 2009), slowly dry cockroaches due to its abrasive effect on the cuticle (Ebeling et al., 1975) and destruct the cellular lining of the fore gut leading to starvation and ultimately death (Cochran, 1995). In addition, Cruz et al., (2010) reported increased larval mortality, cytoplasmic vacuolisation, deterioration of typical morphological structures and increase in the volume of mitochondria of *Apis mellifera* treated with different BA doses. BA has also been shown to be neurotoxic following symptoms of poisoning, decreased acetylcholinesterase activity and increased glutathione S-transferases activity in the German cockroach (Habes et al., 2006). It has also been shown to alter the profiles of different proteins in *Galleria mellonella* larvae (Hyršl et al., 2007). Recently, it was determined that BA induces concentration dependent insecticidal effects in model organism *G. mellonella* (Büyükgüzel

et al., 2013). In the same study, it was determined that lipid peroxidation and the activities of superoxide dismutase, glutathione peroxidase, catalase and glutathione-S-transferase enzymes were altered following larval exposure to lethal and sub-lethal doses of BA.

According to the results obtained from these studies, we think that BA causes toxic effects by altering the physiological and biochemical processes in insects. However, studies showing the effects of BA on the immune system of insects are not available in open literature. Therefore, the hypothesis established in this study was based on the determination of the effects of BA, a natural insecticide on the biological parameters and the cellular immune system of *G. mellonella*. For this purpose, the effects of BA on the hemocyte-mediated defense mechanisms of the model insect and major storage pest *G. mellonella* (Lepidoptera: Pyralidae), were evaluated. Thus, a significant part of the eco-physiological effects that may arise due to the use of BA in pest control studies were explained within the scope of our study.

1.1. The Effects of Various Xenobiotics on Immune System of Insects

1.1.1. The insect immune system

Immune defense systems developed by mammals against xenobiotics are different from those developed by insects. While mammals have both acquired and innate immunity which use somatic gene rearrangement to develop T-and B-immunological cells and antibody-based immune memory (Fearon et al., 1997), insects only have the innate immunity relying on germline-encoded factors to recognize and clear infection. The insect innate immune system consists of both humoral and cellular mediated responses (Hirashima et al., 1990; Faulds, 1991; Canyurt, 1994; Androw et al., 1997; Uçkan and Gülel, 2002). Humoral immunity involves a series of complex enzymatic cascades and the synthesis of antimicrobial peptides like cecropins, attacins and defensins that regulate hemolymph clotting or melanization (Miller et al., 1993; Canyurt, 1994; Uçkan and Gülel, 2002) while the latter is mediated by the hemocytes that phagocytose or capture foreign bodies in multicellular structures called nodules or capsules (Lavine and Strand, 2002).

1.1.2. Insect cellular immune responses

Cellular immune responses are mediated by the circulating hemocytes, which perform defense mechanisms such as phagocytosis, encapsulation and nodule formation (Gupta, 1985; Hirashima et al., 1990; Faulds, 1991; Canyurt, 1994; Uçkan and Gülel, 2002). In insects, hemocytes develop from the head or dorsal mesoderm during embryonic development (Rowley and Ratcliffle, 1981; Tepass et al., 1994) and are continuously produced by the existing dividing cells in the mesodermal hematopoietic organs and/or circulating hemocytes (Jones, 1970; Rowley and Ratcliffle, 1981). In Lepidoptera species larvae, cells in the capsule near the prothoracic spiral have been reported to participate in hemocyte formation whereas in dipteres, the hemopoietic organs are reported to be found in the posterior abdominal segment (Nation, 2002). There is however no data in literature pertaining the presence of hematopoietic organs in the greater wax moth. In a study investigating mitotic indices in *G. mellonella*, the mitotic phase rate of circulating hemocytes was reported to be 1% and this proved sufficient for cell number maintenance during the larval stage and thus no need for hematopoietic organs (Jones and Liu, 1968)

Functionally, unlike vertebrate blood cells, insect blood cells, do not participate in oxygen carriage to the different body parts but have been reported to take part in the production of proteins which are deposited in the insect cuticle and the basal lamina (Chapman, 1998) and some exhibit immune functions (Wojda, 2017). There have been great advances in the techniques and criteria according to which insect hemocytes are classified (Ribeiro and Brehelin., 2006). Using various microscopic techniques and basing on the function, morphology and antigenic properties of the cells, Lavine and strand, (2002) defined and classified lepidopteran species hemocytes as plasmatocytes, granulocytes, oenocytes, spherurocytes and prohemocytes.

Prohemocytes are usually small, regularly shaped cells with a high cytoplasmic to nuclear ratio. Functionally, they are the precursors/stem cells to the differentiating hemocytes. In Transmission electron microscopy (TEM), they appear to possess several small (less than 0.25 mm) cytoplasmic inclusions. They haven’t been proven to spread in monolayers or take part in nodule/capsule formation (Ribeiro and Brehelin, 2006).

Plasmatocytes are regularly shaped oval or spherical and sometimes spindle-shaped cells (20 mm long). In TEM, they appear to enclose polyribosomes, pseudopods, clear vacuoles, rough endoplasmic reticulum and pinocytotic vesicles (Rowley and Ratcliffe, 1981), but lack granules in some species (Rowley and Ratcliffe, 1981; Essawy et al., 1985; Strand, 1994; Ribeiro et al., 1996; Butt and Shields, 1996). They rapidly spread in monolayers a few minutes post incubation and appear as large, thin cells with a large rounded nucleus, numerous pseudopodia and a characteristic fibroblast-like morphology (Lavine and Strand, 2002). Invivo, plasmatocytes are reported to take part in nodule and capsule formation during insect defense. Their role in phagocytosis is however still disputed as some authors (Ratcliffe and Rowley, 1975; Tojo et al., 2000; Ling and Yu, 2006) argue that they are phagocytic whilst for others (Akai and Sato, 1971; Neuwirth, 1974; Beaulaton, 1979), they are not phagocytes.

Granulocytes appear as spherical (diameter from 5 to 8 mm) cells containing granules, numerous pinocytotic vesicles, rough endoplasmic reticulum, thin and long pseudopodia. Among the many functions of granular hemocytes is phagocytosis (Brehelin and Hoffmann, 1980; Horohov and Dunn, 1983; Essawy et al., 1985; Pech et al., 1994; Dunphy, 1995; Ribeiro et al., 1996, Costa et al., 2005). They have also been shown to take part in nodulation and encapsulation as the first cells to make contact with a foreign body during capsule/nodule formation and release granular content (Ratcliffe and Gagen, 1977; Schmit and Ratcliffe, 1977). After they attach to foreign bodies, granulocytes exocytose special inclusions from the golgi apparatus which aid in attracting plasmatocytes (Gillespie et al., 1997) and their consequent binding to build nodules and/or capsules (Pech and Strand, 1996).

Oenocytoids are regularly shaped large (25 mm diameter) cells with a low cytoplasmic to nuclear ratio. In some insect species, the cytoplasm appears to be homogenous (Beaulaton and Monpeyssin, 1977) with an eccentrically located nucleus. In monolayers oenocytoids are usually fragile and easily lyse in vitro (Strand and Noda, 1991), but in species like *G. mellonella* they appear to be more stable (Rowley and Ratcliffe, 1981). They play a huge role in the phenoloxidase cascade as synthesizers of phenoloxidases enzyme which is released into the plasma upon cell lysis (Essawy et al.,

1985; Ribeiro et al., 1996), as demonstrated by Ashida et al., (1988) in oenocytoids of *Bombyx mori*.

Spherule cells are generally rounded, relatively stable in monolayers and contain spherules which give the cell an irregular shape. Spherule cells contain acidophilic inclusions which are readily stained with acid dyes like acridine orange and muco glycoproteins. Their role in immunity is still vague (Lavine and Strand., 2002; Ribeiro and Brehelin., 2006) but have been reported to participate in the transportion of cuticular components (Wojda et al., 2017).

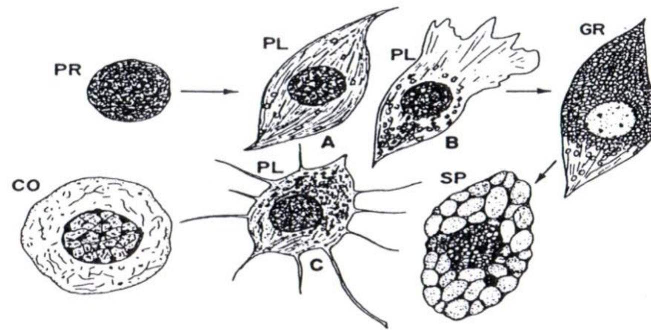

Figure 1. 2. *General view of insect hemocytes. (from Nation, 2002).*
PR: prohemocyte, PL: plasmatocyte, GR: Granulocyte, SP: spherulocyte, CO: Oenocyte.

1.1.2.1. Encapsulation

Encapsulation is an immune reaction where hemocytes bind to large invaders like nematodes, trematodes and parasitoid eggs which cannot be engulfed by a single cell (Richards and Edwards, 2002; Strand, 2008). In Lepidoptera species, plasmatocytes are the primary capsule forming cells, but several studies have reported granulocytes to also take part in encapsulation. While in species like *Manduca sexta* the distribution of plasmatocytes and granulocytes during capsule formation is random (Wiegand et al., 2000), in several others it is highly specialised with granulocytes attaching to the target first and plasmatocytes binding thereafter. Other plasmatocytes in large numbers then attach to the target simultaneously to form a multilayered sheath. Plasmatocytes adhering to targets

following granulocyte attachment is an indication that granulocytes produce factors like plasmatocyte spreading peptide (Clark et al., 1997) that attracts and enables plasmatocyte binding (Strand, 2008). Capsule formation is terminated when granulocytes bind and apoptose on the capsule periphery forming an extracellular sheath (Pech & Strand, 1996; Schmidt et al., 2001; Luo and Pang, 2006). As a result, the capsule periphery transforms into an intact membrane to which plasmatocytes can no longer adhere. Once the capsule has formed, due to factors like asphyxiation, production of antibacterial peptides, toxic quinones and/or hydroquinones and reactive oxygen and nitrogen intermediates, the encapsulated organism dies (Nappi et al., 2000; Schmidt et al., 2001) and the capsules melanise (Strand and Pech, 1995; Schmidt et al., 2001; Wertheim et al., 2005). It is important to note that the morphohology of the formed capsules varies among insect taxa. Also, the rate at which the capsules are formed, the involved hemocytes and whether the capsules are melanised or not varies amongst species (Schmidt et al., 2001).

1.1.2.2. Nodulation

Nodule formation is a prompt response by hemocytes to clear the hemocoel of microorganisms (Arai et al., 2013). During nodule formation, pathogens are recognised by specific molecules after which hemocytes adhere to the pathogen. After the foreign material is completely entrapped by the hemocytes, the nodules begin to melanise (Lavine and Strand, 2002). Post melanisation, the entire nodule is recognized as a foreign body and is removed from the hemocoel (Debnath et al., 2017). In insects, plasmatocytes and granulocytes are reported to actively play a role in nodule formation (Tembhare, 2016). Also, hemocytins and C-type lectins have been reported to take part in the aggregation process just before nodules are formed (Arai et al., 2013).

1.1.2.3. Melanisation

This is a wound healing mechanism and an immune response and that leads to synthesis and deposition of melanin on microbes and wound surfaces. Melanisation leads to the entrapment of pathogens in a dense melanin coat and generation of potentially harmful metabolites that can kill the pathogens (Yassine et al., 2012). According to Santoyo and Aguilar-Cordoba, (2011), during the process of melanogenesis;

- ✓ Phenylalanine hydroxylase converts melanin precursor, phenylalanine into tyrosine.
- ✓ Phenol oxidase then coverts tyrosine to dihydroxyphenylalanine (DOPA).
- ✓ According to the catalysing enzyme DOPA is converted to dopaquinone or dopamine by phenol oxidase or DOPA decarboxylase respectively.
- ✓ In cases where dopamine is synthesized, it is further converted to dopaquinone by phenoloxidase enzyme.
- ✓ Dopaquinone is then converted to 5, 6-dihydroxyindole and then to Indole-5, 6-quinone by phenoloxidase
- ✓ Indole-5,6-quinone is also converted to melanochrome which polymerizes to form melanin.

1.1.2.4. Apoptosis and necrosis mechanisms

Defined as programmed cell death, apoptosis is typified by microvilli loss, cytoplasm and nuclei condensation, cell segmentation, shrinkage and detachment, DNA fragmentation and plasma membrane convolution (Cotter et al., 1992; Taylor et al., 2008; Henry et al., 2013). The regulator enzymes caspases 3 and 6 have been reported to play a role in the above-mentioned apoptosis related morphological changes (Taylor et al., 2008). Apoptosis occurs in almost all animal organs and tissues to remove excess cells and the immunodeficient or the harmful ones (Ellis et al., 1991; Raff et al., 1994). Necrosis on the other hand is referred to as un-programmed or unnatural cell death due to injury, disease or chemicals. It is characterised by chromatin clumping, cellular swelling and lysis leading to the release of the cell constituents (Kim et al., 2001). During necrotic cell death, swelling of the organelles, mitochondria, peroxisome and lysosome membranes leads to dismantling of the cell structural integrity. Basophilic properties decrease due to loss of ribosomes. There is also an observed increase in the number of cytoplasm vacuoles and swelling of mitochondria (Kumar et al., 2003; Halliwell and Gutteridge, 2007).

Whether or not cells undergo necrosis or apoptosis regulated by a variety of internal stimuli or cell intrinsic factors and the environment (Ellis et al., 1991) which either suppress or activate these two cell death pathways. Fluorescence microscopy is commonly used to analyse cell apoptosis and necrosis-specific properties. In general, cell DNA staining acridine orange is used to determine apoptotic index alongside ethidium bromide

which only stains late apoptotic or necrotic cells with impaired membrane integrity. Acridine orange enters both live and dead cells where it binds to the double-stranded DNA and/or single-stranded RNA and lysosomes emitting a green and/or red fluorescence (Kosmider et al., 2004). Ethidium bromide only enters the dead (late apoptotic and necrotic) cells via the disrupted cell membrane and binds to the DNA, emitting a red fluorescence. Tunaz, (2003) and James and Xu, (2011) affirm that immune cellular response occurs as a result of the encounter of insect hemocytes with foreign substances. Whence hemocytes play a very important physiological role in the immune defense mechanism of insects.

Previously, several studies about the effects of various xenobiotics on hemocytes have reported that xenobiotics, in general, alter the number of circulating hemocytes (Shapiro, 1979; Christensen et al., 1989), cell viability, apoptotic or necrotic deaths, lead to morphological changes and elicit cellular responses, such as phagocytosis, nodule formation and encapsulation (Ratcliffe& Rowley, 1975; Gupta, 1979). A study conducted by Er et al., (2016) using *G. mellonella* reported that azadirachtin, a natural insecticide just like BA, decreased total hemocyte counts, hemocyte spreading ability and laminarin-induced nodulation in a time and dose dependent manner. Decreased total hemocyte counts were also reported for β-ecdysone treated *Sporodoptera litura* (Ahmad, 1993) and methoprene treated *Papilio demoleus* larvae (Sendi and Salehi, 2010). The later study also reported significantly reduced plasmatocytes, adipocytes and spherulocyte numbers and severe pathological changes in cytoplasm, cell membrane and nucleus structures of *P. demoleus* larval hemocytes.

In another study conducted using *G. mellonella*, it was determined that gibberellic acid (GA_3), a plant growth regulator, increased the larval hemocyte count, mitotic, late apoptotic and necrotic indices. However, the same study reported that GA_3 altered melanisation and encapsulation responses but had no effect on the different larval hemocyte types (Altuntaş et al., 2012). Indol-3-acetic acid, when administered at different doses to the larvae of the small wax moth *Achoria grisella* has been reported to reduce necrotic and late apoptotic indices, while increasing total hemocyte count and early apoptotic indices in a dose-dependent manner (Çelik et al., 2017). Also, when *G. mellonella* larvae were treated

with potassium nitrate, it was determined that the total hemocyte numbers in the larval hemolymph increased concentration wise (Maguire et al., 2017). Recently Coates et al., (2018) demonstrated that okadaic acid reduces the number of circulating cells and also decreases hemocyte viability in *G. mellonella*. Despite all these researches done with various environmental compounds, no study has been conducted to ascertain the subacute and sublethal effects of BA on larval hemocytes of *G. mellonella*.

1.2. The Greater Wax Moth *Galleria mellonella* L. (Lepidoptera: Pyralidae)

The greater wax moth *G. mellonella* L. (Lepidoptera: Pyralidae) used in this study is an economic pest of honey bees (Apis mellifera). It is commonly found in apiaries, particularly in low-altitude, temperate climatic regions (Allan, 2000) and is recognized as a potential pest worldwide (Sanford, 2003). To protect apiaries against *G. mellonella* infestation, various chemical, biological and physical techniques have been employed (Ritter et al., 1992; Yacobson et al., 1997; Delaware, 2000; Akyol et al., 2009).

In Turkey, it is reported that 5 million honey bee colonies produce approximately 3.500-4.000 tons of beeswax annually (Anon, 2006). The observed fluctuations in productivity are believed to be due to the infestation of the bee colonies by the greater wax moth (Çağlar et al., 2001). In addition, *G. mellonella* larvae are used as model organisms for various immunological, pathogenetic and physiological studies (Cook and Mcarthur, 2013; Fallon et al., 2012). Because this species is both a pest and a model insect, we used it in our research study and are optimistic that the data obtained will make a significant contribution to both the scientific literature and economic realm.

Therefore, in this study we determined the lethal concentrations of BA fed to the 5th instar larvae of *G. mellonella* larvae by force-feeding (gavage) method and also determined the effects of effective BA concentrations on the biological parameters and cellular mediated immune responses of *G. mellonella*. According to the determined lethal concentrations (LC_{30}, LC_{50} and LC_{70}), the sublethal and subacute effects of BA on the survivability of *G. mellonella* larvae, pupae and adults, total and differential hemocyte counts, hemocyte viability, cell spreading, nodulation, encapsulation, melanisation, apoptosis and necrosis responses were determined. Thus, within the scope of our work, the

effects of BA on the hemocyte-mediated immune defense of insects were determined for the first time.

1.3. Thesis Statement

This study aimed to

I. Determine whether force-fed boric acid is toxic to model insect *G. mellonella*,

II. Determine the effects of force-fed boric acid on cellular immune defence mechanisms of *G. mellonella*,

III. Obtain new ecophysiological and cytotoxic data of boric acid on insects using the model organism *G. mellonella,*

IV. Obtain data that will provide beneficial information to the risk assessment and management of boric acid use in integrated pest management programs.

1.4. Hypothesis

Boric acid a naturally occurring compound has for long been used as an insecticide and in various industrial fields. For this reason, BA's presence in the ecosystem is increasing and many living groups, in particular insects which are important components of the ecosystem are exposed to it in their natural environments. Hence, in this thesis, it was hypothesized that effective concentrations of BA may influence various biological parameters and the cell mediated immune responses of model insect *G. mellonella*.

2. METHODOLOGY

2.1. *Galleria mellonella* Culture

The cultivation of *G. mellonella* was carried out in an insectarium (insect room, D51-41) in the animal physiology laboratory, Department of Biology, Faculty of Science, Eskisehir Technical University. Photoperiodical conditions in the insectarium were maintained at 28 ± 2 °C temperature, 60 ± 5% relative humidity in continuous darkness to ensure stock and progressive culture continuity.

In order to establish stock cultures, 5 female and 1 male *G. mellonella* adult individuals were put into 1liter glass jars containing semi-artificial diet (100 g) given in Table 2.1. The jars were covered with gauze and perforated covers (to avoid escaping of the larvae) but in such a way that does not obstruct the ventilation in the culture medium. Adult individuals were removed from the culture medium following mating of the female and male adult individuals. Culture maintenance was done three times a week to check the nutrient needs of the larvae and to remove any wastes. Depending on the density of the larvae, feeds were added to the jars from in sufficient quantities to feed the hatching larvae. If crowded, the larvae were optimally distributed to different jars. During weekly maintenance, the larvae approaching the final larval stage were transferred into 0.5 liter jars containing folded papers to facilitate pupation. Pupa jars were checked 7-10 days later and followed up daily till adult individuals emerged. Mature individuals were then mated to ensure the continuity of the stock cultures.

For experimental purposes, randomly selected 5th instar *G. mellonella* larvae weighing 0.16 ± 0.01 g were transferred to 60 mm petri dishes so that each individual dish contains one individual. Every individual transferred into a petri dish was starved for 3 hours before force feeding, so that the larvae could open their mouth more easily. The semi-artificial diet was mixed according to a modified *G. mellonella* diet recommended by Altuntaş et al., (2012).

Table 2. 1. *G. mellonella larvae artificial diet contents (Altuntaş et al., 2012).*

Feed ingredient	**Quantity**
Bran	250g
Honeycomb	100g
Honey	75 ml
Glycerine	150 ml
Distilled water	75 ml
Pollen	20 g

2.2. Determining The Lethal Concentrations of Boric Acid

Boric acid (H_3BO_3 CARLO ERBA) used in this study was purchased as a pure powder. BA was diluted with distilled water to form solutions in concentrations of 78.125-10,000 ppm. Larvae weighing 0.16 ± 0.01g randomly selected from the stock culture were given 5 µl of the different prepared concentrations via force feeding method (Ramarao et al., 2012; Mukherjee et al., 2013; Dere et al., 2015).

Prior force feeding, the larvae were starved for 3 hours after which, they were kept on ice for 2 minutes to be anesthetized and then 5 µl of boric acid were administered orally to each larva through the oesophagus using a 10 µl hamilton injector (26 gauge). For the control group, 5 µl of distilled water were fed to each larva.

Each of the treated larvae was transferred to a 15 × 60mm petri dish containing 2 g synthetic diet and kept in the insectarium. Both control and experimental groups were observed daily for 30 days and the dead larvae recorded. This way larval mortality rates were determined. The obtained data was tested by probit analysis to determine the lethal concentrations (LC_x) of BA. LC_{30}, LC_{50} and LC_{70} values were used for all biological and hemocyte analyses. In this assay, 20 larvae in four replicates (n =80) were used for the control and all experimental groups.

2.3. Determining The Effects of Boric Acid on Biological Parameters of *G. mellonella*

Following the developmental process of the experimental (LC_{30}, LC_{50} and LC_{70} BA concentration treated larvae) and control groups, larval, pupal and adult life span and the pupal and adult weights of *G. mellonella* were recorded. Follow-ups were done on a daily basis. For both experimental and control groups 15 larvae were used, and all analyses were done in 3 replicates.

2.4. Determining The Effects of Boric Acid on The Hemocyte Mediated Responses of *G. mellonella* Larvae.

Total and differential hemocyte counts, hemocyte viability, cell spreading, nodulation apoptosis, necrosis, encapsulation and melanisation analyses were performed on individual larvae in all control and experimental groups. For purposes of determining the effects of BA on the total and differential hemocyte counts, hemocyte viability cell spreading, apoptosis and necrosis, hemolymph was drawn from the larvae 24 hours post BA treatment by amputating the third fore leg of the larvae with a surgical scissor. Hemolymph was collected using a micro pippete (Sigma, St. Louis, Mo). According to the experiment to be performed, desired amounts of hemolymph were collected from each individual larva.

2.4.1. Total hemocyte count

To determine the effects of the selected concentrations of BA on the total hemocyte count 15 larvae (n = 45 each group) in 3 replicates were used for each treatment and control group. Four microlitres of hemolymph were collected 24 hours post BA administration as described above. The collected hemolymph was transferred to eppendorf tubes containing 36 µl of ice cold anti-coagulant solution (0.098 M Naoh, 0.186 M Nacl, 0.017 M Na_2EDTA and 0.041 M Citric acid, pH 4.5) (Altuntaş et al., 2012). The 1:10 diluted cell suspension was thoroughly mixed with the aid of a micropipette after which 10µl of the cell suspension was drawn and loaded onto a neubauer hemocytometer (Improved Neubauer Hemocytometer; Superior, Germany) with a depth of 0.100 mm. Hemocytes were counted under a Leica DM6000 B Phase-Contrast microscope and expressed as cells x 10^6 cells/ml haemolymph.

Figure 2. 1. *Neubauer hemocytometer*

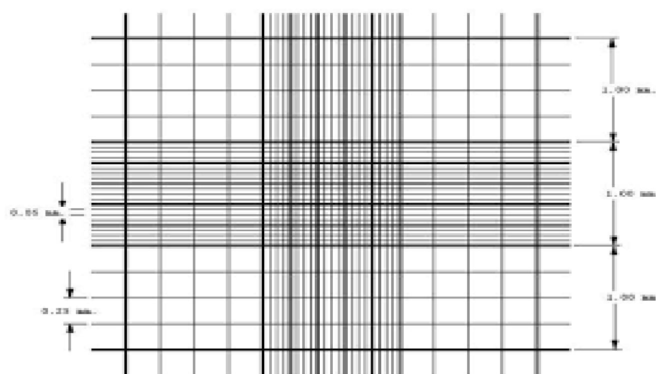

Figure 2. 2. *Presentation of the cytometer area from which cell counts were counted*.

The size of the biggest square (1 mm^2) =1 x 1 x 0.1 = 0.1 mm^3

0.1 mm^3 = 0.0001 cm^3 = 0.0001 ml.

In all experimental groups total hemocytes counts were done by counting the cells in the 16 small squares of each of the 25 medium-sized squares located in the middle of the hemocytometer.

To calculate the total hemocyte counts the formula below was used;

Cell number/ml = Cell counts in large square × the dilution coefficient × 10^4.

2.4.2. Differential hemocyte counts and mitotic indices

For purposes of determining the effects of BA on the different hemocyte numbers of *G. mellonella*, 10 µl haemolymph was drawn from each individual larva as described above 24 hours post forced-feeding. The collected hemolymph was then quickly spread on sterilized microscope slides and allowed to dry at room temperature. After the hemolymph had dried, the slides were dipped in methanol:acetic acid (3:1) solution for 5 minutes to facilitate fixation of the hemocytes then left to dry at room temperature. Freshly prepared Giemsa dye solution was used to stain the hemocytes. The staining solution was prepared by mixing 3 ml of the dye (MERCK Giemsa's Azure Eosine Methylene Blue Solution) with 57 ml of PBS (pH 7.4). The mixture was allowed to stand for 10 minutes before it was used. After fixation, the dried slides were immersed in the giemsa staining solution for 15 minutes after which the slides were rinsed with distilled water and left to dry at room temperature.

The dry slides were layered with cover slips using entellan and examined under a Leica DM6000 B light microscope. Hemocytes were counted from 5 - 7 randomly chosen fields of view and the different hemocyte types recorded. For all experimental groups, 500 cells were counted from each slide. Hemocyte types were classified according to Altuntaş

et al., (2012). From these very slides, hemocytes undergoing mitosis were identified and recorded. This way, the effects of BA on the mitotic indices of *G. mellonella* larvae was determined. For all experimental groups, 15 larvae in 3 replicates (total 45 specimens) were used.

2.4.3. Cell viability

To determine the effects of BA on the viability of *G. mellonella* hemocytes, the trypan blue staining assay according to Coates et al., (2018) was performed with modifications. This technique works on a principal that the cell membranes of dead cells are permeable to trypan blue and so the cytoplasm of dead cells appears blue while the live cells whose cell membranes exclude certain dyes do not assume any colour. From each larva, 6 µl of hemolymph were obtained and mixed with 18 µl of ice-cold 0.02% trypan blue/PBS solution and left to incubate for not more than 5 min. Ten microlitres of the cell suspension was drawn and loaded onto the neubauer hemocytometer (Improved Neubauer haemocytometer; Superior, Germany). Hemocytes were counted under the Leica DM6000 B Phase-Contrast microscope. The stained cells and the total number of hemocytes were counted. The stained cells were expressed as a percentage of the total hemocytes recorded. For each experimental group, 15 larvae were evaluated in three replicates.

2.4.4. Cell spreading

To determine the effects of BA on the spreading ability of hemocytes of *G. mellonella* larvae, 4µl of hemolymph were collected from each larva as described above 24 hours post force-feeding. The collected hemolymph was transferred to an eppendorf tube containing 20 µl ice cold PBS and thoroughly mixed with a micro pipette; 20 µl of hemolymph aliquot was then spread on a microscope slide pre-cleaned with alcohol, placed in a humid chamber (Sigma, H6644) and incubated in the dark at 29 ± 1°C for 30 minutes to facilitate adherence of hemocytes on the microscope slides. After 30 minutes, the slides were rinsed with distilled water and examined under a Leica DM6000 B light microscope. To ascertain the spreading ability of the hemocytes, a total 500 hemocytes were counted from five randomly selected areas and from these, those that display projections were recorded as spread cells and their numbers were recorded.

$$\text{cell spreading} = \frac{\text{spread cells}}{\text{total number of cells}} \times 100.$$

For each experimental and control group, 15 larvae were evaluated in three replicates.

2.4.5. Apoptosis and necrosis

To determine whether the different BA concentrations influence apoptosis and necrosis in the hemocytes of *G. mellonella* larvae, 5 µl of hemolymph was drawn from each larva as described above and transferred to an eppendorf tube containing 5µl acridine orange and 5 µl ethidium bromide solutions and mixed thoroughly. From the aliquot 15 µl were drawn and spread on a microscope slide pre-cleaned with 70% alcohol and the cells analyzed under the Leica DM6000 B fluorescent microscope. These procedures were all carried out in total darkness considering the sensitivity of both stains towards U.V light and safety measures were taken while handling the carcinogenic ethidium bromide.

The hemocytes examined were classified in 4 different ways according to their staining properties as described by Kosmider et al., (2004):

- ✓ Live cells (Green nucleus, cytoplasm may appear orange or red).
- ✓ Early apoptosis (cell membrane integrity is intact, but most often chromatin condensation and fragmentation are observed, the nucleus appears bright green)
- ✓ Late apoptosis (also called secondary necrosis or apoptotic necrosis). Ethidium bromide penetrates the disrupted membranes and enters the cells to stain the nuclei orange. There is also chromatin condensation and fragmentation.
- ✓ Necrosis (a normal/ regularly shaped cell with an orange stained nucleus is observed).

2.4.6. Nodulation

Laminarin (Sigma, St. Louis, Mo) was injected into the larval hemocoel to induce nodulation in both experimental and control larval groups to study the effect of BA on the nodulation responses of *G. mellonella*.

Laminarin (Sigma, St. Louis, Mo) was used to induce nodulation in larvae exposed to the selected concentrations of BA and those in the control group. The assay was carried out according to Er et al., (2017) and Franssens et al., (2006). We conducted preliminary experiments to ascertain the effects of incubation times and laminarin concentrations on the nodulation responses of *G. mellonella* larvae and for this, we studied how G. mellonella larvae respond to different volumes/doses of laminarın at different times. We therefore injected 5µl (50 µg laminarin) and 10 µl (100 µg laminarin) into individual larval hemocoel and analysed the larvae for nodulation responses at 2, 4, 6- and 24-hours post laminarın injection. According to these experiments, we observed that maximum nodulation occurred at the 4h time point when larvae were injected with 5 µl of laminarin. At the 2-hour time

point, significantly lower nodules were counted with both laminarin concentrations. On the other hand, nodule formation was not statistically different at longer incubation times when compared to the 4 h time point. Based on this, all the proceeding experiments in our study were performed by injecting 5 µl laminarin and the larvae were examined 4 hours post laminarin injection.

A stock solution of laminarin was prepared in PBS (Sigma, St. Louis, Mo) at a 10 mg/ml concentration. Twenty-four hours after BA administration, all larvae were anesthetized by chilling on ice for 5 minutes after which 5µl of laminarin was injected into the hemocoel via the first hind leg to induce nodulation. Four hours post laminarin injection, the larvae in all experimental and control groups were chilled/frozen at minus 20°C for 10 minutes, disinfected with 70% alcohol and dissected under the stereo microscope Olympus Leica ZOOM 2000 (Leica, Germany) to evaluate them for nodulation. The darkened nodules in the hemolymph, fat tissue and other visceral organs of the dissected larvae were counted and recorded as "nodules on viscera" and those on the skin were counted and recorded as "on intengument". For each control and experimental group, 10 larvae were evaluated in three replicates.

2.4.7. Encapsulation and melanisation

Sephadex DEAE-25 (40-120µm in diameter) chromatography beads were used as encapsulation targets to study the effects of BA on the encapsulation and melanisation behaviours of *G. mellonella* larval hemocytes. To facilitate the presence of the beads in the larvae, the sephadex DEAE-25 beads were transferred into an Eppendorf centrifuge tube and to them, 1% Coomassie blue solution (Brilliant Blue G Sigma) prepared in PBS was added using a micropipette. The beads were allowed to stain in this solution for 1 hour after which the supernatant was poured off and fresh PBS added to wash the beads. The washing process was repeated three times. Twenty four hours post force feeding, 10µl of the previously prepared stock bead solution containing 15-20 beads was injected into the larvae with the aid of a Hamilton syringe (50 µl and 22 guage sharp injector tip). At both 4 and 24 hour time points post bead injection, each larva was dissected and examined under a stereo microscope to remove the beads and analyse them for encapsulation and melanisation. The whole-body cavity of the larvae was examined, and the found beads were transferred to a slide containing a drop of PBS using a fine-tip dissection needle, covered with coverslips and examined under a phase-contast microscope to analyse the encapsulation and melanisation behaviours of the hemocytes.

Beads extracted from larvae were classified according to whether they were encapsulated, melanized or not as follows;

- ✓ Not encapsulated if the bead is not encapsulated or one layer of hemocytes,
- ✓ Weak encapsulation if there are 2-10 layers of hemocytes on the beads,
- ✓ Strong encapsulation if there are 10 or more haemocyte layers on the beads
- ✓ Melanized if darkened/melanised cells' area covered more than the quarter of the capsule.

15 larvae were used in all experimental and control groups in 3 replicates. (n = 45)

2.5. Data Evaluation

All data were expressed as mean ± standard errors determined using the data obtained from the experiments. The SPSS data analysis software program (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) was used for all statistical analyses. Data for total and differential hemocyte counts, cell viability, cell spreading, nodulation, encapsulation, melanisation, apoptosis and necrosis were Arcsine transformed before analysis. All data from the experimental analyses were tested for normal distribution using Lavene's test. Data obtained from the biological parameters, cell viability and encapsulation assays did not follow normal distribution thus, to compare means, Kruskal Wallis and Mann Whitney tests were performed to determine the statistical differences. On the other hand, data obtained from total and differential hemocyte counts, mitosis, cell spreading, melanisation, nodulation, apoptosis and necrosis analyses were normally distributed so to compare means the ANOVA (one-way analysis of variance) and Tukey's HSD (Honest Significant Difference) test was conducted to ascertain the significant differences. The three-way ANOVA analysis was also carried out to analyse the interactions between BA concentrations (ppm), time and encapsulation levels, and their effects on the encapsulation response in larval hemocytes. All results obtained in the experiments were evaluated as statistically significant at a 95% confidence interval ($p < 0.05$).

3. RESULTS

3.1. Insecticidal Effects

It was observed that the tested BA concentrations had a significant insecticidal effect on *G. mellonella* larvae. Larval mortality increased from 20% at the lowest BA concentration (78.125 ppm) to 100% at 10,000 ppm while only 5% mortality was observed in the control group. As shown in table 3.1, mortality is highly correlated with BA concentrations (x^2 (6) = 8.789; p = 0.186). The probit analysis revealed the LC_{30}, LC_{50} and LC_{70} values for force-fed BA to be 112.44 (75.72 – 153.16), 320.13 (246.28 – 403.09) and 911.48 (727.96 – 1167.44) ppm respectively (Table 3.1).

Considering the sigmoid-shaped mortality-concentration curve, it was decided that all biochemical assays to be carried out in this study should be studied using the LC_{50}, LC_{30} and LC_{70} concentrations since they are appropriate for demonstrating the subacute and sublethal effects of force-fed BA on *G. mellonella* larvae.

Table 3. 1. *Mortality of G. mellonella larvae exposed to eight BA concentrations and the determined lethal concentrations of BA*

BA concentration (ppm)	**Number of dead larvae**	**Mortality (%)**	**Lethal concentrations (ppm)**			
Control	4	5	Lethal concentrations (LC_x)	Probability doses	95% Confidence Interval**	
78.125	16	20			Lower bound	Upper bound
156.25	32	40	LD_{10}	24.820	12.994	40.198
312.50	44	55	LD_{30}	112.438	75.716	153.164
625	52	65	LD_{50}	320.133	246.284	403.094
1,250	56	70	LD_{70}	911.484	727.959	1167.44
2,500	65	81.13	LD_{90}	4129.119	2,941.770	6413.81
5,000	72	90	LD_{95}	8524.646	5,591.730	14935.18
10,000	80	100	LD_{99}	33206.59	18,383.68	73989.76

**80 larvae were used in the control and each experimental group (number of treated larvae).*

*** Values are displayed with the lower and upper confidence limits (Probit = 1.154 × concentrations (ppm) - 2.891).*

3.2. Effects of BA on The Biological Parameters of *Galleria mellonella*

Treating *G. mellonella* larvae with different BA concentrations significantly increased the larval development time at all BA concentrations when compared to the control ($\chi^2(3) = 21.35$; $p = 0.000$). On the contrary however, BA significantly reduced the pupal developmental time ($\chi^2(3)=85.046$; $p=0.000$) and adult longevity of *G. mellonella* ($\chi^2(3) = 42.36$; $p = 0.000$) at all BA concentrations in a concentration-dependent manner when compared to the control (Figure 3.1).

Additionally, BA treatment led to significant reductions in pupal ($\chi^2(3) = 119.987$; $p = 0.000$) and adult ($\chi^2(3) = 116.807$; $p = 0.000$) weights in all BA treated groups when compared to the control. (Figure 3.2).

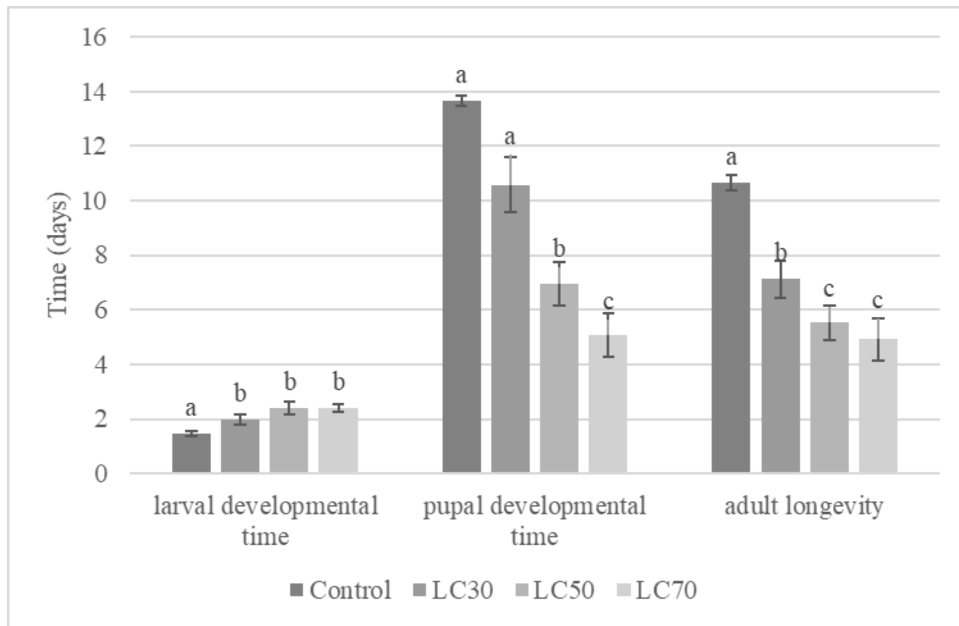

Figure 3. 1. *Boric acid induced changes in the adult longevity and larval and pupal developmental time (days).*

**Each bar represents the mean ± standard error. Significant differences are denoted by different letters (a-d) (Mann Whitney U test, P < 0.05).*

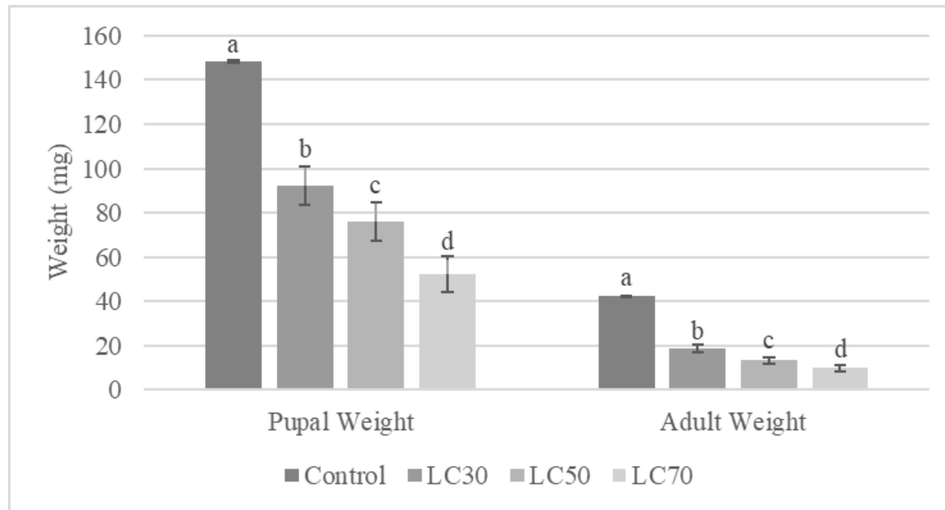

Figure 3. 2. *Effects of BA on the pupal and adult weights of G. mellonella*.

**Each bar represents the mean ± standard error. Significant differences are denoted by different letters (a-d) (Mann Whitney U test, P < 0.05).*

3.3. Total Hemocyte Counts

Results obtained showed that BA significantly reduced the total hemocyte counts of *G. mellonella* larvae in a concentration-dependent manner ($F = 209.519$; $df = 3, 176$; $p = 0.000$) with the lowest counts (13.0222×10^6 cells/ml) recorded at the highest BA concentration (LC_{70}, Figure 3.3).

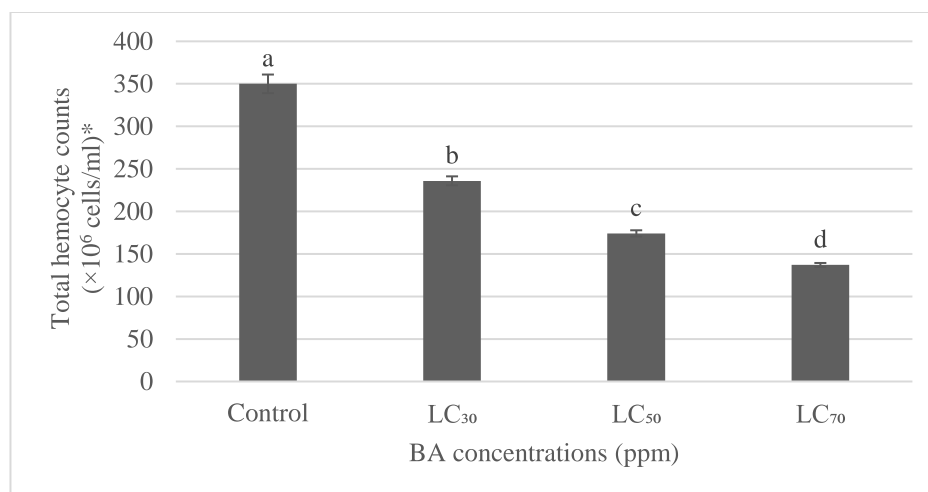

Figure 3. 3. *Total hemocyte counts (x10^6 cell/ml) of G. mellonella larvae treated with different BA concentrations*.

**Each bar represents the mean ± standard error. Different letters (a-d) denote significant differences (Tukey's HSD test, P<0.05).*

3.4. Differential Hemocyte Counts and Mitotic Indices

Treating *G. mellonella* larvae with BA significantly reduced the number of prohemocytes in all BA treated groups ($F = 85.985$; $df = 3, 176$; $p = 0.000$) when compared to the control except at LC_{50} where an increase was observed (Table 3.2). When compared to the control, a change in the number of granulocytes and plasmatocytes was only significant at LC_{50} and LC_{70} respectively ($p < 0.05$). While the number of spherulocytes significantly increased concentration-wise at the 2 highest BA concentrations ($F = 194.089$; $df = 3, 176$; $p = 0.000$), that of oenocytes only significantly increased at LC_{50} when compared to the control ($F = 39.545$; $df = 3, 176$; $p = 0.000$). There was no significant difference observed in the mitotic indices in all BA treated groups in comparison with the control ($p > 0.05$, Table 3.2).

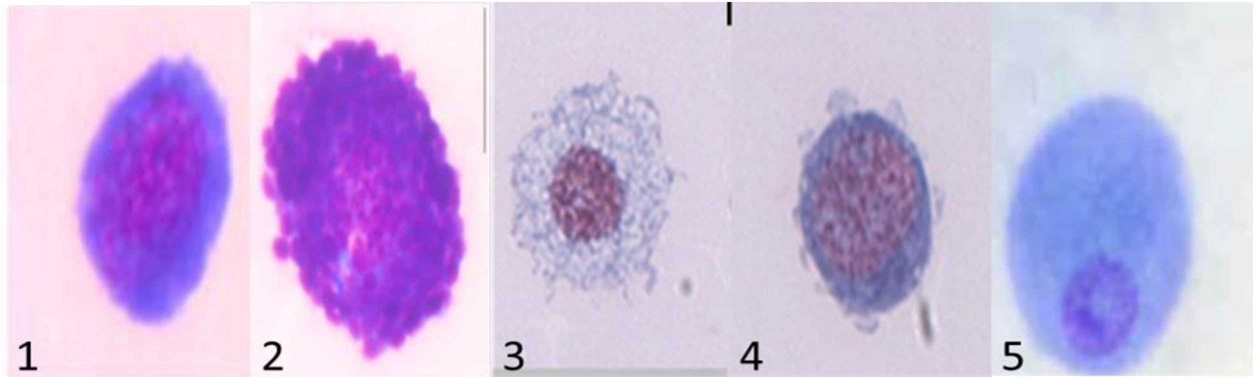

Figure 3. 4. *Different hemocytes of G. mellonella.*

1) Prohemocyte 2) spherulocyte 3) granulocyte 4) plasmatocyte 5) oenocyte.

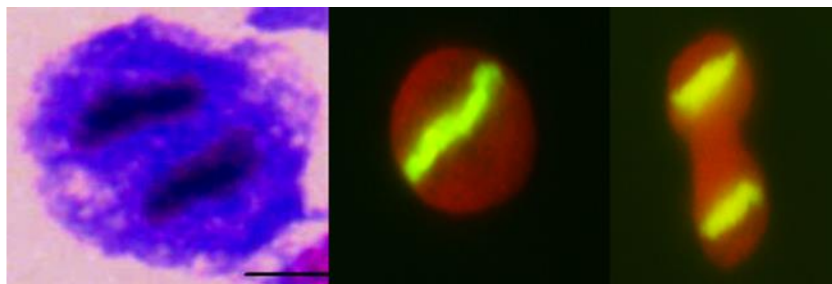

Figure 3. 5. *Mitosis in G. mellonella hemocytes*.

Table 3. 2. *Comparing the differential hemocyte counts and mitotic indices (per 500 cells) of G. mellonella larvae treated with different BA concentrations.*

BA (ppm)	Differential Hemocyte Counts (Mean ± SE %)*					
	PRO	PL	GR	SPE	OEN	Mitosis
Control	1.09 ± 0.06a	47.86 ± 0.3a	48.06 ± 0.3ab	2.84 ± 0.02a	0.14 ± 0.02a	0.09 ± 0.02a
LC_{30}	0.65 ± 0.05b	48..58 ± 0.3a	47.33 ± 0.33a	2.90 ± 0.1a	0.55 ± 0.03b	0.07 ± 0.02a
LC_{50}	1.54 ± 0.07c	47.08 ± 0.2b	47.14 ± 0.2a	4.15 ± 0.1b	0.12 ± 0.02a	0.07 ± 0.02a
LC_{70}	0.40 ± 0.03d	45.15 ± 0.3c	48.61 ± 0.31b	5.76 ± 0.1c	0.10 ± 0.02a	0.05 ± 0.01a

**All data are presented as percentage mean ± standard error. Different letters (a-d) show significant differences (Tukey's HSD test, $P < 0.05$) between experimental groups. Pro: prohemocyte, Pl: plasmatocyte, Gr: granulocyte, Spe: spherulocyte, Oen: oenocyte.*

3.5. Cell Viability and Spreading

Treating *G. mellonella* larvae with BA significantly reduced the viability of their hemocytes ($\chi^2(3) = 165.516$; $p = 0.000$) among the treatment groups in a concentration-dependent manner in comparison with the control with a 50% decrease in viability observed at the highest (LC_{70}) concentration (Figure 3.7).

Treating *G. mellonella* larvae with BA also significantly reduced the spreading ability of the hemocytes. The decrement followed a concentration-dependent manner among experimental groups when compared with the control ($F = 568.189$; $df = 3, 176$; $p = 0.000$) especially at the 2 highest BA concentrations (LC_{50} and LC_{70}) where spreading ability decreased by almost 57% and 79% respectively compared to the control (Figure 3.6 and 3.7).

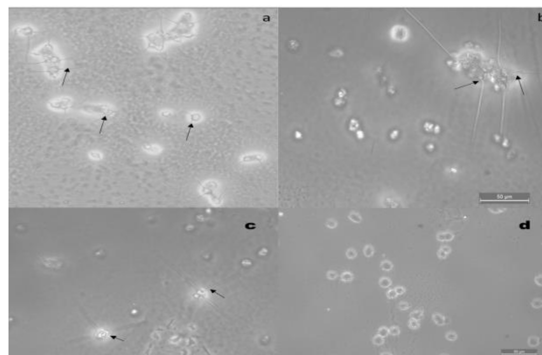

Figure 3. 6. *Cell spreading responses of G. mellonella larval hemocytes post BA treatment. The cells were examined under a Leica DM6000 B brand light microscope using 200x objective (Scale bar: 50 µm). a) Control, b) LC_{30}, c) LC_{50}, d) LC_{70} concentrations of BA. Arrows indicate the spread cells.*

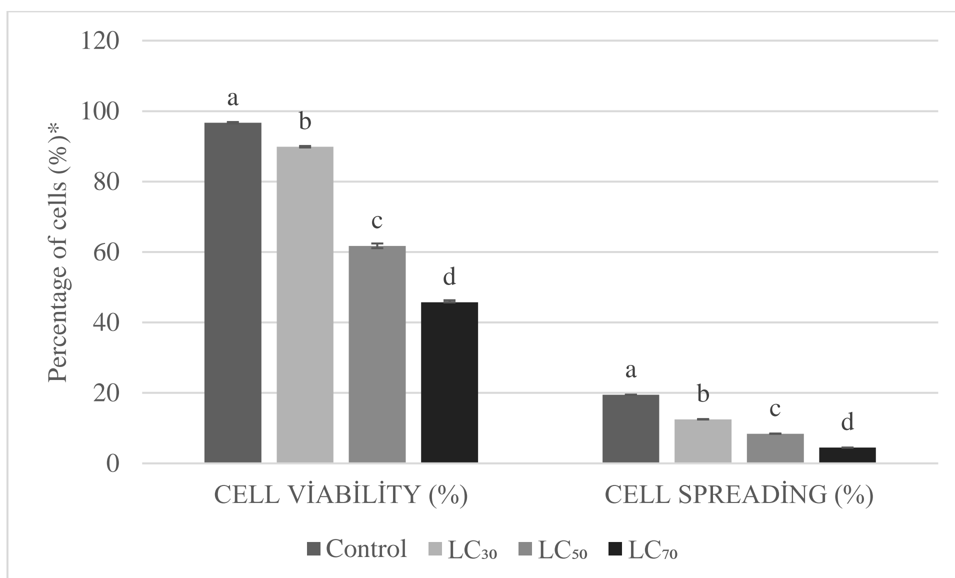

Figure 3. 7. *Percentage of G. mellonella viable and spread hemocytes after treatment with BA.*

**Each bar represents mean ± standard error. The letters (a-d) on each column show a significant difference between among experimental groups (for cell spreading, Tukey's HSD test, P < 0.05) (for cell viability, Mann Whitney U test, P < 0.05).*

3.6. Apoptosis and Necrosis

Exposure of *G. mellonella* larvae to BA significantly reduced the number of live hemocytes in all BA treated groups (F = 1577.286; df = 3,116; p = 0.000) in comparison with the control.

When compared to the control, the ratio of early apoptotic cells increased significantly (F = 259.520; df = 3, 116; p = 0.000) concentration-wise at the 2 lower BA concentrations (LC_{30} and LC_{50}) but drastically decreased to values identical to those observed in the control group at the highest BA concentration, LC_{70}. A change in late apoptotic cell ratios was only significant at LC_{50} and LC_{70} (F = 906.713; df = 3, 116; p = 0.000) compared to the control. Moreover, the values recorded at these concentrations were each almost 7 and 12 times higher than that observed in the control group. The percentage of necrotic cells increased in a concentration-dependent manner in all BA treated groups when compared to the control (F = 565.411; df = 3, 116; p = 0.000, Table 3.3).

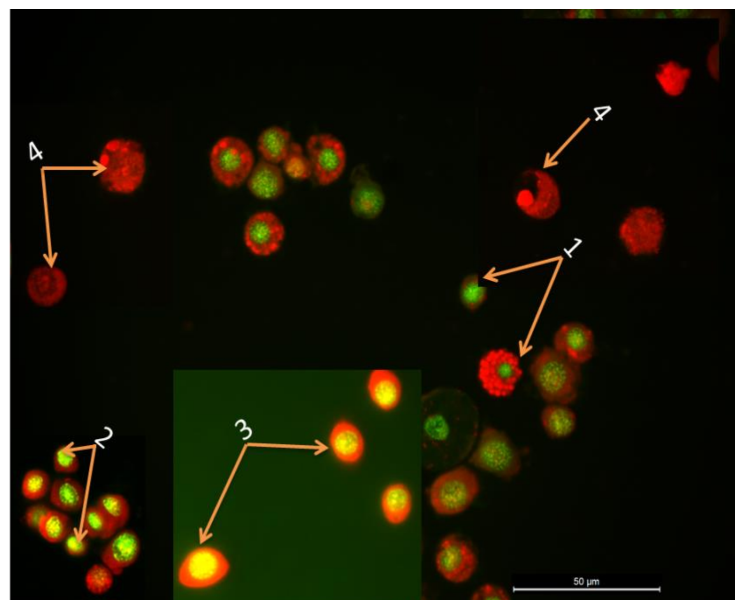

Figure 3. 8. *Apoptosis and necrosis in G. mellonella hemocytes*

1) live cells 2) early apoptotic cells 3) late apoptotic cells 4) necrotic cell

Table 3. 3. *The effects of BA on apoptosis and necrosis in G. mellonella larval hemocytes.*

BA (ppm)	Apoptosis and necrosis (Mean ± SE %)*			
	Live cells	**Early apoptosis**	**Late Apoptosis**	**Necrosis**
Control	91.79 ± 0.2a	5.84 ± 0.14a	2.00 ± 0.12a	0.37 ± 0.06a
LC_{30}	82.63 ± 0.3b	11.61 ± 0.2b	5.17 ± 0.1a	0.56 ± 0.1b
LC_{50}	65.19 ± 0.5c	13.54 ± 0.4c	14.91 ± 0.4b	6.36 ± 0.3c
LC_{70}	50.68 ± 0.7d	6.04 ± 0.2a	24.57 ± 0.5c	18.71 ± 0.4d

**Each data is reported as percentage mean ± standard error. Significant differences among all test groups are denoted by different letters (a-d, Mann Whitney U test, $P < 0.05$).*

3.7. Nodulation

Four hours after injection with laminarin, the larvae in all experimental groups were assessed for nodulation. The number of nodules counted on the integument decreased in a concentration-dependent manner in BA-treated larval groups ($p < 0.05$ Figure 3.9 and 3.10). Moreover, at LC_{50} and LC_{70} nodulation ability decreased by 44.73 and 53.22 % respectively when compared to the control. The nodules attached on the viscera were analysed as 'on viscera" and the number of nodules counted showed an almost similar trend ($F = 22.635$; df = 3, 176; $p = 0.000$) to that observed for those on the integument, though, among the experimental groups the difference was only statistically significant at LC_{30} and LC_{70} when compared to LC_{50} (Figure 3.10).

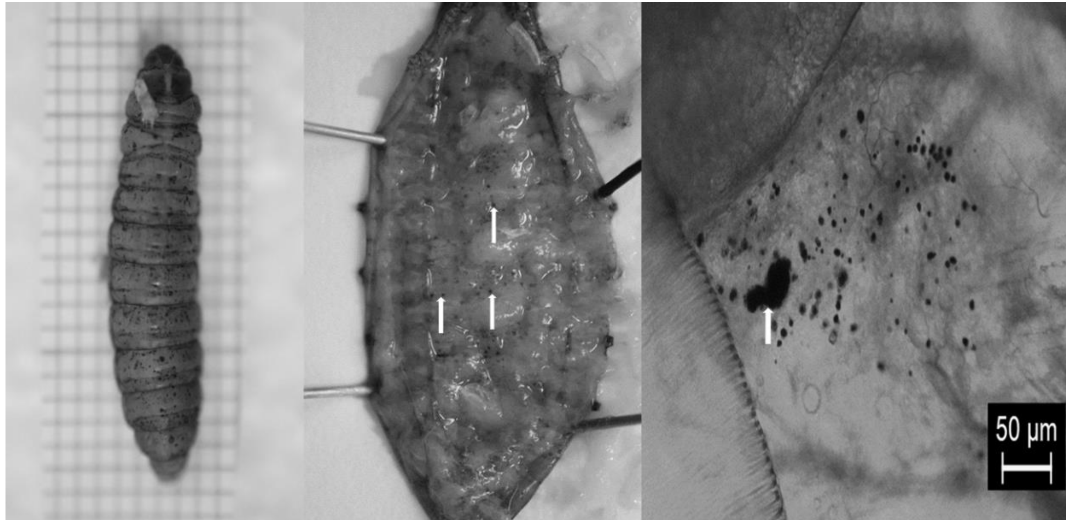

Figure 3. 9. *Nodulation in G. mellonella hemocytes*

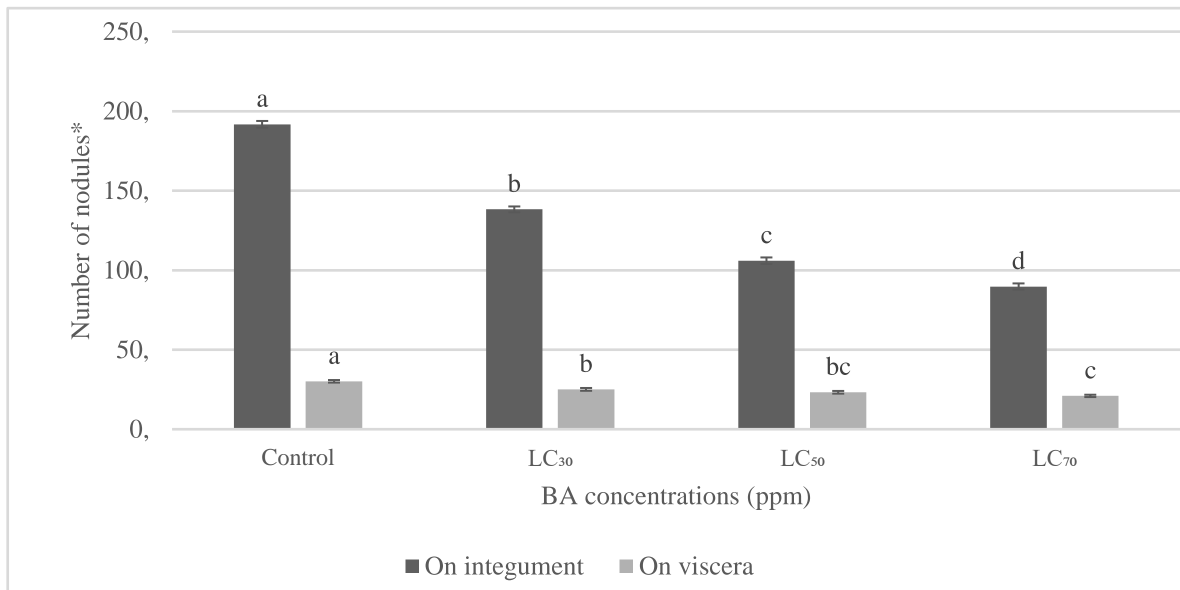

Figure 3. 10. *Effects of BA on nodulation ability of G. mellonella larval hemocytes.*

**Each bar is representative of mean ± standard error. Significant differences among all test groups are denoted by letters (a-d, Tukey's HSD test, $P < 0.05$).*

3.8. Encapsulation and Melanisation

Results obtained in the current study show that BA significantly reduced the encapsulation capabilities of *G. mellonella* hemocytes in a concentration-dependent manner 4 and 24 hours post bead injection into the larval hemocoel when compared with the control (Table 3.4). As is evident from the results, the ratio of strongly encapsulated beads significantly increased with increased exposure time but, reduced with increased BA

concentrations ($p < 0.05$). These ratios remarkably decreased to 47.34% and 26.1% at the 2 highest BA concentrations (LC_{50} and LC_{70} respectively) 24h post bead injection. On the contrary, the ratio of non-encapsulated beads significantly increased with increasing BA concentrations and decreased with reduced exposure time ($p < 0.05$). At the 4-hour time point, there was no significant difference observed in non-encapsulated beads in all BA treated groups except at LC_{70} which showed almost a 3-fold increment in the ratio of non-encapsulated beads when compared to the control ($\chi^2(3) = 32.703$; $p = 0.000$). Nonetheless, when compared to the control, a significant change in the non-encapsulated beads was only significant at LC_{50} and LC_{70} at the 24h time point ($p < 0.05$).

Twenty-four hours post bead injection, a significant increase ($p < 0.05$) in the number of weakly encapsulated beads at all BA treated groups when compared to the control was observed. Four hours post bead injection however, compared to the control a change in the ratio of weakly encapsulated beads was significant only at the highest BA concentration (LC_{70}) ($p < 0.05$, table 3.4).

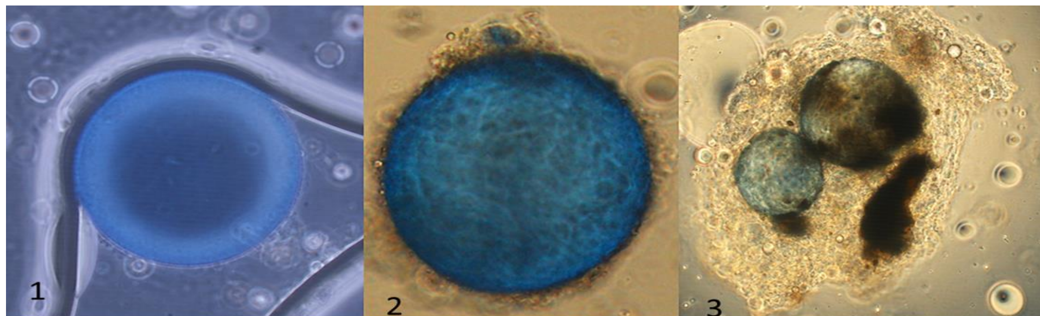

Figure 3. 11. *Encapsulation responses of G. mellonella larval hemocytes.*

1-non encapsulated; 2- weakly encapsulated 3- strongly encapsulated

Table 3. 4. *Comparison of the encapsulation responses of G. mellonella larval hemocytes in all BA treated and control groups.*

Extent of encapsulation (mean ± SE %)*						
	Non encapsulated		Weak		Strong	
Time/ BA (ppm)	**4h**	**24h**	**4h**	**24h**	**4h**	**24h**
Control	7.89 ± 1.6a	4.33 ± 0.1a	73.99 ± 2.4a	13.17 ± 1.7a	17.61 ± 2.0a	82.97 ± 1.7a
LD_{30}	7.84 ± 1.7a	7.40 ± 1.7ab	80.06 ± 2.2a	27.78 ± 1.9b	12.10 ± 1.8b	66.17 ± 1.8b
LD_{50}	12.10 ± 2.1a	8.68 ± 1.4b	77.96 ± 2.6a	43.04 ± 2.40c	9.92 ± 1.8c	47.35 ± 1.9c
LD_{70}	24.46 ± 2.2b	29.52 ± 2.1c	70.10 ± 2.1b	44.5 ± 1.9c	5.68 ± 1.4c	26.13 ± 1.5d

[a]All values are presented as percentage mean ± standard error. Significant differences among all test groups are denoted by letters a-d (Mann Whitney U test, $P < 0.05$). Values with a similar letter are not significantly different ($P > 0.05$). Sephadex DEAE A-25 beads (10-12 beads in 10 µl PBS for each larva) were dissected out and assessed at 4 h and 24 h post injection.

According to Three Way ANOVA analysis, there was no statistically significant interaction between treatment and time ($p = 0.513$). These results also showed a significant interaction between extent of encapsulation and treatment ($p = 0.000$), the extent of encapsulation and time ($p = 0.000$) and a statistically significant three-way interaction between treatment, the extent of encapsulation and time ($F = 16.541$; df = 6, 696; $p = 0.000$).

When compared to the control, there was a statistically significant change ($P < 0.05$) in the percentage of melanised beads in all BA treated groups at the two-time points (4 and 24 h). While, melanisation ratios significantly decreased among all BA treated groups 24 hrs post bead injection ($F = 115.376$; df = 3, 116; $p = 0.000$) no such difference was observed among the BA concentrations at the 4h time point, ($F = 14.451$; df = 3, 116; $p = 0.071$), (Figure 3.12 and 3.13).

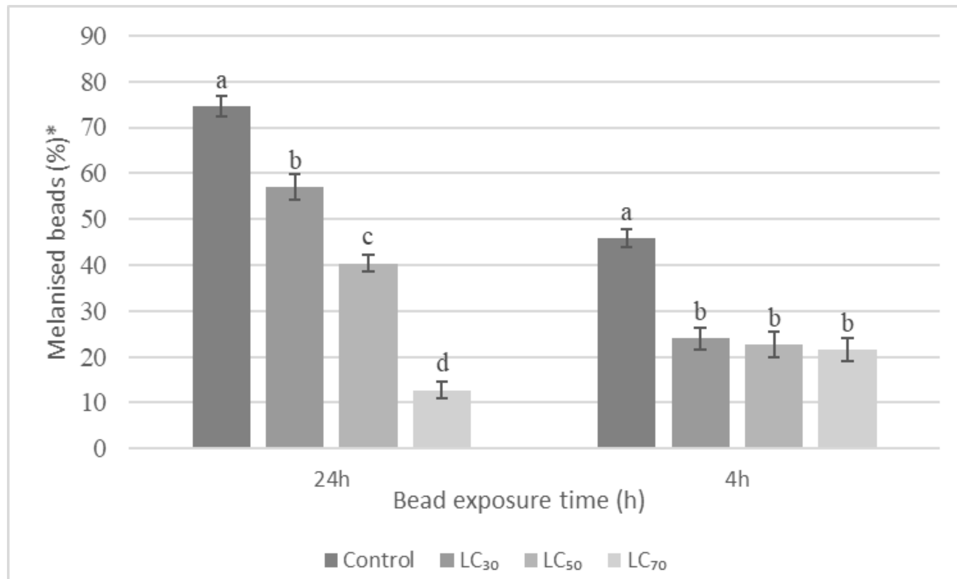

Figure 3. 12. *Effects of BA on melanisation ability of G. mellonella larval hemocytes.*

**Each bar represents percentage mean ± S.E of melanized beads. Significant differences among all test groups are denoted by letters a-d (Tukey's HSD test, P < 0.05). Bars with a similar letter are not statistically different (P > 0.05).*

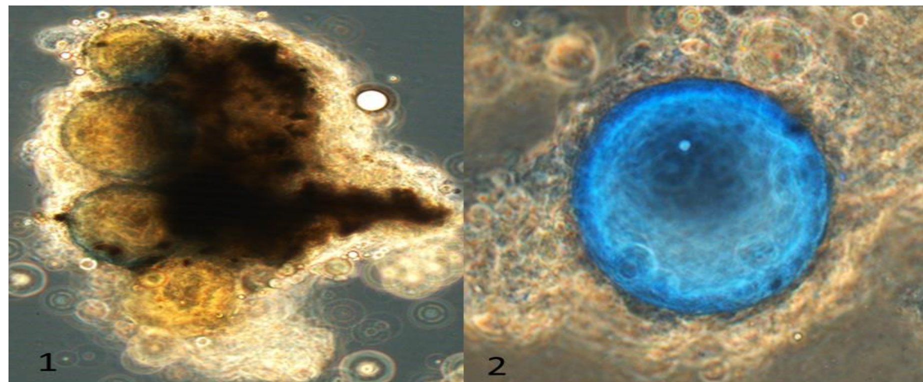

Figure 3. 13. *Melanisation ability of G. mellonella larval hemocytes.*

1- melanised beads 2- non-melanised bead

4. DISCUSSION AND CONCLUSION

BA a widely used inorganic insecticide has proved to be effective against various pests. Recently, insecticidal properties of BA on *G. mellonella* (Hyršl et al., 2007; Büyükgüzel et al., 2013), cockroaches (Cochran, 1995; Habes et al., 2006) and termites (Farid et al., 2015) have been reported. Studies reported that BA induced oxidative stress, altered protein profiles (Hyršl et al., 2007; Büyükgüzel et al., 2013) and also caused cuticular abrasion and destruction of the digestive tract wall (Cochran, 1995; Ebeling, 1995). These physiological effects may be related to BA-induced oxidative stress and suppression of the insect immune mechanisms leading to death. However, it is still unclear how BA affects hemocyte mediated immunity in insects. For the first time in this study therefore, we present the insecticidal and immunologic effects of force-fed BA on model organism *G. mellonella*.

Fifth instar *G. mellonella* larvae were exposed to various BA concentrations (78.125-10,000 ppm) via force-feeding and the larval mortality was monitored. All BA concentrations elicited significantly higher larval mortality rates compared to the control. The increasing mortality rates could be due to increased BA toxicity and also due to suppression of the immune system leaving the larvae prone to infection leading to death. Coupled to the above, the damaging of the intestinal lining in insects as reported in previous studies by Cochran, (1995) and Büyükgüzel et al., (2013) could be one other reason for the increased larval mortality rates as the larvae are starved and eventually die. The results obtained in this study showing the insecticidal effects of BA on the *G .mellonella* larvae are at par with a previous study conducted by Büyükgüzel et al., (2013) who reported increased mortality of *G. mellonella* following BA treatment. Additionally, the determined LC_{50} value (320.126 ppm) in the current study is almost similar to that reported for honey bees and very low compared to that reported for rats (U.S EPA, 2006). However, compared to our values, Büyükgüzel et al., (2013) reported significantly low LC_x values for dietary BA. The difference between the previous and present study may be associated with the mode of administration of BA to *G. mellonella* since we administered BA using gavage feeding and the previous studies administered it together with the diet. Also, the development stage of the specimen used accounts for the differences in the BA lethal concentrations since we administered BA to the 5th instar larvae which require significantly higher doses/mg body to respond to BA as opposed to the 1st instar larvae used

in the aforementioned study. Additionally, in the previous study, 20 larvae were exposed to dietary BA in the same medium at the same time, making it difficult to ascertain how much BA each larva ingested and overlooked the possibilities of cannibalism which is a common phenomenon among *G. mellonella* larvae.

We also observed that administering BA to *G. mellonella* larvae led to significant decrements in both pupal and adult weights. Previous studies reported that BA elicits intestinal wall damaging (Cochran, 1995; Ebeling, 1995). Due to the damaged alimentary canal, it is possible that the BA treated larvae could not efficiently feed or digest and utilise the consumed feeds leading to starvation and subsequent weight loss. Also, weight loss among various animal species as they evolve from one development stage to another is a common phenomenon. Development from one stage to another forinstance from pupa to adult, necesitates a high demand for energy and nutrients and consequently, the specimen rely on the energy reserves which ultimately leads to weight loss. Considering the fact that the pupal and adult stages of *G. mellonella* do not feed, there is no external/supplemetary source of such nutrient and energy requirements; whence they rely on their body reserviours which also accounts for the observed weight losses at the two development stages. On the other hand, force-fed BA prolonged the larval developmental time but shortened adult longevity and pupal developmental time. We suggest that the shortened lifespan of *G. mellonella* pupae and adults post BA treatment could be related to BA's alteration of *G. mellonella* protein profiles (Hyršl et al., 2008) and BA induced lipid peroxidation (Hyršl et al., 2007; Büyükgüzel et al., 2013). Proteins and lipids are the primary components of most hormones and enzymes which regulate/control growth and development and other physiological processes. Therefore any alteration in the composition, amount or functions of proteins and lipids directly affects endocrine system functioning and subsquently intereferes with the normal development of the pupae and adult wax moths whence the observed changes in the growth and development of *G. mellonella* larvae following BA treatment. The prolonged larval developmental time may be related to inadequate feeds and insuffiecient nutrients, lipid peroxidation and their related stress. All these may slow down the normal physiological functions of the larvae post BA treatment thus slowing down normal larval development. Our results are in agreement with the fndings reported by Hyršl et al., (2007).

Insects respond to xenobiotic infestation via the innate immune system which comprises of both humoral and cellular pathways. Cellular immunity, effected by the circulating hemocytes, involves responses like encapsulation, nodulation, melanisation and phagocytosis. Circulating hemocytes also play a role in pathogen recognition, synthesis of proteins, peptides, reactive oxygen and nitrogen species involved in immunity (Strand, 2008; Altuntaş et al., 2012; Mizerska-Dudka and Andrejko, 2012). Due to their direct and immediate involvement in insect defence, hemocytes are a good indicator of systemic toxicity due to xenobiotics. Various researchers have reported effects of several chemicals/xenobiotics using cellular immune responses as bio indicators (Pech and Strand, 2000; Lavine and Strand, 2002; Salem et al., 2014; Altuntaş et al., 2012; Er et al., 2017).

Similar to previous studies the current study identified granulocytes, plasmatocytes, sperulocytes, oenocytes and prohemocytes as the circulating hemocytes of *G. mellonella* larvae. We found that administering BA to *G. mellonella* altered the proportions of DHC. Results showed that there was an increase in the ratio of granulocytes and a decrease in the ratio of plasmatocytes at higher BA concentrations. Since plasmatocytes and granulocytes are the chief immune cells in insects (Strand, 2008), they are prone to be attracted towards xenobiotics, and, are thus, more likely to suffer greater exposure as compared to other cells (Sendi and Salehi, 2010). While both cells face immune challenges, it could be that plasmatocytes are more severely affected when compared to granulocytes following BA treatment or that *G. mellonella* larvae respond towards BA treatment by producing and releasing more granulocytes than plasmatocytes into circulation, thus the observed increased in the number of granolocytes and the decrease in the number of plasmatocytes. Since BA affects insect cuticular structure (Cochran, 1995), we can deduce that the increased ratios of spherulocytes in hemolymph at high BA concentrations could be a larval response to aid the transportation of cuticular components so as to prevent and/or counteract cuticular damage. Additionally, the decrease in the percentage of oenocytes at LC_{50} and LC_{70} may be related with phenoloxidase activity in BA treated larvae, because precursors of phenoloxidases are synthesized in the oenocytes (Lavine and Strand, 2002). It is likely that force fed BA inhibits the phenoloxidase cascade activity due to increased toxicity at higher concentrations and reduces the hemocyte numbers. For this reason, we suggest that the activity of phenoloxidases in BA treated in larval hemolymph should be investigated in future studies. We also observed a decrease in the number of prohemocytes at higher BA concentrations; this decrease was probably due to increased BA toxicity,

which induced autophagy and/or cell death in the prohemocytes. Additionally, since prohemocytes are the "stem cells" of the different circulating hemocytes as they divide and differentiate into different cells (Strand, 2008), we infer that the observed decrease in the prohemocyte ratios may be due to the continuous differentiation of these cells into granulocytes and spherulocytes whose numbers correspondingly increased in the different test groups.

Besides altering the DHC, BA elicited a drastic decrease in the THC and cell viability while increasing the ratio of apoptotic and necrotic cells in force-fed *G. mellonella* larvae. It is well known that cell death pathways (apoptosis and necrosis) post infection aid pathogen or xenobiotic elimination (Lavine and Strand, 2002; Altuntaş et al., 2012; Wu et al., 2015; Coates et al., 2018) and also helps in the elimination of immunocompetent cells (Cho and Kim, 2004). Post BA administration we observed that the number of dead cells increased with increasing BA concentrations with a 50% hemocyte mortality noted at LC_{70}. However, Tran et al., (2011) demostrated that the trypan blue dye stains both live and dead cells by penetrating through the cell membrane pores thus questioning the accuracy of this assay. It is also known that, according to the concentration and toxicity, chemical substances lead to cell death in insects via apoptosis (programmed cell death) and necrosis (unprogrammed cell death). Therefore using a technique where both cell death pathways are clearly distinguished from each other is very vital for chemical toxicity analysis. With reference to the above arguements, we conducted a secondary cell viability test using acridine orange and ethidium bromide fluorescent dyes to ascertain the mechanism by which BA causes cell death. This double staining assay revealed that BA increases both apoptotic and necrotic cell deaths with apoptosis being the dominant cell death mechanism. Caspase enzymes are well known indicators of apoptosis and interestingly, Hyršl et al., (2007) demonstrated that BA influences the presence of certain 260kDa, 45kDa and 18kDa proteins. Perhaps the presence of these proteins in the hemolymph of *G. mellonella* larvae post BA treatment as reported in a previous study by Hyršl et al., (2007) explains the increased apoptotic cell ratios reported in the current study. Other studies reported such similar weighted proteins post xenobiotic administration and suggested that they are indicators of apoptosis (Liu et al., 2005; Chowdhury et al., 2008). Furthermore, BA has been reported to increase oxidative stress in larvae of *G. mellonella* (Hyršl et al., 2008; Büyükgüzel et al., 2013). It is important to note that increased lipid peroxidation leads to increased levels of reactive oxygen species and distortion of cell membranes eventually

leading to cell damage and subsequent cell death (Banakou and Dailianis, 2010; James and Xu, 2011). We attribute the high necrotic cell ratios at the highest BA concentration to BA induced lipid perodixidation and the increased cytotoxicity that lead to the dismantling of the cellular membrane and the subsequent release of cellular contents which are typical characteristics of necrosis in cells.

We also determined that increasing concentrations of BA led to decreased numbers of total circulating hemocytes in *G. mellonella* larvae. Arguably, the observed suppression of cell viability in our study is a viable explanation for the decrement in the circulating THC in *G. mellonella* larvae. Consistent with our findings, recent studies about the effects of several insecticides on the hemocyte numbers of *G. mellonella* larvae have reported reductions in the insects' THC (Kurt and Kayış, 2015; Er et al., 2017; Coates et al., 2018; Yücel and Kayıs, 2018). In insects, mitotic indices are known to influence the number of hemocytes in circulation (Gardiner and Strand, 2000). Interestingly however, despite the observed decrease in THC, no significant change in hemocyte mitotic indices was observed following BA administration in this study. Although studies in literature reported that boron inhibited cell proliferation by inhibition of mitosis (Fail et al., 1998; Ying et al., 2011). We, therefore, suggest that the reported decrements in THC and DHC are solely due to BA induced cytotoxicity.

Cell spreading is one of the immune responses that take place during, and aids phagocytosis, encapsulation and nodulation in insects as immune cells (granulocytes and plasmatocytes) adhere to the foreign bodies. Mizerska-Dudka and Andrejko, (2012) suggested that altering the hemocyte cytoskeletal rearrangement which regulates lamellipodia and filopodia formation a prerequisite for hemocyte adhesion reduces the spreading ability of cells in *G. mellonella* larvae. In vivo activities of several compounds inhibiting hemocyte activity have been linked to reduced hemocyte spreading with cytoskeletal alterations (Vilcinskas et al., 1997; Amiri-Besheli et al., 2000). Additionally, a previous study reported that BA alters protein profiles of *G. mellonella* (Hyršl et al., 2007). Altering the profiles of various proteins including the plasmatocyte spreading peptide which regulates hemocyte binding and spreading may affect the cell spreading activity in hemocytes (Nakahara et al., 2003). It is, therefore, possible that by altering cytoskeletal rearrangement and protein profiles, BA lowers the spreading ability of *G. mellonella* larval hemocytes.

Other important observations recorded from this study were the suppression of nodulation, melanisation and encapsulation the most vital cellular immune responses of the larval hemocytes. Dean et al., (2004) reported that hemocyte attachment to microbes, microorganism agglutination due to granulocyte degranulation, plasmatocyte recruitment, hemocyte spreading and subsequent capsule and nodule formation are the steps involved in nodulation and encapsulation in insects. All these processes are controlled by both the humoral and cellular immune pathways. The mechanism by which BA suppresses hemocyte mediated immunity is not clear but we attribute it to BA induced cytotoxicity as was observed in the cytological assays. It is possible that since BA induces cell death and reduces THC, there are not sufficient hemocytes in circulation to effect nodulation and encapsulation post laminarin and Sephadex beads' injection. Additionally BA-induced reduction in the number of plasmatocytes the main capsule and nodule forming hemocytes and oenocytes which play a huge role in synthesis of phenoloxidase could be the reason for the observed suppression of hemocyte immune responses in the current study. Reduction in the numbers of these hemocytes directly affects the encapsulation, nodulation and melanisation responses of the larvae. Also, inhibiting the spreading abilities of the circulating hemocytes implies that fewer hemocytes could actually bind to encapsulation and nodulation targets. BA induced lipid peroxidation and oxidative stress (Hyršl et al., 2008; Büyükgüzel et al., 2013) could also be one other reason for the suppressed immune responses. Lipid peroxidation alters arachidonic acid metabolism and inhibits the synthesis of eicosanoids which are important precursors of cell spreading and consequential nodulation and capsule formation in insects (Tunaz et al., 2003; Schmidt et al., 2008). Eicosanoids have also been reported to regulate prophenoloxidase activation which is crucial for cellular immune reactions in the wax moth *G. mellonella* (Mandato et al., 1997). Park and Kim, (2000); Dean et al., (2002); Carton et al., (2002) and Tunaz et al., (2003) presented evidence of eicosanoids' participation in cellular immune reactions. Additionally BA increases the activity of antioxidant enzymes SOD and GST (Büyükgüzel et al., 2013) which ultimately reduce melanisation and capsule formation mechanisms in insects. Kumar et al., (2003) and Li et al., (1994) also reported suppressed encapsulation and melanisation in mosquitoes due to ascorbic acid antioxidants. Therefore, the suppression of cell mediated immune responses may be associated with the inhibition of eicosanoids synthesis and increasing oxidative stress in BA treated larval groups. Our results are in agreement with previous studies which reported similar findings for *G. mellonella* larvae exposed to

various environmental compounds (Altuntaş et al., 2012; Mizerska-Dudka and Andrejko, 2012; Er et al., 2017). We, therefore, argue that BA treatment suppresses melanisation, nodule and capsule formation in larval hemocytes.

In conclusion, data presented herein indicate that BA has insecticidal potential, significantly suppresses the hemocyte-mediated immune responses of *G. mellonella* if organisms are exposed to it at high concentrations. Also, the results obtained from this study using model insect *G. mellonella*, could provide information about the environmental risk assessment of BA for terrestrial invertabrates. There is however need for more detailed studies on how BA or other boron compounds interfere with physiological functions of living organisms especially at the cellular level to elucidate its mode of action. We recommend that the metabolism of BA be investigated and tested in natural environments if it is to be used in integrated pest management programme or in the production of insecticides against various pests such as *G. mellonella*. At the same time, the effects of BA on the nervous system of various organisms and the phenoloxidase cascade should be fully researched about to provide conscience information about its mode of action.

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