

Wright, Claire and Kavanagh, Owen
ORCID logo ORCID: <https://orcid.org/0000-0002-2599-8511> (2022)
Galleria mellonella as a Novel In Vivo Model to Screen Natural
Product-Derived Modulators of Innate Immunity. Applied Sciences,
12 (13). p. 6587.

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Galleria mellonella as a Novel In Vivo Model to Screen Natural Product-Derived Modulators of Innate Immunity

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Featured Application: This manuscript reviews the feasibility of replacing animal experimentation with an insect-larvae replacement model for the purpose of screening natural product-derived immunomodulators.

Abstract: Immunomodulators are drugs that either stimulate or suppress the immune system in response to an immunopathological disease or cancer. The majority of clinically approved immunomodulators are either chemically synthesised (e.g., dexamethasone) or protein-based (e.g., monoclonal antibodies), whose uses are limited due to toxicity issues, poor bioavailability, or prohibitive cost. Nature is an excellent source of novel compounds, as it is estimated that almost half of all licenced medicines are derived from nature or inspired by natural product (NP) structures. The clinical success of the fungal-derived immunosuppressant cyclosporin A demonstrates the potential of natural products as immunomodulators. Conventionally, the screening of NP molecules for immunomodulation is performed in small animal models; however, there is a growing impetus to replace animal models with more ethical alternatives. One novel approach is the use of *Galleria mellonella* larvae as an in vivo model of immunity. Despite lacking adaptive antigen-specific immunity, this insect possesses an innate immune system comparable to mammals. In this review, we will describe studies that have used this alternative in vivo model to assess the immunomodulating activity of synthetic and NP-derived compounds, outline the array of bioassays employed, and suggest strategies to enhance the use of this model in future research.

Keywords: *Galleria mellonella*; animal alternative; invertebrate in vivo model; immunomodulation; natural product



Citation: Wright, C.L.; Kavanagh, O. *Galleria mellonella* as a Novel In Vivo Model to Screen Natural Product-Derived Modulators of Innate Immunity. *Appl. Sci.* **2022**, *12*, 6587. <https://doi.org/10.3390/app12136587>

Academic Editor: Catarina Guerreiro Pereira

Received: 18 May 2022

Accepted: 24 June 2022

Published: 29 June 2022

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1. Introduction

Immunomodulators are a class of drugs that work by influencing the immune system positively (immunostimulant) or negatively (immunosuppressant). Immunostimulants potentiate or “boost” immunity in the immunosuppressed and are also licenced for use as vaccine adjuvants, anti-cancer agents and to prevent infections in humans and livestock. Immunosuppressants can counteract immune hypersensitivities, autoimmunity, cancer immunotherapy and organ transplant rejection. These drugs tend to be expensive and exhibit undesirable safety profiles [1], and so new candidate therapeutics are urgently required. These compounds may be synthetic in origin or be derived from natural products (NP) [2,3]. In cancer treatment, immunomodulators can be used prophylactically against cancer initiation or the inhibition of tumour growth or to facilitate the activation of tumour-specific immune response [4,5].

Natural products (NP) are any secondary metabolite molecule produced by a natural source (e.g., plant, microbial or marine) and are the origin of many drugs used in modern human and veterinary medicine [6]. These secondary metabolites help defend the host against detrimental environmental challenges, such as pathogen invasion, and consequently are a good source of antimicrobial molecules.

Humans have been using natural products for medicinal purposes since pre-historic times, with plants being used as medicines traced back 60,000 years [7,8]. The use of traditional medicine has continued across the world, and the World Health Organisation (WHO) estimates that 80% of the world's population uses traditional medicine [9]. The use of traditional medicine has become inseparable from some nation's public health systems [10] and while the success of ancient remedies may have relied on trial and error, the inclusion of natural products in modern medicine has been validated by extensive scientific research and empirical data. The use of natural products in modern medicine continues with 64% of all drugs originating from a diverse range of natural sources or their derivatives [11,12].

With approximately 350,000 known species of plants alone, the potential for the discovery of novel therapeutic compounds is vast. Examples of successful inclusion of natural products in modern medicine are numerous, including analgesics, antihypertensives, hepatoprotective, cardioprotective and many others. The discovery of artemisinin won the 2015 Nobel Prize in Physiology or Medicine. Derived from the plant *Artemisia annua*, the Artemisinin drug and its derivatives are used as a front-line drug across the world for the treatment of malaria and parasitic worm infections [13]. Natural products have also been harnessed for their anti-cancer properties. Taxol and romidepsin are both anti-tumour chemotherapies used for many different cancer types. While Taxol was derived from the Pacific Yew tree *Taxus berifolia* [14], romidepsin was obtained from the bacterium *Chromobacterium violaceum* [15], again illustrating the diverse range of reservoirs for new medicines. Cancer has represented (arguably) the largest modern stimulus for research into immunotherapies and the discovery of novel anti-cancer molecules from natural products; therefore, an ethically appropriate, robust and sustainable model for research is required.

Natural product-derived immunomodulators are gaining interest due to the success of clinically approved immunopharmacological interventions (e.g., fungal derivative cyclosporin A) and the use of herbal and fungal food supplements to boost our immune systems to prevent infectious disease [16]. The NP supplements that are marketed to the general public may not have the underpinning research and evidence to support their medical effectiveness or safety. Consequently, the National Centre for Complementary and Integrative Health (NCCIH), a branch of the U.S. National Institute of Health (NIH), launched their strategic plan which emphasises the need to comprehensively research the biological mechanisms of action as well as the safety profiles of putative NP food supplement interventions [17].

Preclinical animal studies have been an essential component in the pipeline to bring new drugs to market and especially in addressing the issues with NP immunomodulators highlighted by the NCCIH Strategic Plan. These studies are considered expensive, require a high level of maintenance, and most importantly are considered unethical [18,19]. Using the framework established by the 3Rs (replacement, reduction, and refinement) [20] there is a move within the research community to find alternative invertebrate model replacements than can reliably confirm the safety and efficacy of novel NP immunomodulators. Non-invertebrate alternatives, such as the nematode *Caenorhabditis elegans* (*C. elegans*), the fruit fly *Drosophila melanogaster*, and the zebrafish (*Danio rerio*), have been extensively used but the *Galleria mellonella* (*G. mellonella*) larvae possess several key advantages as a replacement preclinical in vivo model.

G. mellonella belongs in the *Pyrilidae* family of the order *Lepidoptera* and can be found in the egg, larval, pupal, or moth form [21]. In the larval stage, *G. mellonella* is found in beehives feeding on the honeycomb of the hive colony before pupation and finally metamorphosing into adult moths. The complete life cycle lasts for 8–12 weeks, of which 5–6 weeks are in the larval stage in which they typically grow up to 3 cm in length and between 300 and 500 g in weight [22]. This larvae stage makes an ideal in vivo model for assessing drug safety and efficacy as well as host–pathogen interactions [23].

G. mellonella are superior to other invertebrate models due to their larger size facilitating experimental manipulation (e.g., drug administration) and the ability to grow at a

wide range of temperatures (20–37 °C). In addition, they confer several key advantages over small animal models due to their short data generation time (24–48 h versus weeks), being relatively inexpensive, being not constrained by stringent ethical requirements, and in many cases, producing comparable data to animal studies [24–26]. Recently, its genome was fully sequenced, opening opportunities for genetic and epigenetic studies [27]. Larvae of the greater wax moth *G. mellonella* are a commonly used in vivo invertebrate infection model to study bacterial, fungal and nematodal infections and for assessing the efficacy of antimicrobial drugs or insecticides, which has been extensively reviewed elsewhere [28–30]. A summary of the advantages and disadvantages can be seen in Table 1.

Table 1. Advantages and disadvantages of *Galleria mellonella* as in vivo model for research activities compared to the small animal model and other invertebrate in vivo models (e.g., *Caenorhabditis elegans* (*C. elegans*), *Drosophila melanogaster* fruit fly, and zebrafish (*Danio rerio*)).

Feature	Small Animal Model (e.g., Murine)	<i>G. mellonella</i>	Other Invertebrate Models
Innate immune system	✓	✓	✓
Genome sequenced	✓	✓	✓
Fast data production time	✗	✓	✓
Standardised protocols	✓	✗	✓
Facilitates reduction of animal testing	✗	✓	✓
Adaptive immune system	✓	✗	✗
Inexpensive (comparatively)	✗	✓	✗

This systematic review describes studies that have used the invertebrate *G. mellonella* in vivo model to assess immunomodulating activity of synthetic and natural product-derived immunomodulators and suggest strategies to enhance the use of this model in future research.

2. *Galleria Mellonella* Immune System

One of the reasons for the success of invertebrates such as *G. mellonella* in scientific research is the presence of an immune system able to recognise foreign “non-self” elements. The key features of the immune system are two-fold in the ability (i) to detect and eliminate a diverse variety of microorganisms but also (ii) to discriminate “self” (host) from “non-self” (foreign) molecules. Vertebrates possess two functionally distinct but overlapping arms to their immune system, where the first line of defence is the germline-encoded innate (not antigen-specific and no memory) system and the second more powerful, adaptive (antigen-specific and memory) immunity, which is generated de novo from somatic recombination [31]. The latter adaptive immunity seems to have formed later in evolutionary terms after the invertebrates separated from vertebrates on the ancestral tree of life [32], as insects lack this more sophisticated immune response but do possess an innate immune system that is conserved among many invertebrates and higher vertebrates (e.g., mice) [33].

A review of the *G. mellonella* non-vertebrate immune system components (e.g., melanisation, nodulation and encapsulation) [21,34], its use in toxicology studies [23] and an in-depth comparison to the mammalian immune system [33,35] is beyond the scope of this review.

3. Comparable Innate Immunity

The insect immune system shares a high degree of structural and functional homology to the mammalian innate immune system, suggesting a shared ancestral precursor [33,35].

Once the physical barriers of the mammalian or larval host are breached, innate immunity mechanisms are triggered by first recognising “non-self” structurally conserved molecules on the microbe surface, called pathogen-associated molecular patterns (PAMPs), using pathogen recognition receptors (PRRs), before mounting a response. Indeed, the Toll-signalling pathway first identified in the fruit fly *Drosophila melanogaster* led to the Nobel prize-winning discovery of the family of human PRR called Toll-like receptors (TLR) which resembles the human interleukin-1 (IL-1) signalling pathway [36].

When the cellular PRR recognise and bind to the PAMP, one of three signalling pathways (Toll, IMD, and JAK/STAT) are activated, resulting in the induction of host immune responses mediated via transcriptional control of specific immune-related genes (e.g., antimicrobial peptides and opsonins) [37]. These insect signal transduction pathways are analogous to mammalian pathways with both sharing the same molecular transducers, such as MyD88, and its downstream NF- κ B master immune transcription factor [33]. Similar to its mammalian counterpart, the *G. mellonella* innate immunity consists of both cellular and humoral (soluble factors) components.

The cellular immune system of insects is mediated by a family of phagocytic cells called haemocytes, which patrol the larvae’s blood (haemolymph) or remain associated with the internal organs and body fat. There are several haemocyte sub-types, but plasmatocytes, granulocytes and oenocytoids have been shown to have a clearly defined role in larvae immune responses [34,35,38,39]. Phagocytosis is a process where certain immune cells (phagocytes) recognise “non-self” PAMPs via cell surface expressed PRR leading to the internalisation of the foreign pathogen, and its subsequent destruction by a combination of lysozymal enzymatic degradation and reactive oxygen species (ROS). Haemocytes (principally, plasmacytes and granulocytes) express mammalian-like PRR, such as apolipoprotein III (apoLp-III) [40,41] and calreticulin [42], which are crucial to produce ROS and the enhancement of engulfed pathogen lysozymal degradation [43]. Other PRR human homologues identified in *G. mellonella* are lepidopterans, haemolin, peptidoglycan recognition proteins (PGRPs), β -1,3-glucan recognition proteins (β GRPs), Gram-negative bacteria binding proteins (GNBs), and C-type lectins [37].

Furthermore, like mammalian macrophages and neutrophils, haemocytes possess the machinery essential for phagocytosis (pseudopodia) and subsequent pathogen destruction (lysosomes and NADPH oxidase system) [35]. For example, the *G. mellonella* haemocyte in vitro phagocytotic activity upon bacterial infection has been shown to increase over the first hour post-infection and then plateau after about 2 h [44]. The same study showed that haemocytes are twice as efficient at engulfing Gram-negative bacteria (*E. coli*) than Gram-positive organisms (*B. subtilis*), even though the mechanism is unclear. Another similarity found downstream of PRR activation in the superoxide-forming complex responsible for the respiratory burst. Insect haemocytes express p47 and p67 proteins, which are homologous to the neutrophil p47phox and p67phox proteins, which promote superoxide production initiated by the NAPPH oxidase complex [45].

The humoral immune responses consist of soluble effectors, such as anti-microbial peptides (AMP), opsonins and coagulation factors. AMPs are small protein molecules produced rapidly “de novo” in response to the detection of PAMPs, which possess anti-microbial killing activity [43]. *G. mellonella* has been reported to produce at least 18 inducible AMP, and these soluble effectors share many similarities with those produced by mammalian macrophages, neutrophils, and epithelial cells [33]. Some of the AMP homologues found in both insects and mammals include, but are not limited to, lysozymes and defensins [33,35]. AMP production is mediated by the Toll- and IMD-signalling pathways [46], which are analogous to the human IL-1 and tumour necrosis factor alpha (TNF α) pathways, respectively [33]. Similar to vertebrates, micro RNAs (miRNA) have also been shown to exert some transcriptional control of AMP expression [47].

A prerequisite for activation of the phagocytic process in insects and mammals is the coating of the invader cell surface with soluble molecules called opsonins [21]. Opsonins are complement-like soluble protein molecules that bind to PAMPs, resulting in increased

engulfment by phagocytes in a process called opsonisation. Similar to mammalian opsonins of the complement system, microbial infection of *G. mellonella* induces the rapid expression of soluble opsonins, such as ApoLp-III and haemolin [41,48].

Coagulation is the first line of defence in response to wounding in invertebrates and vertebrates alike, and there are many commonalities in the structure and function between the two systems. In contrast to vertebrates, insects have an open circulatory system, meaning their coagulation processes serves a very important role in their protection [33]. In response to infection, a particular haemocyte type (oenocytoids) ruptures and releases net-like extracellular nucleic acid coagulation structures similar to human neutrophil extracellular traps (NETs), which are thought to entrap the microbe and trigger the coagulation cascade [38]. The multi-functional protein apoLp-III was identified as the most likely protein to interact with the extracellular nucleic acid, and this has also been identified as the principal coagulogen [34].

4. *G. mellonella* and Natural Product Immunomodulators

In contrast to the extensive use of *G. mellonella* in elucidating host–pathogen interactions [22,23,29], the use of this insect model has not been utilised to the same extent in screening NP immunomodulatory capacity. A search within the NIH PubMed database with the combination, “natural products AND immunomodulation AND Galleria”, yields only 11 articles with a date range of 2002 to 2022. However, the search criteria can be further refined to “Galleria AND immunity” (2214 hits), “Galleria AND natural products” (86 hits), “immunomodulation AND galleria” (82 hits), and “Galleria mellonella AND immunomodulator” (61 hits). The authors were unable to find published studies on natural product immunomodulation in *G. mellonella* including the terms “marine” or “aquatic”. Before addressing the field of research for immunomodulators derived from a natural source, it is necessary to review the use of modulators from non-natural (non-NP) or synthetic sources.

5. Non-NP (Synthetic) Immunomodulators

The synthetic corticosteroid dexamethasone (DEX) is a well-characterised immunosuppressant which acts as an anti-inflammatory agent by inhibiting phospholipase A(2) (PLA₂) in the mammalian eicosanoid pathway. A 50 µM DEX treatment was shown to have suppressive effects on the *G. mellonella* immune system where it significantly ($p < 0.05$) inhibited haemocyte phagocytosis in vitro [49], suggesting that eicosanoid-dependent phagocytosis is a common pathway in invertebrates and vertebrates alike. Consequently, DEX was used as a positive control immunosuppressant when assessing a parasite–host model in larvae [50]. A water-soluble derivative (DEX-21-phosphate (DEX-21P)) was used to suppress *G. mellonella* immunity prior to bacterial challenge with the objective to create a model to mimic the immunosuppressed state of patients suffering from hospital-acquired infections [51]. Treated larvae (200 µg DEX-21P per larva ($n = 40$) for 10 min prior to bacterial challenge) rendered the insects more susceptible to bacterial infection and significantly increased the subsequent lethality in a dose-dependent manner. The DEX-21P was shown to be non-toxic in this model, and the immunosuppressant effect of this corticosteroid was confirmed by an approximate 50% reduction in vitro phagocytosis of fluorescent-labelled bacteria by haemocytes incubated with a clinically equivalent dose of DEX-21P (5 mg/mL).

The majority of published studies featuring in this review focus on immunomodulation primarily in the context of how the immune system can be harnessed to enhance the anti-microbial activity of the compound and not how it can serve as an *ipso facto* immunomodulator. For example, studies have used *G. mellonella* to primarily assess the efficacy of anti-microbial photodynamic therapy (PDT) with the secondary outcome being the examination of the treatment effect on the insect immune system. In one study, methylene blue (MB)-PDT was shown to confer higher survival rates (75%) in larvae challenged with the human periodontal bacterial pathogen *Porphyromonas gingivalis* and enhance immunity by inducing a 2.62-fold increase in the total haemocyte count (THC) [52]. In a more recent example, the safety of MB and another frequently used photosensitiser 5-aminolevulinic acid

(ALA) were firstly assessed following topical treatment of larvae ($n = 15$). ALA-mediated PDT was found to be safer than MB even at high doses (500 mM) and the anti-fungal efficacy of this photosensitiser-PDT therapy to alleviate fungal dermatological lesions was assessed in this insect model [53]. ALA-mediated PDT almost doubled the larvae lifespan following the fungal challenge (*Fonsecaea monophora*) and induced an enhanced host innate immunity typified by an almost 2-fold increase in THC compared to the untreated group. Interestingly, the PDT-treated haemocytes stimulated in vitro with either bacteria (*S. aureus*) or fungus (*C. albicans*) started to develop morphological changes compatible with enhanced phagocytic activity. The authors concluded that the anti-microbial activity was mediated indirectly via enhancing insect innate immunity.

Transition metal chelates containing phenanthroline exert anti-fungal properties through multiple mechanisms of action. Gandra et al. (2020) [54] investigated the effects of these compounds on the *G. mellonella* immune system and found that they exert at least part of their anti-fungal activity by acting as an immunostimulator. Corroborating previous human primary cell culture observations, copper chelates were found to be more toxic than other metals in *G. mellonella* and manganese compounds exhibited the optimal selectivity index. Different metal chelates ($15 \mu\text{g}/\text{larvae}$; $n = 10$) elicited significant rises ($p < 0.05$) in AMP mRNA levels in a metal-dependent manner (manganese chelates increased transferrin, IMPI and gallerimycin; silver increased galliomicin expression) and the manganese chelates also doubled THC levels (7.6×10^7 to 14.9×10^7 cells/mL) compared to the sham control.

6. NP Immunomodulators

6.1. Bacterial-Derived Probiotics

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [55]. They can consist of commensal bacteria of the healthy gut microbiome which are administered as food and, in some cases, are well-characterised immunomodulators. Over the last decade, the efficacy of *G. mellonella* as an alternative in vivo model for the preclinical assessment of putative probiotic immunomodulators has been demonstrated. *Lactobacillus rhamnosus* (*L. rhamnosus*) is an example of a probiotic which exerts its immunomodulatory effect by stimulating pro-inflammatory mammalian (murine) cytokine levels [56]. In the same study, the prophylactic inoculation of larvae ($n = 16$) with *L. rhamnosus* strain ATCC 7469 increased haemocyte number (1.5-fold versus negative control) and reduced pathogenic fungal (*C. albicans*) load following experimental challenge 24 h later ($p < 0.0001$). In another study, the supernatant from a different *L. rhamnosus* strain (ATCC9595) was shown to cause an 80% increase in the survival of larvae ($n = 16$; $p > 0.0001$ versus negative control) challenged with the same lethal fungal inoculum, which was attributed, at least in part, to a 2.1-fold increase in haemocytes [57].

The same group found that inoculation with another species belonging to the *Lactobacillus* genus, *L. paracasei* was superior to *L. rhamnosus* in eliciting a greater level of protection in the experimental candidiasis *G. mellonella* model with an almost 2-fold increase in survival rate (27% versus 14% survival 120 h post-challenge) [58]. The optimal inoculum (10^7 cells/larvae) of *L. paracasei* when administered alone demonstrated a 2-fold increase in haemocyte number and a significant increase in AMP gene expression (the anti-fungal peptides galiomicin (6.67-fold) and gallerimycin (1.68-fold)) immediately post-infection, compared to the negative control. The accumulation of evidence gathered in these studies seems to point to the probiotic fungicidal prophylactic effect being due to their ability to stimulate the host immune system rather than any direct anti-fungal effect.

G. mellonella has been shown to be an effective model to study the immunomodulating properties of probiotic food supplements and as a screen for new immunomodulatory strains and secretory components. This model could also contribute to the discovery of other genera of immunomodulating probiotics, thus extending the knowledge in the prevention of disease.

6.2. Bacterial-Derived Proteins & Extracts

The immunomodulatory activity of *Enterococcus faecalis*-derived virulence factor extracellular gelatinase (GelE) protease was assessed in *G. mellonella* with a view to determine how this bacterium can evade insect defences. GelE was found to completely abolish the cell-free haemolymph antibacterial activity of immunised larvae presumably via proteolytic degradation, as they illustrated in vitro with an insect AMP (Gm cecropin) [59]. Most interestingly, this abrogation of bactericidal activity was also observed with GelE-treated human serum, which was attributed to proteolytic degradation of the complement system, as they demonstrated the in vitro proteolysis of some key complement mediators responsible for initiating the complement cascade. These observations suggest some commonality between mammalian and insect innate immune soluble immune effectors as well as a shared immune evasion mechanism of *E. faecalis*.

Photorhabdus luminescens (*P. luminescens*) bacteria, an insect pathogen, infect and kill their host by producing toxins, thus allowing their symbiont entomopathogenic nematodes (EPN) to propagate in the cadavers. Larvae injection with purified recombinant *P. luminescens* PirA₁B₁ (and to a lesser extent PirA₂B₂) toxin elicited immunosuppression following injection into larvae ($n = 15$). This was measured by a significant inhibition ($p < 0.05$; numerical data not shown) in haemocyte in vitro phagocytosis of fluorescent bacteria (53% reduction compared to the negative control vs. PirA₂B₂ = 29% reduction), reduced pseudopod formation and a 58% decrease in THC, compared to the negative control [60,61]. The authors attributed the potent immunosuppressant activity of *P. luminescens* to PirA₁B₁ toxin-mediated disruption of the cytoskeleton and subsequent haemocyte lysis.

6.3. Fungal Metabolites

The natural product cyclosporin A (CyA) is a small lipophilic cyclic peptide produced by the entomopathogenic fungus *Tolypocladium inflatum*, which functions to promote mycotic growth by severely compromising its insect host's immune system. CyA has similar effects on the mammalian immune system by inhibiting antigen receptor signal-transduction pathways conserved in both lymphocytes and haemocytes [62] and by blocking transcription of pro-inflammatory mammalian cytokines [63]. CyA also inhibits apoptosis in human macrophage/monocyte populations and inhibits neutrophil functions in vitro. It has a long clinical history of use as a potent immunosuppressant in humans to prevent organ transplant rejection and is also used to suppress autoimmune hypersensitivities, such as rheumatoid arthritis [64]. The insecticidal effect of CyA is not observed in *G. mellonella* [65], making this an ideal model to study this immunomodulator.

In vivo treatment of *G. mellonella* larvae (7th instar; $n = 10$) with CyA elicited an increase in humoral defence systems in the haemolymph (anti-microbial lysozyme release (numerical data not published) but inhibited cellular immune processes in isolated haemocyte (in vitro phagocytic activity) in a dose-dependent manner [65]. The conflicting results can be attributed either to (i) the injection process triggering an inflammatory response [66], (ii) the difference in treatment regimens (in vivo work involved injection of CyA whereas "in vitro" studies involved ex vivo incubation with naïve haemocytes), or (iii) the different CyA doses employed (in vivo = 10–30 µg; in vitro = 100 µg). In another study, larvae (7th instar; $n =$ unpublished) inoculated with bacterial-derived LPS (*Pseudomonas aeruginosa*; 39 ng/larvae) induced high levels of humoral anti-bacterial activity which was completely abrogated by the co-administration of CyA [67]. This immunosuppressive effect of the insect humoral defences correlated with a loss in protective immunity (30%) directed against *E. coli* challenge, resulting in a higher mortality rate in CyA-treated bacterially challenged insects compared to the negative controls. The authors then compared the effect of CyA (22.5 µg/larvae) on LPS-inoculated pupae (2–3 days post-cocoon; $n =$ unpublished) in the initial phase (CyA + LPS together) and in the effector phase (LPS + CyA 18 h later) of the *G. mellonella* immune response. CyA was more effective in inhibiting LPS-induced haemolymph lysozymal activity (40% reduction) and reducing bacterial (*E. coli*) infection load (50% reduction) in the initial phase. CyA administration in the effector phase was less

effective, causing only 20% reduction in lysosomal activity and a 24% in bacterial load compared to LPS-inoculated controls [68]. Taken together, these studies demonstrate that the well-characterised immunosuppressant effects of CyA fungal derivative in humans can be replicated in *G. mellonella* immunity and entomopathogenic infection models and that these properties are used by the fungi to disable insect immunity to facilitate host–pathogen interactions. Although CyA has been shown to exert its immunosuppressant effect by inhibiting human and insect phagocytes, the very real possibility exists that the insecticidal activity of CyA in insects (postulated to be via blocking of insect detoxification P-glycoprotein efflux pumps) is via a different mechanism than described in humans (calcineurin inhibition).

CyA has a very successful clinical history but, at high doses, is associated with severe adverse effects, so there is an urgent need to find new, less toxic NP immunosuppressant. The entomopathogenic fungal-derived cyclic lipophilic amino acid metabolite myriocin (ISP-1) has demonstrated more potent immunosuppressive effects than CyA in vitro (myriocin IC₅₀ = 0.0056 vs. 0.03 µg/mL CyA) and in vivo (myriocin 10-fold > CyA in lymphoproliferation in mice) [69]. A comprehensive study was undertaken using the *G. mellonella* model to determine if the dual anti-mycotic and immunosuppressant properties of myriocin might act synergistically to provide sufficient protection against invasive fungal infections, such as candidiasis (de Melo et al., 2013 [66]). Like humans, myriocin exhibited general immunosuppressive effects following the injection (0.5 ng/larvae) of larvae (5th instar; *n* = 20) with a significant (*p* < 0.001) decrease in haemocyte number (THC approximately 30% less versus control) recorded and a concomitant suppressed humoral immune response. Myriocin treatment only had a moderate effect on immune-mediated gene expression, as of the eight genes screened (six AMP genes), only a significant response (*p* < 0.001 versus negative control) was detected in two genes (transferrin AMP downregulation and IMPI mRNA transcription upregulation). The overall effect of myriocin treatment of *G. mellonella* was a general immunosuppressant effect and a lack of protection against subsequent pathogenic *C. albicans* challenge. In other words, the dual immunosuppressant and anti-fungal activity of myriocin observed in humans did not translate to the insect model when administered during the early-stage immune phase, but more studies need to be done in the late (effector) stage infections. As an addendum to this work, poor solubility and concerns about safety profile of myriocin led to the development of the synthetic analogue fingolimod (FTY720), which is a clinically approved immunosuppressant used in treating the autoimmune disease multiple sclerosis [70].

Oosporein (Op) is a benzoquinone-derivative secondary metabolite natural product produced abundantly by some insect- and plant-pathogenic fungi, and Op-expressing fungal strains have shown promise as a potential insect pest bio-control agent. Feng et al. (2015) [71] showed that following the injection of *G. mellonella* with heat-killed fungal spores (conidia) containing oosporein (10–40 µg) resulted in a dose-dependent inhibition of invertebrate-specific humoral defence mechanisms, including the down-regulation of gene transcription of the anti-fungal AMP gallerimycin (data not shown). Mc Namara et al. (2019) [72] isolated Op from a different fungus (*Beauveria caledonica*) and observed an approximate 50% reduction in haemocyte density compared to the negative control following the injection of *G. mellonella* (instar unpublished; *n* = 10) with purified Op (0.125 µg). Both studies demonstrated that insects pre-treated with purified Op had significantly higher infection loads following fungal infection challenge than control larvae. Taken together, the *G. mellonella* model was used successfully to deduce that Op mediates fungal virulence by dampening insect host immune defences rather than directly killing the insect.

6.4. Nematodes and Nematocomplexes

Entomopathogenic nematodes (EPN) are parasitic nematodes (e.g., nematode family Steinernematidae) which form mutualistic nematocomplexes with certain bacteria (*Xenorhabdus* genus) [73]. This synergistic relationship elicits a damaging immunocompromising effect in the insect host and consequently has been exploited as potent bio-insecticides in pest control. Juvenile nematodes carry the symbiont bacterial host and

together they infect and overcome their insect host immune system which, in turn, kills the insect, allowing the nematode to reproduce in the cadaver and serve as a vector for continued spread of the bacteria. *G. mellonella* are a commonly used model to test this insecticidal activity but by inference also endorses the potential of this model to study and elucidate host–pathogen immune responses.

Infection of *G. mellonella* (last (7th) instar; $n = 30$) with the *Steinernema feltiae* (*S. feltiae*) EPN alone reduced the in vitro haemocyte phagocytic activity of bacteria (*E. coli*) by approximately 50% which was attributed to sequestering soluble haemolymph immune molecules (e.g., opsonins and PRR such as LPS-binding protein) by binding to the lipid-rich cuticle surface [44]. The role of the *Steinernema* cell surface cuticle in dampening immune responses was further demonstrated when Liu et al. (2012) [74] showed an 80% reduction in vivo phagocytosis of fluorescent beads coated with crude extract of nematode surface coat proteins; however, this property was lost when purified cuticle-derived recombinant Sg-ENOL was used. The authors demonstrated that rSg-ENOL was a virulence factor accelerating bacterial symbiont infection (*X. poinarii*) by accelerating plasmin-mediated tissue infiltration in the insect host.

In another study, a putative mechanism of immunosuppression was elucidated in response to injection of purified nematode cuticles (from *S. carpocapsae* or *H. bacteriophora*) into *G. mellonella* (early 5th instar; $n = 20$). Administration with either cuticle type caused a significant reduction in haemocyte density (approximately 50–70% versus negative control; $p < 0.05$) and a similar reduction in haemocyte in vitro phagocytosis of fluorescent bacteria [50]. Haemolymph plasma taken from the cuticle-treated insects failed to prevent infection in another bacterial model, suggesting that haemolymph soluble anti-microbial molecules (e.g., opsonins and AMPs) were depleted by EPN pre-treatment corroborating the observation by Brivio et al. (2010). This inhibitory effect was seen following treatment with the PLA₂-inhibitor DEX (see previous section) and was blocked by the eicosanoid substrate arachidonic acid, which strongly implicates the eicosanoid biosynthesis pathway in this broad-spectrum immunosuppression. The immunosuppressive effect of extracts from the nematode *S. carpocapsae* outer layer proteins (SoP) and its symbiont bacteria *X. nematophila* (XoP) cell wall were evaluated in *G. mellonella* (late-stage caterpillars; $n =$ unpublished). The study determined that prior treatment of larvae with bacterial extracts (XoP) alone was responsible for the marked inhibition of in vivo haemocyte phagocytosis of fluorescent bacteria (*S. aureus*) ($p < 0.0001$), resulting in a halving of the phagocytosis ability [75].

Use of the *G. mellonella* in vivo model demonstrated that the EPN were rapidly acting, broad spectrum immunosuppressive agents in insects whose activity is mediated by the eicosanoid pathway common to vertebrates and invertebrates alike. The nematodal cuticle plays a role in immune evasion by sequestering soluble immune molecules (e.g., opsonins and AMPs) from the haemolymph, and both the nematode and symbiont bacteria directly compromise haemocyte phagocytic activity. Indeed, in a recent study, EPN and other bacterial feeding nematodes were found to ingest and destroy haemocytes [76].

6.5. Plant-Derived Extracts and Antioxidants

Iqbal et al. (2022) investigated the antimicrobial and immunomodulatory properties of extracts from *Sida cordifolia* plant with the objective of finding novel antibiotics. A polysaccharide-enriched fraction extracted from the root (SCAF5) was identified using a combination of screening with a murine macrophage cell line (RAW267.4) and ex vivo lymphocyte assays. *G. mellonella* (last (7th) instar; $n = 10$) administered SCAF5 (10 µg/larvae) elicited a statistically significant 2.6-fold increase in haemocyte count (THC; $p < 0.001$ compared to the negative control) 24 h post-administration, which was comparable to the LPS positive control. Interestingly, SCAF5-treated larvae had a 98% reduction in bacterial load when challenged with an antibiotic-resistant strain (methicillin-resistant *S. aureus*). The use of *G. mellonella* not only confirmed the immunostimulant effects of the NP extract in the murine model, but was used to demonstrate its potential as an antibiotic alternative.

Other putative plant-derived immunomodulators, including resveratrol, curcumin and epigallocatechin (EGCG), have been administered to *G. mellonella* but only to assess drug toxicity or to simulate infection models, and no immune responses were recorded [77–79].

7. Available Techniques to Evaluate Natural Product-Derived Immunomodulators in *Galleria mellonella* Insect Model of Innate Immunity

The *G. mellonella* invertebrate model is supported by a surprising array of investigative assays, varying from the most basic (e.g., haemocyte counts) to the more complex quantitative chemical and molecular approaches. For example, the mammalian innate immune system can effectively destroy phagocytosed pathogens by generating reactive oxygen species (ROS, e.g., the superoxide radical O_2^-) via a process called oxidative stress. Similarly, insect haemocytes phagocytosing pathogens express the same defensive mechanism to generate oxidative stress (e.g., NAD(P)H oxidase complex) to destroy the microbe and the enzyme systems to control it (e.g., catalase and superoxide dismutase).

Markers of this defence system include the oxidative modification of the DNA (deoxyguanosine converted to 8-hydroxy-2-deoxyguanosine (8-OHdG)), lipid peroxidation (generation of malondialdehyde (MDA)) (see Table 2). Kazek et al. (2020) expanded the potential of the *G. mellonella* model of immunity by quantifying this oxidative stress in terms of changes in 8-OHdG and MDA levels, following fungal infection [80]. The same group also demonstrated that the destruction of haemocytes was caused by two forms of programmed cell death (autophagy and apoptosis) and infection increased eicosanoid (prostaglandin) biosynthesis [81], although the significance of the latter feature needs further elucidation. The author's focus was in defining the insecticidal mechanism of this fungal biological control agent, but these assays have value in studies evaluating ROS-generating or immune cell damaging mechanisms when screening for NP immunomodulatory compounds.

Table 2. Available techniques to evaluate natural product-derived immunomodulators in *Galleria mellonella* insect model of innate immunity.

Immunological Determinant	Assay Characteristics	Comments	Reference
Total haemocyte count (THC)	Haemocytometer count	Easiest method to measure larvae immune response	[54,57,58,66]
Haemocyte density	Haemocytometer count per μ L haemolymph		[50,72]
Haemocyte viability	MTT colorimetric assay	Measures cell viability through formation of formazan crystals	[75]
Transcriptomics	RT-PCR	Measures expression of AMP or Immunity genes	[48,54,58,66,71,81,82]
Proteomics	Label-free quantitation (LC-MS or MS-MS)	Un-targeted analysis of complete haemolymph protein profile	[82–84]
	2D electrophoresis	Protein identification by electrophoretic pattern	[44,48,67]
Haemolymph enzyme activity	Colorimetric, zone-of-clearance,	Measures insect enzymes involved in immunity (e.g., lysozyme and superoxide dismutase)	[66,85]
	Bacterial (<i>Micrococcus luteus</i>) agar diffusion assay	Haemolymph lysozyme	[65,67]
Phagocytosis assay (in vitro)	Fluorescent microscopy	Measure phagocytosis of fluorescent-labelled bacteria.	[50,51,74,75]
Phagocytosis assay (in vivo)	Fluorescent microscopy	Inoculate with fluorescent-labelled bacteria or beads, examine haemolymph by fluorescent microscopy	[39]
Macrophage activation (RNS)	Greiss Assay	Measures reactive nitrogen species (RNS) burst	[26]

Table 2. Cont.

Immunological Determinant	Assay Characteristics	Comments	Reference
Macrophage activation (ROS)	- DNA Damage ELISA (8-OHdG) - Lipid Peroxidation assay (MDA) - Catalase Fluorometric resorufin assay - Cu/Zn-Superoxide Dismutase (SOD) ELISA	Measures aspects of reactive oxygen species (ROS) burst and regulatory enzymes (catalase and SOD) (MDA is biomarker of oxidative stress and 8-OHdG is a biomarker of DNA oxidative damage)	[80]
Coagulation assay (in vitro)	Fluorescent microscopy	Haemolymph incubated with fluorescent-labelled bacteria	[38]
Cytoskeleton morphisms (Phagocytosis mechanism and pseudopod formation)	Fluorescent microscopy	Examines haemocyte cytoskeleton with fluorescent-labelled phalloidin	[60,80]
Autophagy	- LC3 immunofluorescence - cationic amphiphilic tracer (CAT) assay	- Measures the autophagosomal marker LC3 in haemocytes - CAT dye selectively labels autophagic vacuoles	[80]
Apoptosis	- Caspase measurement - Annexin V measurement by flow cytometry	Caspase is measured using carboxyfluorescein derivative of VAD-FMK and annexin V is a marker of apoptosis	[81]
Eicosanoid biosynthesis	Measurement of eicosanoids (e.g., prostaglandins) by ELISA		[81]
miRNA expression levels	Using an insect-specific miRNA microarray	Employs >2000 mRNA probes	[66]
Epigenetic gene regulation	Measures gene-associated methylation and global histone acetylation by Illumina transcriptome sequencing	Extraction of genomic DNA, RNA and histones.	[86]

Abbreviations; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); RT-PCR (reverse transcription polymerase chain reaction); LC-MS (liquid chromatography–mass spectrometry); MS-MS (tandem mass spectrometry); 8-OHdG (8-hydroxy-2-deoxyguanosine); MDA (malondialdehyde); VAD-FMK (valylalanylaspatic acid fluoromethyl ketone).

8. Concluding Remarks

The innate insect immune system shares many similarities with its mammalian counterpart, including PAMP-sensing PPRs, signal transduction pathways, immune gene expression transcription regulation, phagocytic mechanisms and the production of humoral immune effectors, such as AMP and ROS. The majority of studies reviewed herein examined *G. mellonella* immune responses as a secondary consideration to their focus on developing anti-microbial infection models or screening for novel bio-insecticides. However, the work with corticosteroids, probiotics and cyclosporin demonstrates the emerging recognition and feasibility of *G. mellonella* as a novel pre-clinical approach to discover and characterise new immunomodulating compounds. *G. mellonella* has attributes that lend it to the preliminary research of drug effects on the innate immune system with an extensive array of data outputs (see Table 2). It is compatible with the concept of the 3Rs in that it is a suitable replacement for animals in screening experiments for NP immunomodulators.

Despite *G. mellonella* lacking antigen-specific, memory-based adaptive immunity, there is emerging evidence that larvae immune responses are greatly enhanced in response to re-infection. Furthermore, this immune “memory” is epigenetically inherited by subsequent insect generations [87]. Due to the specificity and ability to form “memory” this phenomenon, termed immune priming, has been suggested as being analogous to the mammalian adaptive immune system. However recent studies suggest both systems are functionally distinct [83] and that immune priming more closely resembles the phenomenon of “trained immunity” of vertebrate innate cells [88] rather than adaptive immunity *per se*.

One principal limitation was the lack of whole genomic sequence data, which is essential to carry out inter-species homology studies; however, this was alleviated by the recent sequencing of the entire *G. mellonella* genome [27]. Another limitation that has yet to

be addressed is the lack of standardised larval growth conditions amongst suppliers [21], as differences in light source, temperature and diet could affect laboratory experimentation and variability of results [89,90]. Another important feature is the observation that the haemocyte composition fluctuates during the larvae life cycle, highlighting the importance of the standardisation of the larvae stage in experiments to ensure the reproducibility of results. The mature or late larval stage (6th or 7th instar weighing 250–350 mg) lacking cuticle pigmentation is recommended, where plasmacytes and granulocytes predominate and a larvae number of 10–20 is recommended for each experimental treatment [21,60].

Animal welfare and subsequent ethical considerations in biomedical research are constantly under revision. In April 2022, new legislation come into UK law with the Animal Welfare (Sentience) Act 2022, which extended new regulations to protect decapods (e.g., crabs) and cephalopods (e.g., octopus) [91]. The need for animal welfare and protection is evident to all those who work in research that necessitates animal models, but the restrictions mean that robust, reliable, and reproducible alternatives, such as *G. mellonella*, must be established.

In light of the COVID-19 pandemic, public interest in dietary NP immunomodulating supplements to “boost” immune protection to disease has increased, meaning this larval model will be integral to ensure compliance with the NCCIH Strategic Plan [17] to verify the safety and efficacy profiles of these home-administered NP interventions. Our review highlights the feasibility of using *G. mellonella* as a novel in vivo approach to replace pre-clinical animal studies for the screening and characterisation of NP immunomodulators.

Author Contributions: Literature search for the manuscript was performed by C.L.W. and O.K.; the manuscript content was organised by O.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors have no conflict of interest.

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