Modeliranje utjecaja ekstrakta ličinke Hermetia illucens na cijeljenje rana in vitro

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In vitro modelling of the infuence of extracts from *Hermetia illucens* larvae on wound healing

Graduation thesis

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This Graduation thesis was conducted in the Laboratory for Zoology and Developmental Biology at the Department of Biology, Technische Universität Dresden under the leadership of Prof. Dr. Herwig O. Gutzeit. This Graduation thesis is submitted to evaluation to the Department of Biology at Faculty of Science, University of Zagreb in order to acquire the Master of Molecular Biology title.

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Modeliranje utjecaja ekstrakata ličinke *Hermetia illucens* na cijeljenje rana *in vitro*

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Ličinke crne vojničke muhe (Black soldier fly), *Hermetia illucens,* su strvinari koji žive u izrazito neprijatnom okolišu, poput gnojiva i komposta, naseljenima bakterijama i gljivicama. Strvine uginulih životinja i trule biljke mogu se razgraditi ličinkama crne vojničke muhe. Ovakve biološke karakteristike upućuju na to da su ličinke vrste *Hermetia illucens* bogate antimikrobnim peptidima (AMP) i ostalim tvarima koje posjeduju aktivnost za borbu protiv rezistentnih bakterijskih sojeva.
Cijeljenje rana je složen proces koji se sastoji od četiri preklapajuće ali strogo definirane faze: hemostaze, upalne faze, faze cijeljenja (proliferacije) i faze remodelacije (maturacije i epitelizacije). Sve faze procesa cijeljenja rana ovisne su o lučenju različitih staničnih spojeva poput: faktora rasta, kemokina, citokina, proteinaza, proteina izvanstaničnog matriksa. *In vitro* proces cijeljenja rana može se pratiti uporabom ovih različitih staničnih spojeva kao molekularnih markera. Fokus ovog istraživanja je obogaćivanje ekstrakata ličinki vrste *Hermetia illucens* u svrhu dobivanja pročišćenih AMP-ova i ostalih antibiotskih spojeva te *in vitro* praćenje utjecaja ekstrakata iz ličinki vrste *H. illucens* na rast bakterija te na citotoksičnost ljudskih stanica. Ekstrakti su pokazali visok kapacitet inhibicije bakterijskog rasta, posebice vrste *Pseudomonas fluorescens.* Štoviše, većina esktrakata koji su inhibirali bakterijski rast nisu se pokazali citotoksičnim za ljudske stanice.

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Ključne riječi: Crna vojnička muha (*Hermetia illucens),* ekstrakti ličinke, HepG2- ljudska stanična linija raka jetre, normalni ljudski fibroblasti kože, antimikrobini efekt, cijeljenje rana

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In vitro modelling of the infuence of extracts from *Hermetia illucens* larvae on wound healing

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Larvae of black soldier fly, *Hermetia illucens*, are scavengers who live in extremely unpleasant and harsh environments, such as manure and compost, populated by bacteria and fungi. Carcasses of dead animals and rotting plants could be degraded by larvae of black soldier fly. These biological characteristics suggest that the larvae of black soldier fly are rich in antimicrobial peptides (AMPs) and other substances which possess the activity to fight against resistant bacterial strains.

Wound healing is a complex process consisting of four overlapping but strictly defined phases: hemostasis, inflammatory phase, proliferative phase and the remodelling phase (maturation and epithelialization). All phases of wound healing are dependent on the secretion of a variety of cellular compounds such as growth factors, chemokines, cytokines, proteinases, and extracellular matrix proteins. *In vitro* process of wound healing could be monitored using these different cellular compounds as molecular markers. Focus of this research is enrichment of extracts from *H. illucens* larvae in order to obtain purified AMPs and other antibiotic compounds and *in vitro* monitoring of the impact of extracts from *H. illucens* larvae on bacterial growth and cytotoxicity of human cells. Extracts showed high capacity of inhibition of bacterial growth, especially species *Pseudomonas fluorescens*. Moreover, majority of extracts which inhibited bacterial growth did not show citotoxic effect on human cells.

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Key words: Black soldier fly (*Hermetia illucens*), larval extracts, Hep G2-human liver cancer cell line, Normal human dermal fibroblasts (NHDF), antimicrobial effect, wound healing

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Abbreviations

AMP – Antimicrobial peptide

ACN – Acetonitrile

Ang-1 – Angiopoietin-1

aSMA – a-smooth muscle actin

B. subtilis – Bacillus subtilis

C. dificile – Clostridium difficile

cDNA – complementary DNA

$CHCl_3$ – Chloroform

DEPC – Diethyl pyrocarbonate

DMEM – Dulbecco's Modified Eagle's medium

DMSO – Dimethyl sulfoxide

E. coli – Escherichia coli

ECM – Extracellular matrix

EDTA – Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

EtOAc – Ethyl acetate

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

FN – Fibronectin

GF – Growth factor

H. illucens – Hermetia illucens

H_2O – Water

HCl – Hydrochloric acid

HepG2 – name of human hepatocellular carcinoma cell line

HPLC – High-performance liquid chromatography

IL-1 – Interleukin-1

LB medium – Luria-Bertani medium

LPS – Lipopolysaccharide

LTA – Lipoteichoic acid

M. luteus - Micrococcus luteus

MDT – Maggot debridement therapy

MF – Myofibroblast

MMP – Matrix metalloprotein

MRSA – methicillin-resistant Staphylococcus aureus

MSSA – methicillin-sensitive Staphylococcus aureus

NaOH – Sodium hydroxide

NHDF – Normal Human Dermal Fibroblasts

OD – Optical density

P. fluorescens – Pseudomonas fluorescens

PCR – Polymerase chain reaction

PDGF – Platelet-derived growth factor

PMN – Polymorphonuclear

RPMI medium – Roswell Park Memorial Institute medium

RT-PCR – Reverse transcription polymerase chain reaction

TGF-β –Transforming growth factor beta

TGF-β1 –Transforming growth factor beta 1

VEGF – Vascular endothelial growth factor

1. Introduction

1. 1. Wound healing

Wound healing is a complex and well-orchestrated process, where numerous factors are activated or inhibited in a sequence of steps. It is the process of restoration in injured skin tissue and one of the most important therapeutic targets. Furthermore, it is an evolutionarily conserved, multi-cellular, multi-molecular process involving co-ordinated inter-play between complex signaling networks.

Immediately after the infliction of damage, the repair of wound starts (Kasuya & Tokura 2014). A wound comprises a break in skin epithelial continuity and is characterized by disruption of structure and function of underlying tissues. After injury, skin integrity must be restored promptly in order to re-establish homeostatic mechanisms, prevent infection and minimize fluid loss. This is achieved through a complex biological process where multiple parallel and interrelated pathways are activated and synchronized to induce wound repair. Once complete, they must be shut down in a precise order to prevent exaggerated or delayed responses (Greaves et al. 2013).

As a normal biological process in the human body, wound healing is achieved through four precisely and highly programmed overlapping phases: hemostasis, inflammatory phase, proliferative phase, and tissue remodeling phase or restoration (Fig 1.). These phases and their bio-physiological functions must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity. There are many factors that can affect wound healing which interfere with one or more phases in this process, thus causing improper or impaired tissue repair.

Wounds that exhibit impaired healing, including delayed acute wounds and chronic wounds, generally have failed to progress through the normal stages of healing. Such wounds are characterized by a prolonged inflammation, persistent infections, formation of drug-resistant microbial biofilms and the inability of dermal and/or epidermal cells to respond to regenerative stimuli (Fromm-Dornieden & Koenen 2013). Most chronic wounds are ulcers that are associated with ischemia, diabetes mellitus, venous stasis disease, or pressure (Guo & DiPetro 2010; Mathieu et al. 2006).

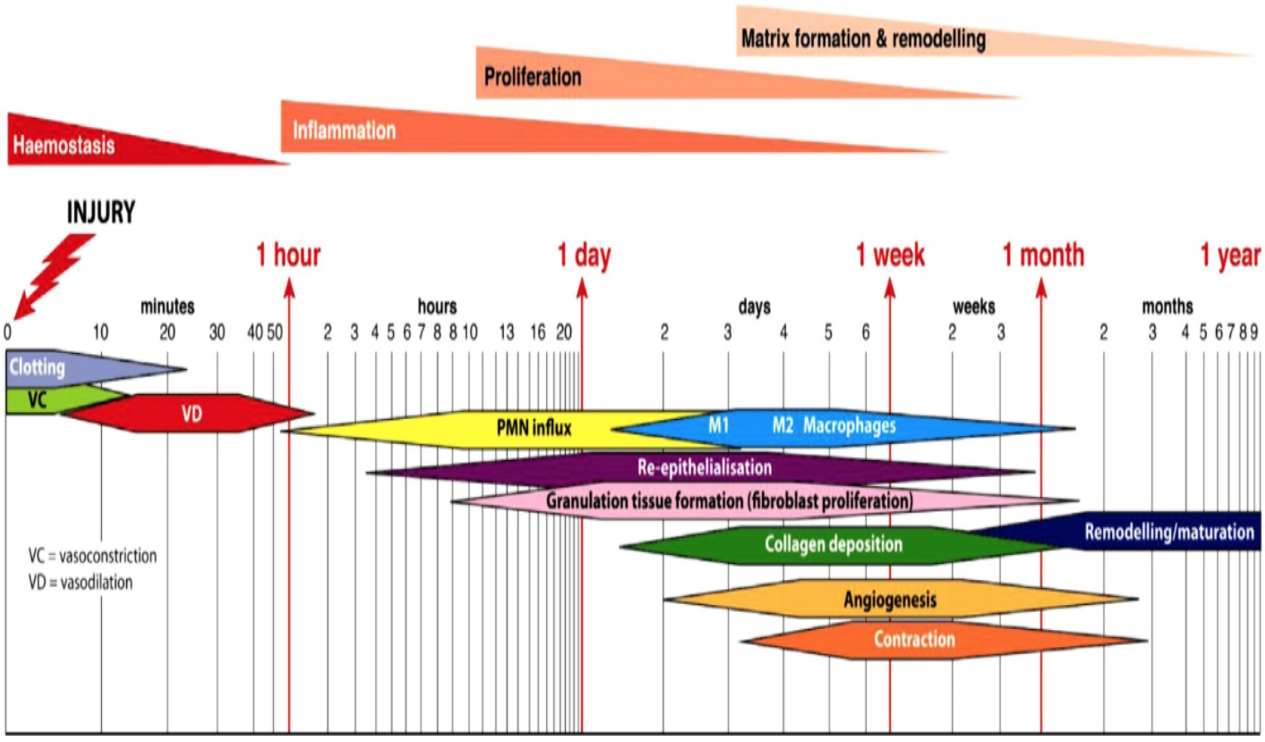


Figure 1. Overview of overlapping 4-phase model of acute wound healing and its timeframe. Phase 1 – hemostasis. After cutaneous injury vasoconstriction, clotting cascades and platelets act together to prevent prolonged hemorrhage. Once fibrin clot forms there is vasodilation enabling PMN extravasation and migration to the wound site; phase 2 – inflammation. An initial influx of neutrophils is later replaced by macrophages which have an early inflammatory (M1) and later reparative (M2) phenotype. There is phagocytosis of bacteria and wound debris with concurrent secretion of multiple growth factors, chemokines and cytokines which drive fibroblast and endothelial cell recruitment to the wound bed; phase 3 – cellular proliferation. Migratory endothelial cells and fibroblasts proliferate resulting in ECM deposition, angiogenesis and granulation tissue formation. ECM forms a scaffold for further cellular influx and is remodelled by a variety of enzymes so that its composition changes throughout wound healing; phase 4 – remodelling. Wound maturation occurs with continued ECM remodelling into a predominantly collagenous structure, fibroblast differentiation into myofibroblasts, wound contraction and gradually reducing cellularity with eventual scar formation. Re-epithelialisation occurs concurrently beginning within hours of injury until there is restoration of epithelial continuity. PMN, polymorphonuclear cells; ECM, extracellular matrix. Source: Greaves et al. 2013.

1.1.1. Hemostasis

Hemostasis is an essential step for the initiation and continuation of the wound healing process and is characterized by stopping of a flow of blood in the healing wound. It is well established that the major stages of hemostasis are vasoconstriction, platelet degranulation and aggregation, and fibrin

deposition (Pakyari et al. 2013). Injury to damaged blood vessels stimulates the clotting cascade resulting in proteolytic cleavage of fibrinogen by the enzyme thrombin and formation of an insoluble fibrin clot (Diegelmann & Evans 2004). As the barrier function of the skin is compromised at the wound site, the clot holds damaged tissues together and functions as a temporary shield. Structurally, it provides a provisional matrix for cell migration (Clark 2001). The first subset of cells that enter the injury site are platelets. Platelets release many different growth factors and inflammatory cytokines, such as platelet-derived growth factor (PDGF), TGF-β1, epidermal growth factor (EGF), and fibroblast growth factor (FGF), all of which promote the inflammatory phase and some of which function as chemoattractants.

1.1.2. Inflammatory phase

This phase is characterized by increased capillary permeability and cell migration into the wound site. Vasodilation that follows the early vasoconstriction is the result of capillary leaks that are mediated by histamine, leukotrienes, and prostaglandins (Richardson 2004). Neutrophils are among the first cells infiltrating the wound site, followed by monocytes and lymphocytes (Zaja-Milatovic & Richmond 2008). These proinflammatory cells are necessary to remove debris and kill bacteria through phagocytosis and free radical production. Neutrophils have innate antimicrobial functions, in addition to releasing proteases to eliminate the denatured ECM components. A critical function of neutrophils is the clearance of invading microbes and cellular debris in the wound area. Leukocyte migration into the injured tissue is mediated by factors such as TGF-β, PDGF, IL-1 and many other cytokines and growth factors. Monocytes transform into macrophages as they enter the wounded area under the influence of TGF-β, ECM, complement particles and serum factors. Macrophages clear the area of debris and microbes and release many important cytokines and factors, such as FGF, TGF-β, PDGF, and EGF, which in turn initiate the formation of granulation tissue (Behm et al. 2012). Macrophages also act as antigen-presenting cells (Rennekampff et al. 2000). Macrophages even stimulate angiogenesis and fibroplasia and ECM production, thereby linking between the inflammatory and proliferation phases. However, it is notable that excess inflammation may delay the process of wound healing, because prolonged edema disturbs progression to the next step, and pro-inflammatory cytokines and toxic free radicals are harmful for skin constituents (Kasuya & Tokura 2014).

As inflammatory phase declines, fewer inflammatory factors are secreted, existing ones are broken

down, and remaining neutrophils and macrophages are reduced at the wound site. These changes indicate that the inflammatory phase is ending and the proliferative phase is underway (Opalenik & Davidson 2005). *In vitro* evidence, obtained using the dermal equivalent model, suggests that the presence of macrophages actually delays wound contraction and thus the disappearance of macrophages from the wound may be essential for subsequent phases to occur (Akasaka et al. 2004).

Because inflammation plays roles in fighting infection, clearing debris and inducing the proliferation phase, it is a necessary part of healing. However, inflammation can lead to tissue damage if it lasts too long (Bayat et al. 2003). Thus the reduction of inflammation is frequently a goal in therapeutic settings. Inflammation lasts as long as there is debris in the wound. Thus, if the individual's immune system is compromised and is unable to clear the debris from the wound and/or if excessive detritus, devitalized tissue or microbial biofilm is present in the wound, these factors may cause a prolonged inflammatory phase and prevent the wound from properly commencing the proliferation phase of healing. This can lead to a chronic wound.

1.1.3. Proliferative phase

Cellular proliferation represents the third phase of the 4-stage acute wound healing model. Proliferation of cellular and structural components is triggered by factors secreted during the preceding inflammatory phase. The main and leading events that happen during this phase include re-epithelialization, angiogenesis, and fibroplasia.

It begins 3–4 days after injury and continues for 2–4 weeks. During this time there is fibroblast and endothelial cell proliferation, phenotypic alteration and migration as well as ECM deposition, and granulation tissue formation (Singer & Clark 1999; Bauer et al. 2005). Fibroblast derived ECM provides support for further cellular influx, adhesion, and differentiation (Gurtner et al. 2008; Clark 2001). After its formation, ECM undergoes continuous synthesis and remodelling, reaching a steady state 21 days after wounding (Diegelmann & Evans 2004). Initially disorganized fibrin is later remodeled with hyaluronan, proteoglycans and fibronectin (FN) before a predominantly collagenous final structure (mostly types I and III) is formed (Clark 2001; Welch et al. 1990; Clark et al. 1995). Remodelling is achieved by specific matrix metalloproteins (MMPs) influenced by

transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), interleukin-1 (IL-1) and epidermal growth factor (EGF), which are tightly regulated. Blood vessel sprouts invade the

wound concurrent with fibroblast in-growth. Neovascularization occurs in response to pro-angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiogenin, angiotropin, and angiopoietin-1 (Ang-1) released by infiltrating macrophages and keratinocytes (Folkman & D'Amore 1996; Iruela-Arispe & Dvorak 1997; Risau 1997). The dense population of fibroblasts, macrophages and neovasculature, embedded within a loose matrix of collagen, FN and hyaluronic acid is termed granulation tissue. It is a response to abundant growth factors (GFs) and cytokines found in the wound from 4 days post-injury onwards (Diegelmann & Evans 2004; Pilcher et al. 1999; Parks 1999). In later stages of the proliferative phase, fibroblasts assume the myofibroblast (MF) phenotype which contains a-smooth muscle actin (aSMA) (Gurtner et al. 2008; Opalenik & Davidson 2005). During fibroplasia, fibroblasts migrate, proliferate, and produce ECM components, which result in formation of granulation tissue within the wound site. Fibroblast migration is mediated by TGF-β1 and PDGF in this phase.

Granulation tissue formation and fibroblast migration enable wound contraction from day 6 post injury but is GF- dependent relying heavily on PDGF and TGF-β (Macri & Clark 2009; Greaves et al 2013). Following robust proliferation and ECM synthesis, wound healing enters the final remodelling phase, which can last for years. (Gosain & DiPietro 2004; Campos et al. 2008).

1.1.4. Remodelling phase

As the final phase of wound healing, the remodelling phase is responsible for the development of new epithelium and final scar tissue formation. Synthesis of the extracellular matrix in the proliferative and remodelling phases is initiated contemporarily with granulation tissue development. This phase may last up to 1 or 2 years, or sometimes for an even more prolonged period of time (Pilling et al. 2006; Ignos & Massague 1986). The remodelling of an acute wound is tightly controlled by regulatory mechanisms with the aim of maintaining a delicate balance between degradation and synthesis, leading to normal healing. Along with intracellular matrix maturation, collagen bundles increase in diameter and hyaluronic acid and fibronectin are degraded (Garcia-de-Alba et al. 2010; Tomasek et al. 2009). The tensile strength of the wound increases progressively in parallel with collagen collection (Tomasek et al. 2009; Hinz et al. 2001). Collagen fibers may regain approximately 80% of the original strength compared with unwounded tissue. The acquired final strength depends on the localization of the repair and its duration, but the original strength of the

tissue can never be regained (Singer & Clark 1999; Hinz et al. 2001).

Synthesis and breakdown of collagen as well as extracellular matrix remodelling take place continuously and both tend to equilibrate to a steady state about 3 weeks after injury (Hartlapp et al. 2001; Hinz et al. 2001). Matrix metalloproteinase enzymes, produced by neutrophils, macrophages, and fibroblasts in the wound, are responsible for the degradation of collagen. Their activity is tightly regulated and synchronized by inhibitory factors. Gradually, the activity of tissue inhibitors of metalloproteinases increases, culminating in a drop in activity of metalloproteinase enzymes, thereby promoting new matrix accumulation (Iruela-Arispe et al. 1997; Hinz et al. 2001; Namiki et al. 1995).

Although the initial deposition of collagen bundles is highly disorganized, the new collagen matrix becomes more oriented and cross-linked over time. Its subsequent organization is achieved during the final stages of the remodelling phase, to a greater extent by the wound contraction that has already begun in the proliferative phase. The underlying connective tissue shrinks in size and brings the wound margins closer together, owing to fibroblast interactions with the extracellular matrix. The process is regulated by a number of factors, with PDGF, TGF-β, and FGF being the most important (Hinz et al. 2001; Monaco & Lawrence 2003). As the wound heals, the density of fibroblasts and macrophages is further reduced by apoptosis (Ehrlich & Rajaratnam 1990; Ohtani et al. 2007). With time, the growth of capillaries stops, blood flow to the area declines and metabolic activity at the wound site decreases (Hinz et al. 2001; Stavri et al. 1995). The end result is a fully matured scar with a decreased number of cells and blood vessels and a high tensile strength.

1.2 Larvae

Maggot (larval) therapy is highly successful in cleansing infected and necrotic wounds (Sherman et al. 2000) and has been practiced since the 1930s when William Baer's observations confirmed the reduction of bacterial load at the wound site by the use of maggots (Baer 1931). The main actions of fly maggots on wounds may be categorized into three modes: debridement, disinfection and bacterial death, and stimulation of tissue granulation and repair. However, the use of maggot therapy declined as antibiotics were developed and surgical techniques improved. Nevertheless, new strains of pathogenic fungi and bacteria developed primarily in response to overdose usage of antibiotics and thus were found commonly in hospitals and the wider community (Kerridge et al. 2005), renewing the interest in maggot therapy in the search for effective methods to treat non-healing wounds and control the evolution of resistance (Bexfield et al. 2004). Recently, several

studies reporting antimicrobial activity of the hemolymph and maggot extract, either in the whole body or in the excreta/secreta, have provided impetus for the development of alternative antimicrobial products into therapeutically valuable anti-infective agents. It is well known that insects have a well-developed innate immune system, subdivided into cellular and humoral defense responses, the latter of which involve production of antimicrobial peptides (AMPs) that are synthesized in the fat body and subsequently secreted into the hemolymph (Bulet et al.1999; Hoffmann & Reichhart 2002).

1.2.1 *Hermetia illucens* larvae

The larvae of the black soldier fly, *Hermetia illucens,* are scavengers that can live in extremely harsh environments, such as manures and compost, inhabited by bacteria and fungi. The torpedo-shaped larvae can grow up to 27 mm in length and about 6 mm in width (Diclaro & Kaufman 2009; Tomberlin et al. 2001).

Animal wastes and rotten plants can be decomposed and recycled by larvae. Additionally, the black soldier fly is sometimes found in carrion. These biological characteristics suggest that the soldier fly may be rich in different types of AMPs and other substances possessing activity against drug-resistant "superbugs". Recently, the antibacterial effect of *H. illucens* larval extract against Gram-negative bacteria was evaluated (Choi et al. 2012). However, insects infected with the microorganism could contain more biologically and pharmacologically active chemicals and evidence has been reported that antibacterial activity of the extract from induced larvae was much stronger than that of native larvae (Hou et al. 2007). The aim of the present study is to get antimicrobial substances from *H. llucens* larvae and to test antibacterial activity of its extracts against a range of Gram-positive and Gram-negative bacteria.

1.2.2 Antimicrobial substances in larvae

Antimicrobial peptides have been shown to be an crucial component of the insect's innate defense system, giving powerful resistance to a broad range of harmful parasites and pathogens (Boman. 1995; 2000). The majority of those identified have been shown to be inducible, low molecular weight, cationic peptides (Bulet et al. 1999). Seven different families of antimicrobial peptides have been described in insects: attacins, cecropins, defensin, diptericins, drosocin, drosomycins and

metchnikowin. Attacins, diptericins and drosocin are active against Gram-negative, while defensin against Gram-positive bacteria. Drosomycins are antifungal peptides, whereas cecropins and metchnikowin exert antibacterial as well as antifungal activity (Hoffmann and Reichhart. 2002; Hultmark. 2003; Tzou et al. 2002; Uvell and Engström. 2007). Among them are members of linear α-helical peptides (cecropins and moricin-like peptides), cysteine-stabilized peptides (defensins), proline-rich peptides, and glycine-rich peptides (gloverin). An important role in insect humoral immune response against pathogens is also played by lysozyme, constitutively present in the hemolymph (Hultmark. 1996; Yu et al. 2002). Moreover, it was shown that bacterial cells became more susceptible to antimicrobial peptides in the presence of lysozyme, and such synergistic action of lysozyme with cecropin B, attacin and defensin, against *Escherichia coli* was demonstrated (Chalk et al. 1994; Engström et al. 1984).

A

B

Figure 2. *Hermetia illucens* (Black soldier fly)A) adult (photography); B) larvae (photography);
Source: Schmelzer & Gurib-Fakim, 2013.

1.3. Aims and objectives

The aim of this study was to evaluate an extract from insect *Hermetia illucens* (Black soldier fly), larvae for their influence on wound healing process *in vitro*. Concentration was on inflammatory phase of wound healing process because of well known anti-microbial properties of Black soldier fly larvae. For this purpose, methanol extracts as well as chloroform and ethyl acetate extracts of the larvae and different fractions of those extracts would be examined on four different bacterial species which include Gram-positive and Gram-negative bacteria.Four different bacterial species are: *Escherichia coli* BL21 (DE3)*, Micrococcus luteus, Pseudomonas fluorescens* BL915 and *Bacillus subtilis*. The principal investigation method would be bacterial growth assay where inhibition of bacterial growth will be examined. Furthermore, cytotoxicity of three main extract phases would be verified using Neutral Red assay on HepG2 and NHDF cell lines.

2. Materials and methods

2.1.Materials

2.1.1. Chemicals

Table 1. List of used chemicals

Chemical	Manufacturer
Acetic acid	Roth, Karlsruhe (D)
Bacto tryptone	Roth, Karlsruhe (D)
Chloramphenicol	AppliChem, Darmstadt (D)
Chloroform	Roth, Karlsruhe (D)
Dimethyl sulfoxide (DMSO)	Roth, Karlsruhe (D)
Ethyl acetate	Roth, Karlsruhe (D)
Ethylenediamine tetraacetate (EDTA)	Roth, Karlsruhe (D)
Ethanol	Roth, Karlsruhe (D)
Non-essential amino acids	Biochrom, Berlin (D)
Glucose	Roth, Karlsruhe (D)
Glycerin	AppliChem, Darmstadt (D)
Yeast extract	Roth, Karlsruhe (D)
Methanol	VWR Pro Labo Chemicals, Darmstadt (D)
Sodium chloride	Roth, Karlsruhe (D)
Gentamicin	Biochrom, Berlin (D)
Peptone	Roth, Karlsruhe (D)
Trypsin/EDTA Solution	Biochrom, Berlin (D)
Hank´s salt solution	Biochrom, Berlin (D)
RPMI 1640 Media	Biochrom, Berlin (D)
Dulbecco´s MEM Media	Biochrom, Berlin (D)

FBS	TU Dresden, Dresden (D)
Neutral red powder	Sigma-Aldrich, St. Louis (USA)
Isopropyl alcohol	Roth, Karlsruhe (D)
TriFast	PeqLab, Erlangen (D)
5x GoTaq Flexi reaction buffer	Promega, Madison (USA)
Magnesium chloride	Roth, Karlsruhe (D)
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich, St. Louis (USA)
DNase I	Fermentas, Toronto (CA)
RevertAid Reverse Transcriptase	Thermo Fisher Scientific, Waltham (USA)
Sodium hydroxide	Roth, Karlsruhe (D)
Hydrogen chloride	Roth, Karlsruhe (D)

2.1.2. Devices

Table 2. Equipment with the associated manufacturing firms

Equipment	Manufacturer
Stickmixer OPTIPRO 600 W	Moulinex, Alencon (FRA)
Ultrospec 2000 spectrophotometer	Pharmacia Biotech, Uppsala (SWE)
Vacuum rotary evaporator	Buchi, Essen (D)
Centrifuge Avanti J-26 XP	Backman Coulter, Brea (USA)
Centrifuge Universal 30F	Hettich, Tuttlingen (D)
NanoDrop 1000 spectrophotometer	Thermo Scientific
Microplate Reader Infinite F200	Tecan
Analysis software NanoDrop 1000	Thermo Scientific
Inverted microscope ID03	Carl Zeiss, (D)

CO_2 incubator	Sanyo
Centrifuge 1-14 (bench centrifuge)	Sigma-Aldrich, Steinheim (D)
Analysis software Magellan	Tecan
Refrigerated Centrifuge 5702R	Eppendorf
Sterile filter	Eppendorf
syringe	Eppendorf

2.1.3 Microorganisms used

Table 3. Microorganisms used with short name and origin

Strain	Short term	Reference / origin
Escherichia coli BL21 (DE3)	*E. coli*	Invitrogen, Carlsbad (USA)
Micrococcus luteus	*M. luteus*	Strain Collection AG Gutzeit, TU Dresden
Pseudomonas fluorescens BL915	*P. fluorescens*	HILL *et al.* 1994
Bacillus subtilis	*B. subtilis*	DSMZ, Braunschweig (D)

2.1.4 Cell lines

Two human cell lines (TU Dresden) were used in this study; human hepatocellular carcinoma (HepG2) and normal human dermal fibroblasts (NHDF) . The HepG2 cells are epithelial carcinoma cells derived from the liver (ATCC, 2004). NHDF are derived from the dermis of normal human neonatal foreskin or adult skin.

2.1.5 Media and antibiotics

For the cultivation and killing of the microorganisms below listed media and antibiotic were used.

To adjust the pH of media NaOH or HCl was used. For sterilization the culture media were autoclaved for 20 min at 121°C.

LB medium (Sambrook & Russell 2001)

10 g	NaCl
10 g	Bacto tryptone
5 g	Yeast extract
1 L	H_2O

pH was adjusted to 7.4 before autoclaving

RPMI 1640 Media supplemented with Gentamicin and FBS

500 mL	RPMI 1640 Media
5 mL	Gentamicin
50 mL	FBS

Gentamicin and FBS were mixed together in sterile beaker and then sterilized through sterile filter into bottle with RPMI 1640 Media

Dulbecco´s MEM Media supplemented with Gentamicin, FBS and non-essential amino acids

500 mL	Dulbecco´s MEM Media
5 mL	Gentamicin
100 mL	FBS
5 mL	non-essential amino acids

Gentamicin, FBS and non-essential amino acids were mixed together in sterile beaker and then sterilized through sterile filter into bottle with Dulbecco´s MEM Media

Chloramphenicol stock solution (c= 10 mg/mL)

50 mg	solid chloramphenicol
5 mL	96% Ethanol

Chloramphenicol used for bacterial growth assay (c= 2.88 mg/mL,V= 10 mL)

2.88 mL chloramphenicol stock solution

7.12 mL distilled H_2O

2.2 Methods

2.2.1 Storage of microorganisms

For long-term storage of bacterial cultures sterile glycerol cultures of created strains, whose retention was carried out at –80°C, were used.

2.2.2 Cultivation of microorganisms

2.2.2.1 Cultivation of *Escherichia coli*

The cultivation was carried out in liquid cultures in LB medium in 13 x 100 mm borosilicate glass culture/test tubes with caps. 10 µL of *Escherichia coli* BL21 (DE3) from glycerol culture was inoculated into 4 mL of LB media and left incubating and shaking over night on 30°C and 180 rpm.

2.2.2.2 Cultivation of *Micrococcus luteus*

The cultivation was carried out in liquid cultures in LB medium in 13 x 100 mm borosilicate glass culture/test tubes with caps. 10 µL of *M. luteus* from glycerol culture was inoculated into 4 mL of LB media and left incubating and shaking over/night on 30°C and 180 rpm.

2.2.2.3 Cultivation of *Pseudomonas fluorescens*

The cultivation was carried out in liquid cultures in LB medium in 13 x 100 mm borosilicate glass culture/test tubes with caps. 10 µL of *P. fluorescens* BL915 from glycerol culture was inoculated into 4 mL of LB media and left incubating and shaking over/night on 30°C and 180 rpm.

2.2.2.4 Cultivation of *Bacillus subtilis*

The cultivation was carried out in liquid cultures in LB medium in 13 x 100 mm borosilicate glass culture/test tubes with caps. 10 µL of *B. subtilis* from glycerol culture was inoculated into 4 mL of LB media and left incubating and shaking over/night on 30°C and 180 rpm.

2.2.3 Raising of *Hermetia illucens*

2.2.3.1 Breeding

The breeding of *Hermetia illucens* is carried out in a separate breeding station. The breeding facility is located on the site of a Biogas plant in Grimma, near Leipzig, Germany. In the breeding system feeding the larvae was carried out with shredded food waste (non-vegetarian) of different compositions. The larvae were transported in the plastic boxes or plastic bags, partly with small amounts of already implemented substrate, partly without substrate, from the breeding station and further cultured in the laboratory. There was the need for a regular replenishment of larvae, as in the laboratory no established breeding was present.

2.2.3.2 Keeping the larvae in the laboratory

The larvae were kept in standard plastic boxes. Several hundreds grams of larvae were held in large boxes (30 x 20 x 20 cm). All boxes were provided with air holes and with a commercial insect net to prevent an exodus of larvae.

The larvae were always stored in the incubator at 26°C during the cultivation time. The optimum temperature for culturing *Hermetia illucens* is between 27°C and 30°C (Tomberlin *et al.* 2009).

2.2.3.3 Feeding of larvae

All the larvae were fed with regular larval food which contain: corn, wheat, soy extract, calcium carbonate, sunflower extract, wheat bran, distilled grains and vegetable fatty acids. Additionally, larvae were fed with organic waste because it is hypothesized that it would activate greater production of anti-microbial substances within the larvae compared to larvae fed with regular food.

2.2.3.4 Killing of larvae

After collection of the larvae from the food they were washed using a commercial kitchen sieve under cold water to remove food residues and after short drying on paper towels transferred to plastic bags. The killing of the larvae was carried out by cold, the larvae were frozen at -196°C in liquid nitrogen . The larvae were kept at -20°C in freezer.

2.2.4 Extraction of larvae

2.2.4.1 Mixing and centrifuging

Frozen larvae were taken out of the freezer and thawed at the room temperature for three hours. Larvae were weighed and the weigh of thawed larvae was: m (thawed larval population)= 818 g. After that, larvae have been transferred into eight 1L sterile beakers and in each sterile beaker contained m=125.25 g of larvae. Larvae in each sterile beaker were poured with 258.3 mL of methanol, 25.83 mL of distilled water and 2.87 mL of acetic acid. All this components were mixed with hand mixer until all larvae smashed into small pieces so there was no whole larvae. Duration of mixing procedure was about five minutes. Then, all sterile beakers with larval mixture were covered with aluminum foil and left on stirrer over night. Next morning, larval mixtures from eight sterile beakers were transferred to centrifugation tubes (Volume of each centrifugation tube was 500 mL) and centrifuged on 6681 x g; 20°C for 15 minutes. After centrifugation of larval mixtures, supernatant was decanted from centrifugation tubes to two round bottom flasks.

Same extraction procedures as above were used to prepare extracts of larvae fed with organic waste. Extracts were also prepared from feces of larvae fed with organic waste since it is well known that majority of important antimicrobial compounds which larvae possess could be found in larval gut.

2.2.4.2 Concentrating of larval extracts

Larval mixtures in round bottom flasks (which were decanted after centrifugation) were evaporated in vacuum rotary evaporator under reduced pressure. Half of round bottom flask with larval mixtures was sank into warm bath (on 40°C) while it was rotating at the same time on vacuum rotary evaporator. Pressure starting point on the beginning of the procedure of concentrating of the larval extracts was 240 mbar and it was slowly decreased until 100 mbar. Process of evaporation was carried out for roughly two hours. In this procedure methanol was fully evaporated and leftover liquid (which contained larval extracts) was used for further purification and extraction.

2.2.4.3 Separating funnel extraction

Concentrated leftover liquid (extract; which was concentrated on the rotary evaporator) was transferred to a separating funnel. After that, 50 mL of chloroform was added to separating funnel

and whole mixture was shaken while separating funnel was closed to prevent leakage of content. Then, separating funnel was turned upside down and tap was opened to release the gas which accumulated after shaking. After approximately three minutes of incubation, two phases appeared in separating funnel: upper- which was aqueous phase and lower which was chloroform phase. Lower chloroform phase was let to flow through into Claissen dish by opening the tap on the separating funnel. To aqueous phase, which was still in the separating funnel, 50 mL of ethyl acetate was added and the whole procedure was repeated: separating funnel was turned upside down and tap was opened to release the gas which accumulated after shaking. After approximately three minutes of incubation two phases appeared in separating funnel: upper- which was now ethyl acetate phase and lower- which was aqueous phase. Each of these two phases (ethyl acetate and aqueous phase) were carefully let to flow through to two different Claissen dishes.

For all three phases which were separated on separating funnel concentrating on rotary evaporator was conducted: Half of round bottom flask with larval mixtures was sank into warm bath (at 40°C) while it was rotating at the same time on vacuum rotary evaporator. Pressure starting point on the beginning of the procedure of concentrating of the chloroform phase was 400 mbar and it was slowly decreased until 300 mbar. Pressure starting point on the beginning of the procedure of concentrating of the ethyl acetate phase was 200 mbar and it was slowly decreased until 150 mbar. Pressure starting point on the beginning of the procedure of concentrating of the aqueous phase was 300 mbar and it was slowly decreased until approximately 40 mbar.

All three extract phases (aqueous, ethyl acetate and chloroform) were kept in fridge at 4°C and were used for further experiments such as bacterial growth assay and cell toxicity tests.

2.2.5 Bacterial growth assay

2.2.5.1 Measuring of the OD of bacteria

Fifty mL of *E. coli* bacteria was added to plastic cuvette and mixed with 950 mL of LB medium, it was thoroughly resuspended pippeting several times up and down. Same procedure was used for other three bacterial species: *M. luteus, P. fluorescens* and *B. subtilis* in three different cuvettes. Nine hundred fifty mL of LB medium was also added to one separate cuvette. Then, the OD was determined on spectrophotometer at 600 nm. First, OD was measured for LB medium only and that value was used as a reference for measurement of OD of bacterial species. After that, OD was measured for each of four bacterial species. Measured values were used to calculate the volume of each of bacteria and LB medium that should be used in order to get the OD= 0.05 which was later

on used in bacterial growth assay. To calculate the volumes of each bacteria following formula was used:

$$V_{final} = \frac{OD_{final} \times V_1}{OD_{measured}}$$

where V_{final} is volume of bacteria that should be mixed with LB medium subtracted by the same number in order to get OD=0.05 of bacteria, OD_{final} is 0.05, V_1 is always 50mL and $OD_{measured}$ is measured OD.

When final volumes for each of bacteria were calculated, they were used to prepare mixture of bacteria and LB medium for all of four different bacterial species in four different Eppendorf tubes in order to be used in bacterial growth assay.

2.2.5.2 Preparation of master mix tubes

For each of 4 bacterial species 8 different Eppendorf tubes with master mixes were prepared.

In following scheme volumes of used substances in general master mix preparation for *E. coli* bacteria used in bacterial growth assay is showed:

Negative control

225 µL	distilled H_2O	300 µL
25 µL	peptone	
50 µL	*E. coli,* OD= 0.05	

Positive control

175 µL	distilled H_2O	300 µL
25 µL	peptone	
50 µL	*E. coli,* OD= 0.05	
50 µL	chloramphenicol, c= 2.88 mg/mL	

6 different Eppendorf tubes with different dilutions of different *Hermetia llucens* extracts

175 µL distilled H_2O

25 µL peptone

50 µL *E. coli,* OD= 0.05

50 µL of different extracts in each tube

} 300 µL in each of six Eppendorf tubes

5 0 µL of extract in each tube actually serves as a substitute for antibiotic, and its antimicrobial activity is measured.

Same procedure is repeated with all of the three bacterial species left: *M. luteus, P. fluorescens* and *B. subtilis*. After preparation of master mix tubes there was 32 master mix tubes which were used in preparation of the 96-well plate for bacterial growth assay.

2.2.5.3 Plate preparation

For 96-well preparation 32 master mix tubes with bacteria, antibiotic, extracts and negative controls were used.

For each sample in plate preparation three repetitions (three wells of 96-well plate) were used and following pipetting order (Table 1.) was applied: 1 . *E. coli*- growth control (negative control) → antibiotic control (positive control) → extract 1. → … → extract 6.

60 µL of each sample was pipetted in each well of the 96-well plate.

Table 3.Pipetting scheme on the 96-well plate for bacterial growth assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	*E.coli*- negative control, 60 µL	*E.coli*- extract 1, 60 µL	*E.coli*- extract 4, 60 µL	*M.luteus*- negative control, 60 µL	*M.luteus*- extract 1, 60 µL	*M.luteus*- extract 4, 60 µL	*P.fluorescens*-negative control, 60 µL	*P.fluorescens*- extract 1, 60 µL	*P.fluorescens*- extract 4,, 60 µL	*B.subtilis*- negative control, 60 µL	*B.subtilis*- extract 1, 60 µL	*B.subtilis*- extract 4, 60 µL
B	*E.coli*- negative control, 60 µL	*E.coli*- extract 2, 60 µL	*E.coli*- extract 4, 60 µL	*M.luteus*- negative control, 60 µL	*M.luteus*- extract 2, 60 µL	*M.luteus*- extract 4, 60 µL	*P.fluorescens*-negative control, 60 µL	*P.fluorescens*- extract 2, 60 µL	*P.fluorescens*- extract 4, 60 µL	*B.subtilis*- negative control, 60 µL	*B.subtilis*- extract 2, 60 µL	*B.subtilis*- extract 4, 60 µL
C	*E.coli*- negative control, 60 µL	*E.coli*- extract 2, 60 µL	*E.coli*- extract 5. 60 µL	*M.luteus*- negative control, 60 µL	*M.luteus*- extract 2, 60 µL	*M.luteus*- extract 5, 60 µL	*P.fluorescens*-negative control, 60 µL	*P.fluorescens*- extract 2, 60 µL	*P.fluorescens*- extract 5, 60 µL	*B.subtilis*- negative control, 60 µL	*B.subtilis*- extract 2, 60 µL	*B.subtilis*- extract 5, 60 µL
D	*E.coli*- positive	*E.coli*- extract 2,	*E.coli*- extract 5,	*M.luteus*- positive	*M.luteus*- extract 2,	*M.luteus*- extract 5,	*P.fluorescens*- positive	*P.fluorescens*- extract 2,	*P.fluorescens*- extract 5,	*B.subtilis*- positive	*B.subtilis*- extract 2,	*B.subtilis*- extract 5,

	control, 60 µL	60 µL	60 µL,	control, 60 µL	60 µL	60 µL	control, 60 µL	60 µL	60 µL	control, 60 µL	60 µL,	60 µL
E	*E.coli*-positive control, 60 µL	*E.coli*-extract 3, 60 µL	*E.coli*-extract 5, 60 µL	*M.luteus*-positive control, 60 µL	*M.luteus*-extract 3, 60 µL	*M.luteus*-extract 5, 60 µL	*P.fluorescens*- positive control, 60 µL	*P.fluorescens*- extract 3, 60 µL	*P.fluorescens*- extract 5, 60 µL	*B.subtilis*-positive control, 60 µL	*B.subtilis*-extract 3, 60 µL	*B.subtilis*-extract 5, 60 µL
F	*E.coli*-positive control, 60 µL	*E.coli*-extract 3, 60 µL	*E.coli*-extract 6, 60 µL	*M.luteus*-positive control, 60 µL	*M.luteus*-extract 3, 60 µL	*M.luteus*-extract 6, 60 µL	*P.fluorescens*- positive control, 60 µL	*P.fluorescens*- extract 3, 60 µL	*P.fluorescens*- extract 6, 60 µL	*B.subtilis*-positive control, 60 µL	*B.subtilis*-extract 3, 60 µL	*B.subtilis*-extract 6, 60 µL
G	*E.coli*-extract 1, 60 µL	*E.coli*-extract 3, 60 µL	*E.coli*-extract 6, 60 µL	*M.luteus*-extract 1, 60 µL	*M.luteus*-extract 3, 60 µL	*M.luteus*-extract 6, 60 µL	*P.fluorescens*- extract 1, 60 µL	*P.fluorescens*- extract 3, 60 µL	*P.fluorescens*- extract 6, 60 µL	*B.subtilis*-extract 1, 60 µL	*B.subtilis*-extract 3, 60 µL	*B.subtilis*-extract 6, 60 µL
H	*E.coli*-extract 1, 60 µL	*E.coli*-extract 4, 60 µL	*E.coli*-extract 6, 60 µL	*M.luteus*-extract 1, 60 µL	*M.luteus*-extract 4, 60 µL	*M.luteus*-extract 6, 60 µL	*P.fluorescens*- extract 1, 60 µL	P.fluorescens- extract 4, 60 µL	*P.fluorescens*- extract 6, 60 µL	*B.subtilis*-extract 1, 60 µL	*B.subtilis*-extract 4, 60 µL	*B.subtilis*-extract 6, 60 µL

After preparation of 96-well plate it have been put to Tecan Infinite 200 spectrophotometer. Spectrophotometer parameters were then set up to 25 kinetic cycles with an interval time of one hour per kinetic cycle. Through this 25 cycles absorbance was measured on 600 nm.

After 24 hours of plate reading in the spectrophotometer plate was taken out and discarded into toxic waste bin and measured data was evaluated.

Bacterial growth assay was set up every day throughout the period of time of two months. Sometimes, same samples were prepared for several times since bacteria in control wells did not grow or some of the wells were contaminated.

2.2.6 Cell culture

2.2.6.1 Cells storage

The HepG2 and NHDF cells which have been used in this research had previously been stored by freezing in liquid nitrogen.

2.2.6.2 HepG2 and NHDF cells cultivation

Cell aliquots of HepG2 and NHDF cell lines were taken out from cryogenic tank and warmed up at room temperature until they were completely thawed. Aliquots were then transferred into centrifuge tubes and 10 ml of selective culture media was added to it. For cultivation of HepG2 cells RPMI 1640 Media supplemented with Gentamicin and FBS was used and for cultivation of NHDF cells Dulbecco´s MEM Media supplemented with Gentamicin, FBS and non-essential amino acids was used. Then, cells were centrifuged at 100 x g on room temperature (20°C) for 5 minutes. After

centrifugation, supernatant was discarded while pellet was resuspended in 1 ml selective media. From such cells/media suspension two aliquots of 500 µL were taken and transferred to two 75 cm^2 cell culture flasks. Following that, 20 mL of selective media was added to each flask and flasks with cells were put to incubator. Incubator temperature was 37°C with a humidified atmosphere of 5% CO_2 in air.

All the used growth media were stored in refrigerator at 4°C and prior to use were warmed shortly in a water bath at 37°C.

2.2.6.3 HepG2 and NHDF cell line splitting

When approximately 80% confluency had been reached, which was after seven days of incubating, cells were split (*i.e.*, subcultured).

Firstly, old medium was removed by using sterile glass pipette and cells were rinsed with 10 ml of Hank´s salt solution (*i.e.*,PBS). After that, PBS was taken out and 1 mL of PBS and 1 mL of 10x Trypsin/EDTA was added and cells were incubated in incubator for five minutes. Following incubation, cells were observed under the microscope to check if most of the cells had detached. In some cases, only small amount of cells had detached, so the incubation was extended for two minutes which resulted in larger amount of detached cells. After that, detachment of the cells was stopped by addition of 10 mL of growth medium. The cell suspension was then transferred into 15 ml centrifuge tube and centrifuged at 100 x g at room temperature (20°C) for 5 minutes. Following centrifugation, supernatant was discarded while pellet was resuspended in 1 ml growth medium. From both centrifuged cell cultures (HepG2 and NHDF) 10 µL of cell suspension was transferred into a Neubauer counting chamber and cells were counted. The appropriate amount of thus obtained cell suspension was then transferred into 75 cm^2 cell culture flasks and 20 mL of culture medium was added. Cells were passaged in $^1/_8$ ratio and it took 7 days to reach appropriate confluency for both cell cultures.

2.2.7 Neutral Red assay

This method is based upon protocol of Borenfreund and Puerner, 1985. The uptake of the vital dye Neutral Red into the lysosomes/endosomes and vacuoles of living cells is used as a quantitative indication of cell number and viability. This protocol was used for both cell lines; HepG2 and NHDF.

First, cell suspension of $1x10^5$ per mL in culture medium was prepared in a sterile reservoir for multi-channel pipette. Using a multi-channel pipette 100 µL of cell suspension was dispensed into

wells of first eleven columns of 96-well tissue culture microtiter plate (= $1x10^4$ cells per well), last column was left empty for subsequent blank measurement. Prepared 96-well plates were left in incubator in order to establish confluency in each well of 96-well plate where cells were dispensed.
After incubation time of three days, pre-prepared dilutions of *Hermetia illucens* extracts were added to each well which contained cells. Extracts were prepared by dilution of extracts obtained by separating funnel extraction in sterile media which was used for cell culture. For control of non-toxic activity water diluted in growth media was used and it was pipetted into first column of each plate. Extracts were added by pipetting 100 µL of diluted extract to wells of 96-well plate which already contained 100 µL media. Then, whole content in each well was resuspended by going up and down with pipette several times, and finally 100 µL of that content was taken out with pipette and transferred to waste while volume in each well was still 100 µL. After extracts addition to each plate, plates were incubated for various periods of times. Standard times used for incubation of extracts with cells were 4 hours and 24 hours.
After incubation of extracts whole content of each plate was discarded into waste (while cells were still attached to the bottom surface of each well) and each well was washed two times by adding 200 µL of Hank´s salt solution. After washing, 100 µL of Neutral Red dye was added to wells of first eleven columns of the 96-well plate. After that, plates were put to incubator for incubation for three hours. After that, Neutral Red dye was discarded and 100 µL of lysis buffer was added to each well in the 96-well plate. After waiting period of fifteen minutes absorbance was measured using Ultrospec 2000 spectrophotometer and obtained data was evaluated.
Neutral red assay was also used in testing toxicity of control substance with both HepG2 and NHDF cells. Control substance was Pentabromopseudilin which was known for its cytotoxicity. Dilutions of Pentabromopseudilin were prepared by diluting the substance in DMSO. Also, DMSO diluted in PBS was used as a control for non-toxic activity.

2.2.8 Statistical analysis

All measurements were performed at least in triplicates and the results were expressed as mean value ± standard deviation (SD). Calculation of mean values and standard deviation was preformed using Open Office software.

Statistical analysis, which involved one-way ANOVA and Tukey´s test, was carried out using Origin software in order to deduce statistical significance of the acquired results. Mean values of

three independent experiments were used and the results were considered relative to control (H_2O) . An obtained p-value ≤ 0.05 (*) was considered significant, while p-values ≤ 0.01 (**) and p ≤ 0.001 (***) were considered as highly significant.

3. Results

First, three phases of extracts were applied to *E. coli* bacteria and bacterial growth inhibition was measured using bacterial growth assay during period of 19 hours (Fig. 3).

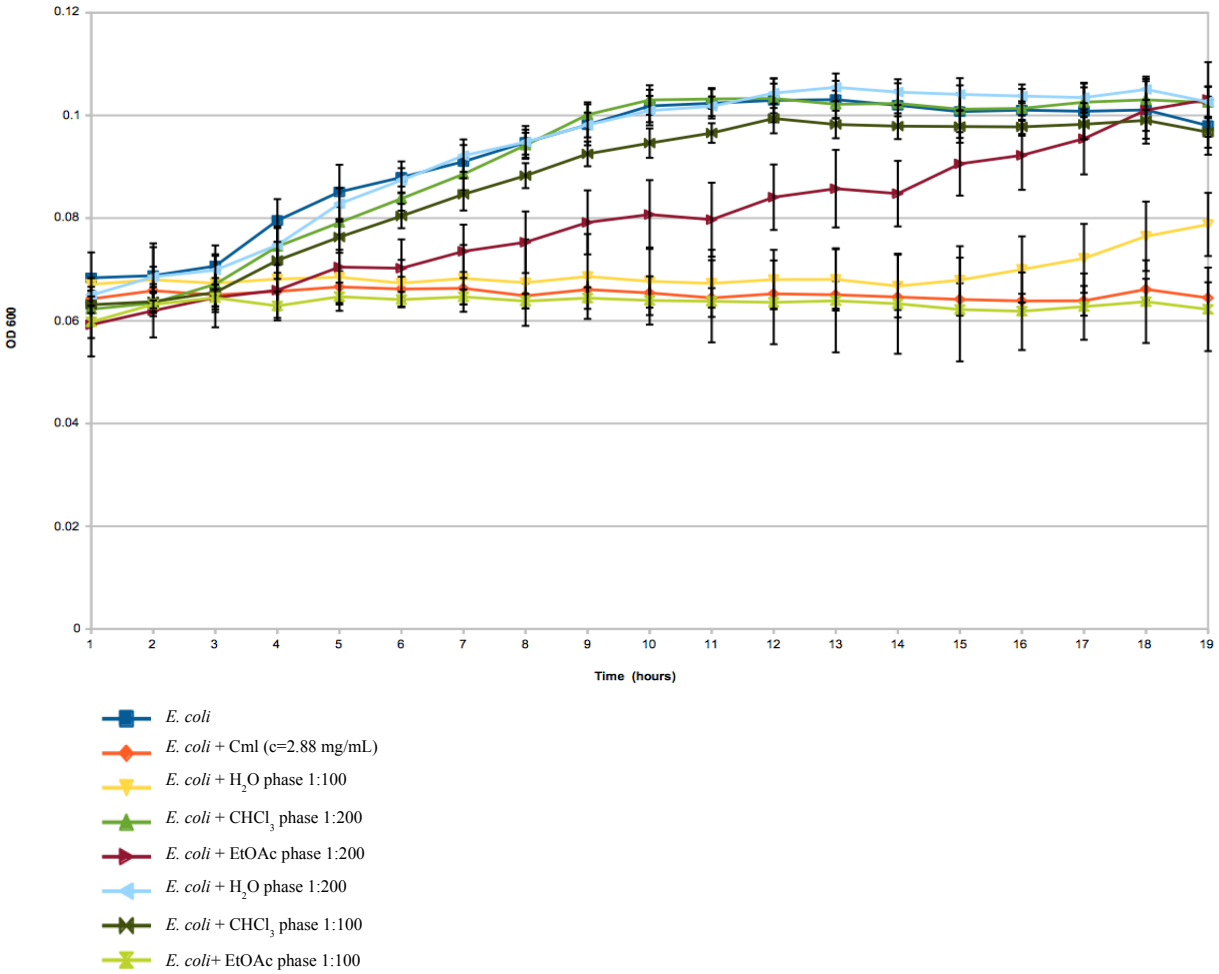


Figure 3. Effects of three different regular food fed *Hermetia illucnes* larvae extracts on growth inhibition of *E. coli* bacteria during 19 hours.

Dark blue curve represents negative control (dH_2O) for growth inhibition of *E. coli* bacteria where none of the extracts were applied. Orange curve represents positive control for growth inhibition of *E. coli* bacteria where chloramphenicol antibiotic was applied in the

concentration of c=2.88 mg/mL. Yellow and light blue curves represent growth inhibition of *E. coli* bacteria where H_2O phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Medium green and darkest green curves represent growth inhibition of *E. coli* bacteria where $CHCl_3$ phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Red and lightest green curves represent growth inhibition of *E. coli* bacteria where EtOAc phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Data of three independent experiments are expressed as the mean ± standard error.

As seen in figure 3. growth curve of *E. coli* bacteria during period of 19 hours, when none of the extracts were applied, is consistent with exponential growth and thus could be described as normal bacterial growth. For negative control, instead of any extracts, distilled water was used together with other substances essential for bacterial growth. As expected, growth of bacteria was completely inhibited in presence of antibiotic Chloramfenicol (c=2.88 mg/mL) which was used as positive control. When H_2O phase extract was applied in higher dilution of 1:200 to *E. coli* bacteria growth was not inhibited and the growth curve was as same as negative control. Lower dilution (*i.e.* higher concentration) of the same H_2O phase extract of 1:100 showed significant inhibition during first 14 hours of measurement and remarkable inhibition in the next 5 hours when compared to lower concentrated sample. Moreover, there was no inhibition of *E. coli* growth when $CHCl_3$ phase extract was applied in higher dilution of 1:200 and there was just minimal inhibition when same extract was applied in higher concentration. EtOAc phase extract applied to *E. coli* bacteria preformed impressive inhibition in lower dilution of 1:100 and it was as same as antibiotic control. Lower concentration of the same extract also had an inhibitory effect which was approximately in between of positive and negative controls.

It is known (Steiner et al. 1981) that insects have a well-developed innate immune system which involves production of anti-microbial peptides. Those AMPs are synthesized in the fat body and later on secreted into the hemolymph of an insect (Bulet et al. 1999; Hoffmann & Reichhart 2002). Accordingly, *Hermetia illucens* larvae extracts were applied to both Gram-negative and Gram-positive bacteria, the latter of which were also included in this research (Fig. 4., Fig. 5.)

Figure 4. shows influence of three different extracts in two dilutions of *Hermetia illucnes* larvae on growth inhibition of *M. luteus* bacteria. Again, distilled water and chloramphenicol antibiotic were used as negative and positive control respectively. Aqueous phase of the extract applied to *M. luteus* bacteria in both dilutions of 1:100 and 1:200 showed inhibiting influence on bacterial growth but still not as inhibitory as antibiotic did. Furthermore, growth of the same bacteria when chloroform phase extracts were applied showed even greater inhibition. Interestingly, this phase did

not show any notable difference when applied in two different dilutions. Once more, ethyl acetate phase extract exhibited remarkable inhibition when applied to *M. luteus* bacteria. Both dilutions (1:100 and 1:200) of the ethyl acetate phase displayed similar outcome and it was as same as antibiotic control.

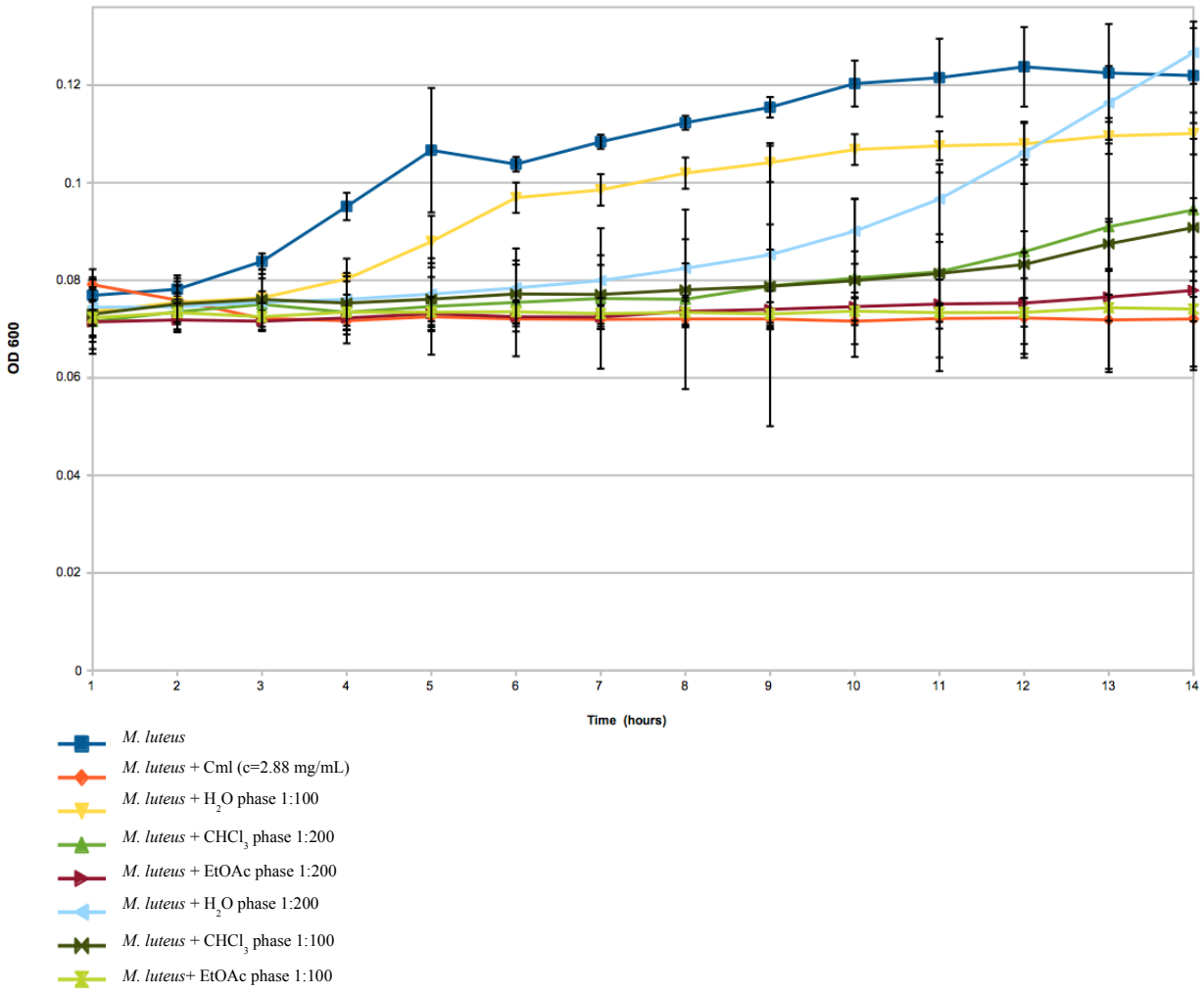


Figure 4. Effects of three different regular food fed *Hermetia illucnes* larvae extracts on growth inhibition of *M. luteus* bacteria during 14 hours.

Dark blue curve represents negative control (dH_2O) for growth inhibition of *M. luteus* bacteria where none of the extracts were applied. Orange curve represents positive control for growth inhibition of *M. luteus* bacteria where chloramphenicol antibiotic was applied in the concentration of c=2,88 mg/mL. Yellow and light blue curves represent growth inhibition of *M. luteus* bacteria where H_2O phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Medium green and darkest green curves represent growth inhibition of *M. luteus* bacteria where $CHCl_3$ phases of *Hermetia illucens* extracts were

applied in different dilutions of 1:100 and 1:200 respectively. Red and lightest green curves represent growth inhibition of *M. luteus* bacteria where EtOAc phase of *Hermetia illucens* extract were applied in different dilutions of 1:100 and 1:200 respectively. Data of three independent experiments are expressed as the mean ± standard error.

The next step was to look at the influence of the same extracts in same dilutions on another Gram-positive bacteria, *Bacillus subtilis* (Figure 5.).

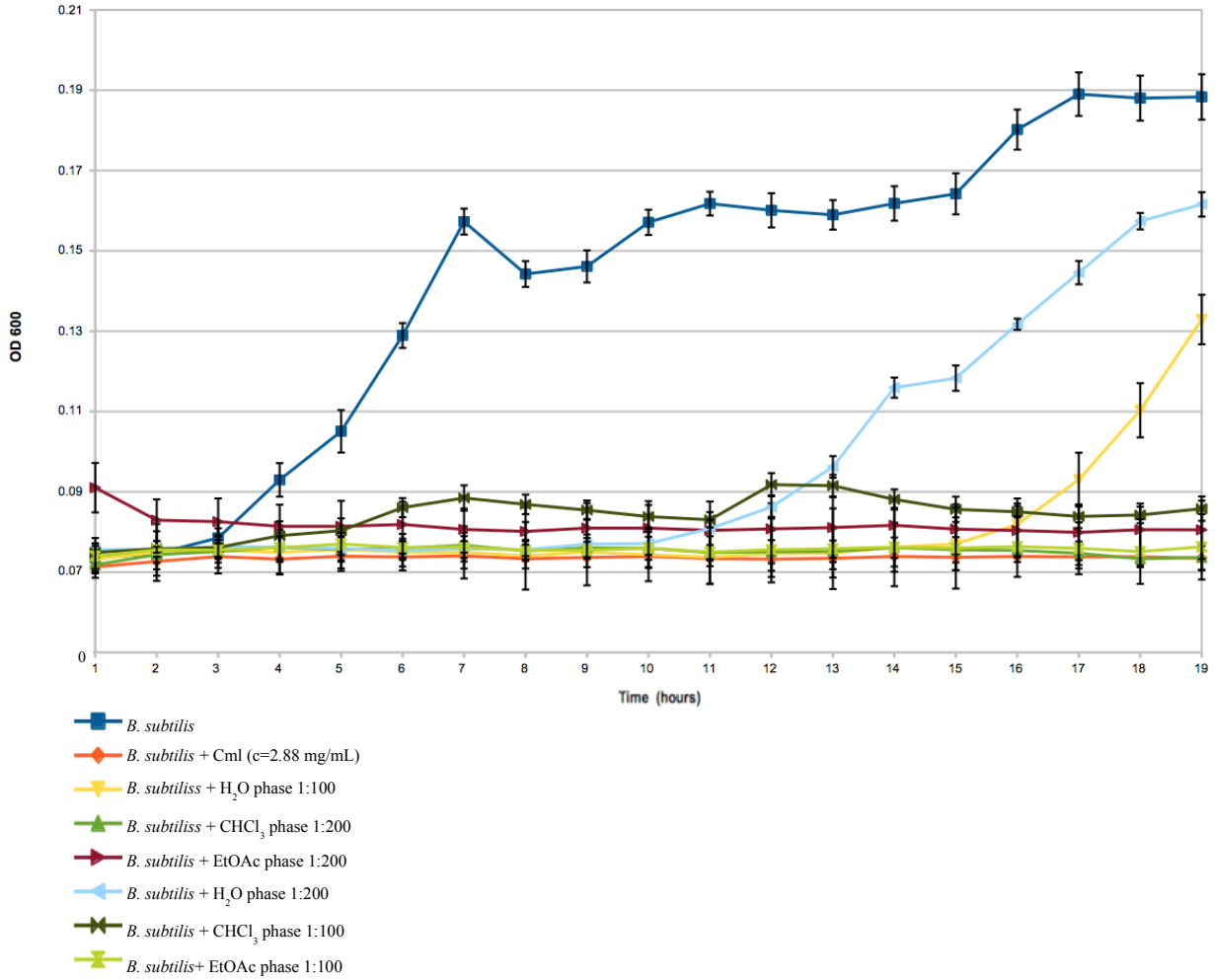


Figure 5. Effects of three different regular food fed *Hermetia illucnes* larvae extracts on growth inhibition of *B. subtilis* bacteria during 19 hours.

Dark blue curve represents negative control (dH_2O) for growth inhibition of *B. subtilis* bacteria where none of the extracts were applied. Orange curve represents positive control for growth inhibition of *B. subtilis* bacteria where chloramphenicol antibiotic was applied. Yellow and light blue curves represent growth inhibition of *B. subtilis* bacteria where H_2O phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Medium green and darkest green curves represent growth inhibition of *B. subtilis* bacteria where $CHCl_3$ phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Red and lightest green curves represent growth inhibition of *B. subtilis* bacteria where EtOAc phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Data of three independent experiments are expressed as the mean ± standard error.

It could be observed that when same extracts were applied to *B. subtilis* bacteria they preformed impressive inhibition (Figure 5.). Chloroform and ethyl acetate phase in both dilutions were as inhibiting as positive control. While, H_2O phase in higher dilution showed growth delay during first eleven hours of measurement and after that time point bacteria starts to grow but it is still quite

inhibited. Lower dilution of the same phase showed even more delayed growth which lasts during first fifteen hours of measurement.

Finally, all the extracts were applied to *P. fluorescens* bacteria in order to identify their anti-microbial properties on another Gram-negative bacteria (Figure 6.).

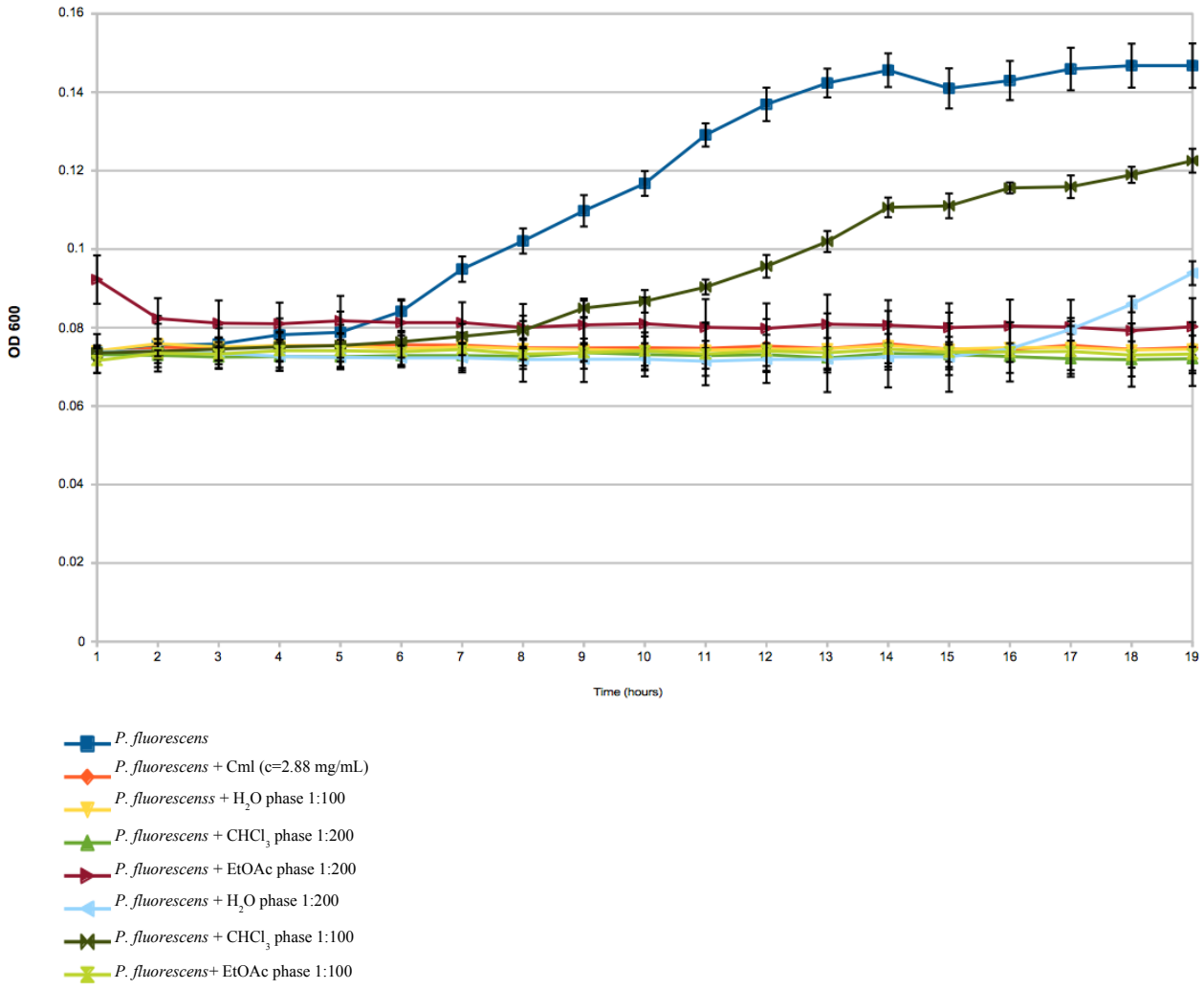


Figure 6. Effects of three different regular food fed *Hermetia illucens* larvae extracts on growth inhibition of *P. fluorescens* bacteria during 19 hours.

Dark blue curve represents negative control (dH_2O) for growth inhibition of *P. fluorescens* bacteria where none of the extracts were applied. Orange curve represents positive control for growth inhibition of *P. fluorescens* bacteria where chloramphenicol antibiotic was applied. Yellow and light blue curves represent growth inhibition of *P. fluorescens* bacteria where H_2O phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Medium green and darkest green curves represent growth inhibition of *P. fluorescens* bacteria where $CHCl_3$ phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Red and lightest green curves represent growth inhibition of *P. fluorescens* bacteria where

EtOAc phases of *Hermetia illucens* extracts were applied in different dilutions of 1:100 and 1:200 respectively. Data of three independent experiments are expressed as the mean ± standard error.

In Figure 6. growth of *P. fluorescens* bacteria with different compounds applied was shown and it could be seen that all three different phases of *Hermetia illucens* extracts preformed striking growth inhibition. All the extracts showed reduced bacterial growth which is consistent with antibiotic control except chloroform and aqueous phase in higher dilution. Bacteria with chloroform phase put on in higher dilution exhibited delayed growth during first 7 hours of growth monitoring after which it starts to grow but still not as bacteria alone. When H_2O phase was applied to bacteria in lower concentration it started to grow slightly, although just in last three hours of measurement and in much lower manner than negative control.

In Figure 7. influence of both extracts, from larvae fed with organic waste and from larval faeces after application to afore mentioned bacterial species, is depicted .

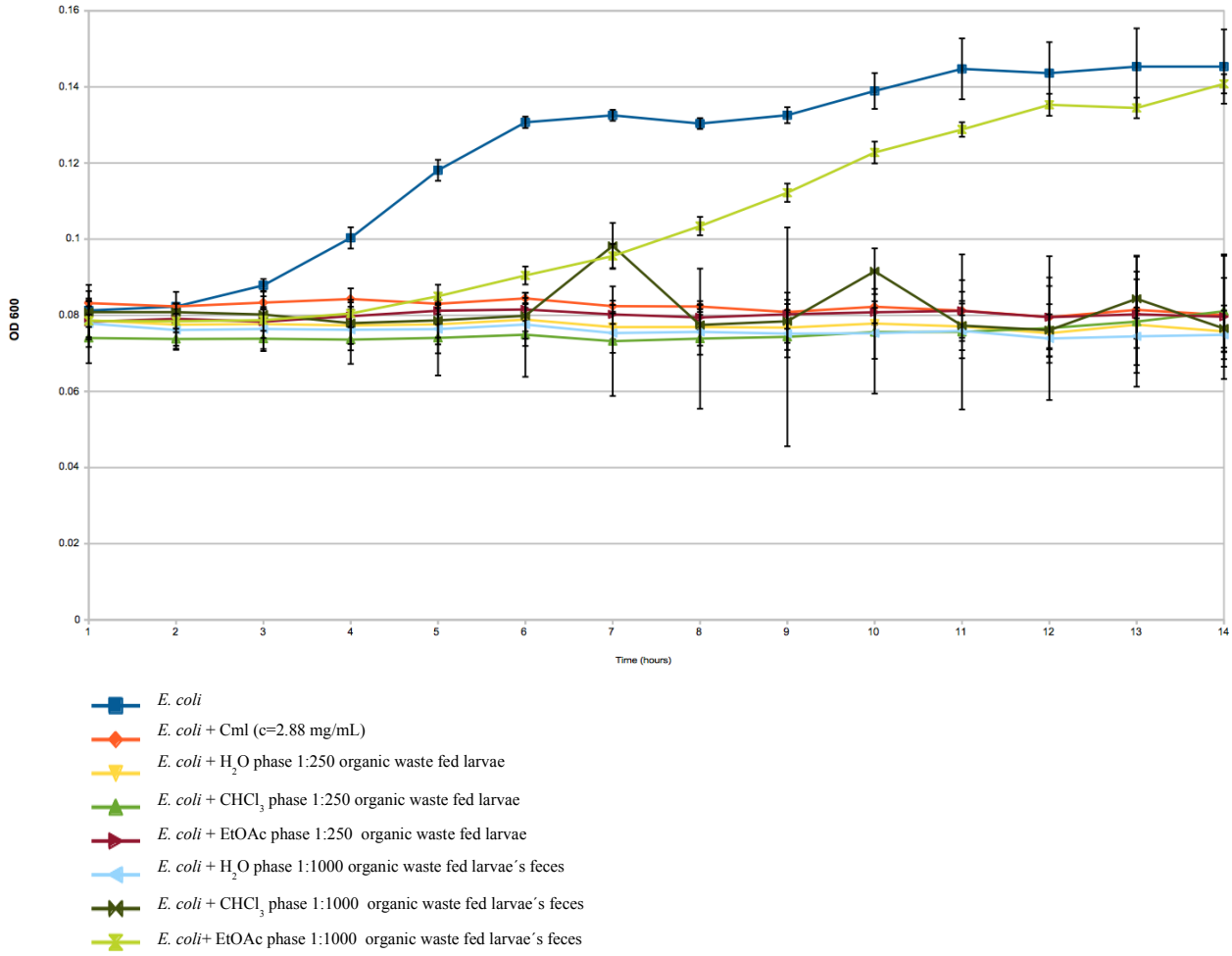


Figure 7. Effects of different *Hermetia illucens* larvae and larval faeces extracts on growth inhibition of *E. coli* bacteria during 14 hours.

Dark blue curve represents negative control (dH_2O) for growth inhibition of *E. coli* bacteria where none of the extracts were applied. Orange curve represents positive control for growth inhibition of *E. coli* bacteria where chloramphenicol antibiotic was applied. Yellow curve represents growth inhibition of *E. coli* bacteria where H_2O phases of *Hermetia illucens* organic waste fed larvae extract was applied in dilution of 1:250. Medium green curve represents growth inhibition of *E. coli* bacteria where $CHCl_3$ phase of *Hermetia illucens* organic waste fed larvae extract was applied in dilution of 1:250. Red curve represents growth inhibition of *E. coli* bacteria where EtOAc phase of *Hermetia illucens* organic waste fed larvae extract was applied in dilution of 1:250. Light blue curve represents growth inhibition of *E. coli* bacteria where H_2O phase of *Hermetia illucens* organic waste fed larvae´s feces extract was applied in dilution of 1:1000. Dark green curve represents growth inhibition of *E. coli* bacteria where $CHCl_3$ phase of *Hermetia illucens* organic waste fed larvae´s feces extract was applied in dilution of 1:1000. Light green curve represents growth inhibition of *E. coli* bacteria where EtOAc phase of *Hermetia illucens* organic waste fed larvae´s feces extract was applied in dilution of 1:1000. Data of three independent experiments are expressed as the mean ± standard error.

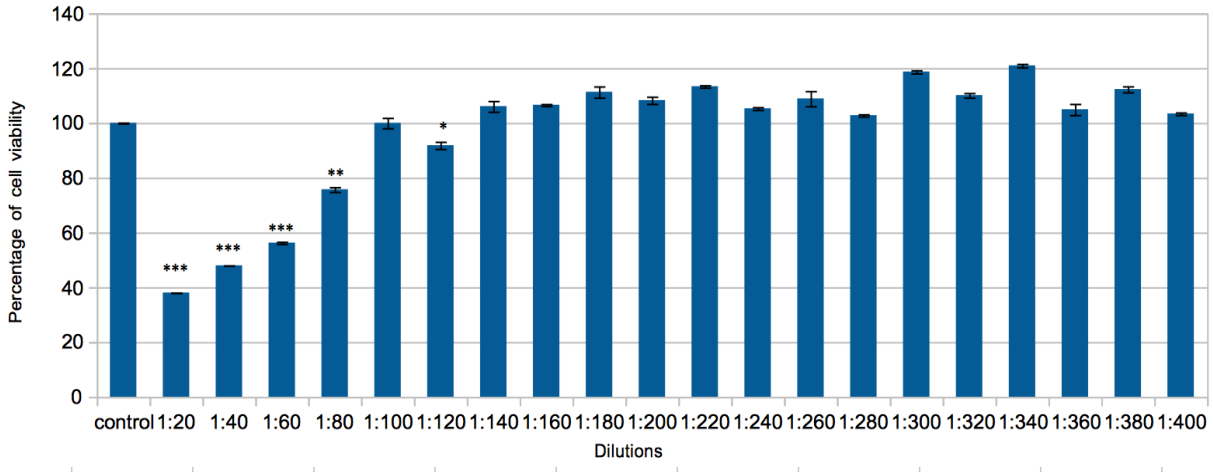
As seen in Figure 7. *E. coli* bacteria without any extracts or antibiotics applied grows exponentially while on the other hand same bacteria with antibiotic added displays no growth. When larvae fed with organic waste in H_2O and EtOAc extracts were applied growth was completely inhibited while

$CHCl_3$ extract of the same larvae shows inhibition half way in between of positive and negative control. Moreover, organic waste fed larvae´s feces applied in all three phases (H_2O, $CHCl_3$ and EtOAC) in dilution of 1:1000 perform complete inhibition which are in line with the positive control.

Except of testing the antimicrobial properties and potential of *Hermetia illucens* larvae´s extracts their cytotoxic effects on human cells was assessed using Neutral Red assay. That is of certain importance before any further investigation of mechanisms of action of these substances and before investing in their development as a pharmaceutical for wound healing.

Figures 8.-11. display cytotoxic effects of different *Hermetia illucens* larvae extracts when applied to human cells (HepG2 and NHDF) *in vitro* and their viability potential.

A



B

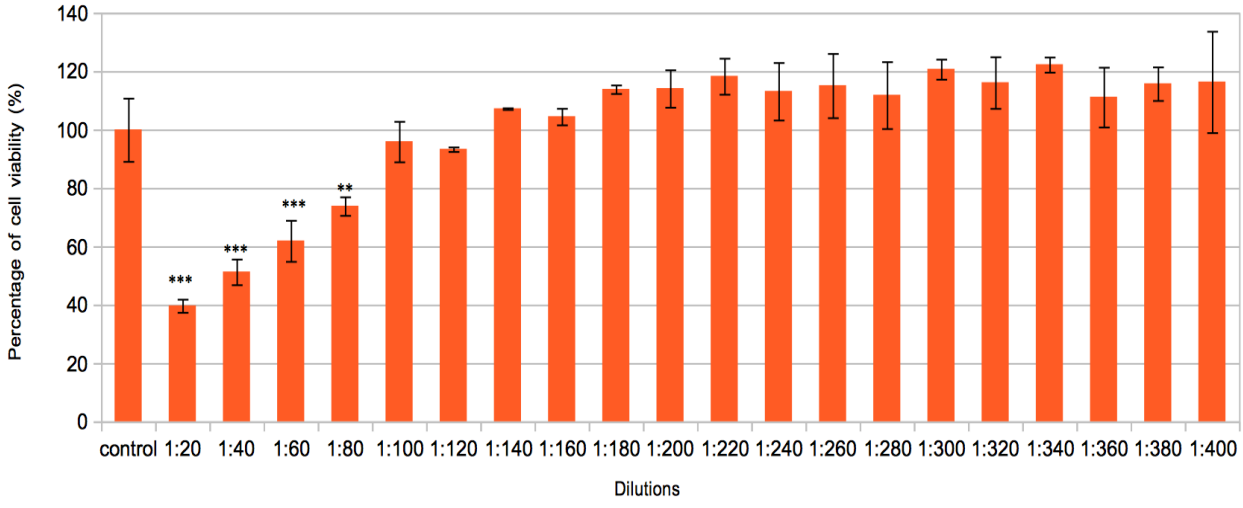


Figure 8. Effect of *Hermetia illucens* normal fed larvae H_2O phase extract and its different dilutions on the cell viability determined by Neutral Red assay in HepG2 cells during 4 hours (A) and 24 hours (B)

Data of three independent experiments are represented relative to the H_2O control (100%) and expressed as the mean ± standard error. Each treatment group was performed in triplicates. Statistical significance is indicated relative to H_2O control as * $p \leq 0,05$, ** $p \leq 0,01$,*** $p \leq 0,001$ and it was calculated using one-way ANOVA and Tukey´s test

Cytotoxicity of *Hermetia illucens* normal fed larvae extract was tested, when HepG2 cells were incubated with H_2O phase extract for four (Figure 8. A) and 24 hours (Figure 8. B), by Neutral Red assay and the results are presented in Figure 8. Treatment included H_2O phase extract and different dilutions of the same extract from 1:20 to 1:400. Some of the dilutions used in this experiment were also used for bacterial growth assay. Control used was water mixed in ratio 1:20 with cell culture media. After dilution of 1:120 non of the higher dilutions show cytotoxic effect. Furthermore, when the extract was incubated with the cells for 24 hours, least diluted extracts, as expected, showed significant cytotoxicity and the first dilution which shows non-toxic influence is 1:140, while dilutions of 1:100 and 1:120 show minor toxic effect.

Next, cytotoxicity of *Hermetia illucens* normal fed larvae $CHCl_3$ phase extract was tested, after incubation with HepG2 cells for four and 24 hours (Figure 9.)

When the $CHCl_3$ phase extract was applied to HepG2 cells (Figure 9.), different dilutions of extract were used when compared to previous phase extract in Figure 8. This was done for experimental convenience because every phase of the three different *Hermetia illucens* extracts needed to be filtered through a syringe filter in order to be sterilized. Since that the $CHCl_3$ phase extract, after the evaporation on the rotary evaporator, was filled with lots of solids and was quite dense it needed to be diluted with sterile water to be suitable for filtering through syringe filter. As a consequence, 1 mL of the $CHCl_3$ phase extract was diluted in 2 mL of sterile water. After that different dilutions, of already diluted extract, were prepared and they were applied to cells. Starting dilution was 1:60 and the final, out of 20 dilutions, was 1:1200. Again, some of the dilutions used in this experiments were already used for bacterial growth inhibition in the bacterial growth assay. Starting dilution of the $CHCl_3$ phase extract showed just slight cytotoxicity and the cell viability compared to control was around 90 percent for both time points. First concentration which didn´t display any cytotoxic effect on the cells was 1:540 and it was same for both 4 hours and 24 hours. All of the different dilutions of the $CHCl_3$ phase extract did not show any significant cytotoxic effect neither during 4 hours nor during 24 hours of incubation with HepG2 cells.

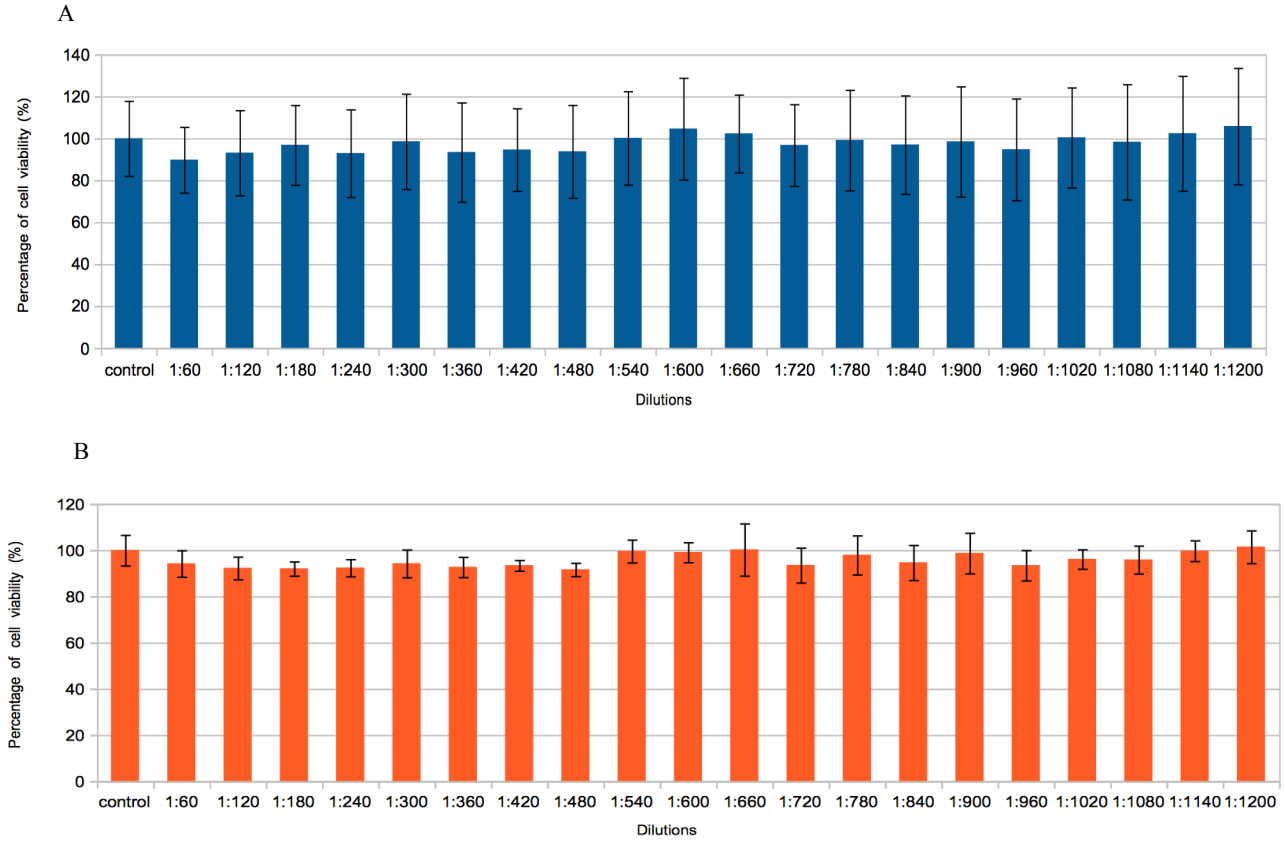


Figure 9. Effect of *Hermetia illucens* normal fed larvae $CHCl_3$ phase extract and its different dilutions on the cell viability determined by Neutral Red assay in HepG2 cells during 4 hours (A) and 24 hours (B)

Data of three independent experiments are represented relative to the H_2O control (100%) and expressed as the mean ± standard error. Each treatment group was performed in triplicates. Statistical significance is indicated relative to H_2O control as * $p \leq 0,05$, ** $p \leq 0,01$,*** $p \leq 0,001$ and it was calculated using one-way ANOVA and Tukey´s test

Cytotoxic effects of *Hermetia illucens* normal fed larvae EtOAc phase extract were examined after incubation with HepG2 cells for four and 24 hours (Figure 10.) Again, EtOAc phase extract was also diluted prior to making serial dilutions for application to the HepG2 cells and it was for the same reason as pervious phase extract. First couple of dilutions (1:60,1:120) of this phase of extract applied to HepG2 cells showed cytotoxicity of 80% compared to control and it was the case in 4 hour and 24 hour incubation experiments. Subsequent dilutions showed decreased toxic effects which was about 90%. First extract which was consistent with control was 1:780 for 4 hours and 1:600 for 24 hours of incubation. It is important to notice that some of the upcoming dilutions, which were after first non-toxic dilutions, showed toxic result even though they were less concentrated.

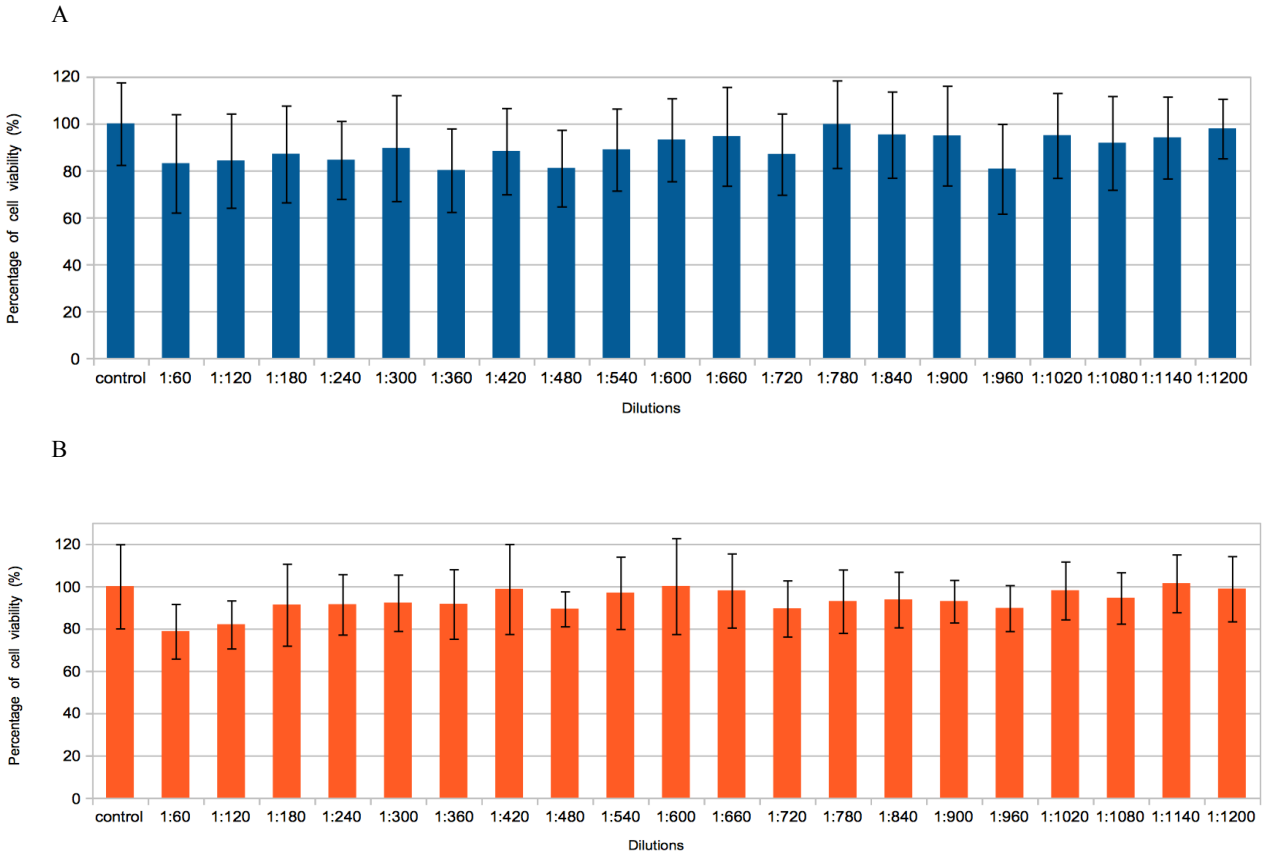


Figure 10. Effect of *Hermetia illucens* normal fed larvae EtOAc phase extract and its different dilutions on the cell viability determined by Neutral Red assay in HepG2 cells during 4 hours (A) and 24 hours (B). Data of three independent experiments are represented relative to the H_2O control (100%) and expressed as the mean ± standard error. Each treatment group was performed in triplicates. Statistical significance is indicated relative to H_2O control as * $p \leq 0,05$, ** $p \leq 0,01$,*** $p \leq 0,001$ and it was calculated using one-way ANOVA and Tukey´s test

Viability of the NHDF cells after the application of different phases of *Hermetia illucens* extracts was also analyzed using the Neutral Red toxicity assay. NHDF cells are human dermal fibroblast cells and in this project they should have been model for testing the effects of the extracts on the expression of different molecular markers characteristic for the wound healing process. In order to proceed with that cytotoxicity was assessed.

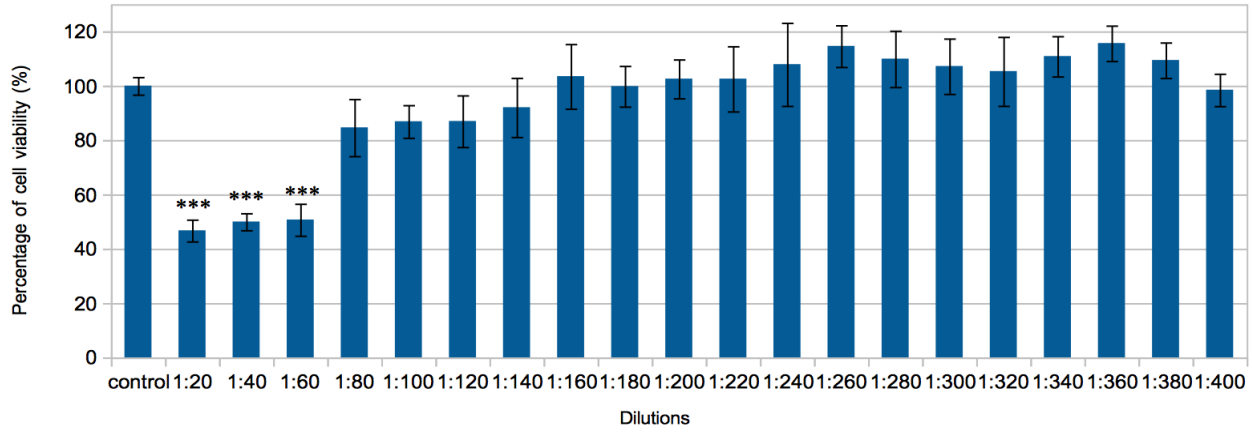


Figure 11. Effect of *Hermetia illucens* normal fed larvae H_2O phase extract and its different dilutions on the cell viability determined by Neutral Red assay in NHDF cells during 24 hours. Data of three independent experiments are represented relative to the H_2O control (100%) and expressed as the mean ± standard error. Each treatment group was performed in triplicates. Statistical significance is indicated relative to H_2O control as * $p \leq 0,05$, ** $p \leq 0,01$,*** $p \leq 0,001$ and it was calculated using one-way ANOVA and Tukey´s test

Figure 11. displays behavior of the NHDF when normal fed *Hermetia illucens* larvae H_2O phase extract was applied in the different dilutions starting form 1:20 and ending with the 1:400 dilution. First three dilutions (1:20, 1:40, 1:60) of this extract show significant toxicity on the cells and it is for all the three dilutions around 50 % when compared to control. Following four dilutions (1:80-1:140) showed less toxic effects on the cells. Finally, first dilution which does not show cytotoxic influence on the NHDF cells is 1:160 and all the next dilutions afterwards did not show any cytotoxic impact.

Also, two other phases ($CHCl_3$ and EtOAc) of *Hermetia illucens* extract were tested for their cytotoxicity effects on the NHDF cells and the results were in line with the results for the same phases on HepG2 cells.

4. Discussion

4.1.Examination of antimicrobial potential of *Hermetia illucens* larvae extracts

In this study, *Hermetia illucens* larvae extract, and its different phases in different concentrations were evaluated for antimicrobial properties and that was first of two main goals of this research. It is well known that the larvae of black soldier fly, *Hermetia illucens*, exist in harsh surroundings, proposing that the soldier fly may be rich in generations of AMPs and similar substances possessing activity against microorganisms, including MRSA (Choi et al. 2012).

Second goal of this project was to determine the effect of the same extracts on the human cells *in vitro* and to test their cytotoxic properties.

The acidic methanol extraction protocol was used for the extraction of antimicrobial substances from the larvae, since the acidic methanol can precipitate and denature large polypeptides and proteins, and at the same time, efficiently extract small peptides such as AMPs. (Meylears et al. 2002). The acidic methanol extract of the larvae was sequentially extracted with chloroform and ethyl acetate to remove lipids and to get the high density of anti-microbial substances.

Aqueous phase of the normal fed *Hermetia illucens* larvae extract, which was actually concentrated leftover liquid after evaporation of the methanol on the rotary evaporator, contained all of the antimicrobial substances which inhibit bacterial growth. For that reason, aqueous phase (H_2O) extract preformed a broad-spectrum antimicrobial activity against a range of Gram-positive and Gram-negative bacteria. H_2O phase extract applied to *E. coli* bacteria (Figure 3.) showed dilution-dependent growth manner. Dilution of 1:200 of this extract did not show any inhibiting properties on the growth of *E. coli* bacteria but dilution of 1:100 showed the inhibiting effect which was almost as good as positive control. It has been previously reported that an extract of another larvae species, housefly (*Musca domestica*), also showed antibacterial effects against *E. coli* and other Gram-negative bacteria (Hou et al. 2007). While the H_2O phase in higher dilution applied to *E. coli* bacteria had a minimal inhibitory effect on the growth of bacteria, same phase applied to another Gram-negative bacteria, *P. fluorescens,* inhibited its growth even more (Figure 6.). Both dilutions of H_2O phase extract applied to *P. fluorescens* bacteria, that of 1:100 and 1:200, inhibited its growth completely and the effect was comparable to the antibiotic control.

On the other hand, Gram-positive bacteria, or sometimes referred as Gram-variable bacteria , *M. luteus*, displayed interesting results when treated with H_2O phase extract. Higher dilution of 1:200 applied to this bacteria showed inhibiting properties and growth was delayed at the beginning of the measurement, while the less diluted extract (*i.e*. more concentrated) that of 1:100, showed better growth of bacteria, especially during the first hours of measurement. Interestingly, in the couple of the last hours of growth measurement of *M. luteus,* H_2O phase extract in dilution of 1:100 starts to display better inhibiting effects than the 1:200 dilution of the same extract. This variability, when the more concentrated extract showed less potent inhibiting outcome probably lies in the morphology of the the cell wall of the specific bacteria and on other bacterial mechanisms which can affect influence of anti-microbial substances (Madigan & Martinko, 2005).

This results, however, should be interpreted with caution since statistical analysis has not been made for this particular set of the data, so experimental error should be considered.

Another Gram-positive bacteria, *B. subtilis*, treated with the H_2O phase extract showed inhibiting growth in the both dilutions of the H_2O phase extract. Also, results were, as expected, concentration-wise, meaning that higher concentration had a larger effect on bacterial growth. When the results of the Gram-negative and Gram-positive bacteria for the H_2O phase extract are compared it could be seen that Gram-negative bacteria show slightly more sensitive effect when treated with this phase of extract. This observation is not completely in accordance with the results from other researches, but also on another species of larvae (*Musca domestica*), which reported that their larvae extracts showed higher antibacterial activity against Gram-positive bacteria than against Gram-negative bacteria (Hou et al. 2007). Also, it has been stated that the difference in susceptibility between Gram-negative and Gram-positive bacteria might be explained by the variations in morphological constitutions between these microorganisms. Gram-negative bacteria have an outer phospholipidic membrane comprising lipopolysaccharide components, on the contrary, the Gram-positive bacteria only have an external peptidoglycan layer which is not as an successful permeability barricade as the previous (Nostro et al., 2000).

However, results from this study show that Gram-negative bacteria are slightly more susceptible to larvae extracts, contrary to other published results, which is most likely due to the different species of larvae used in this research.

Since the aqueous (H_2O phase), used in bacterial killing assays, was used as a phase which contained majority of the AMPs, it is strongly believed that most of the anti-bacterial effects in the bacterial killing assays belong to efficient performance of AMPs. AMPs consist of 12-50 amino

acids and are capable of killing broad range of pathogens (Zasloff, 2002). These peptides are heterogenous group of molecules, showing high diversity in structure as well as speed and strategy of killing microbes. Furthermore, they are all of amphipathic and cationic nature, which is optimal for bacterial killing and increases solubility, and thus bioavailability, in aqueous body fluids like blood, urine and saliva. The positive charge allows the peptides to selectively bind to the bacterial membrane by electrostatic interaction with compounds like lipopolysaccharide (LPS, Gram-negative), phospholipids, lipoteichoic acid (LTA, Gram-positive) and peptidoglycans. Thereafter, the amphipathic nature allows them to incorporate into the lipid bilayer of the bacterial surface, which destroys the integrity of the bacterial membrane and the membrane potential thus ultimately results in bacterial lysis and killing (Yeaman & Yount, 2003).

Hermetia illucens larvae may be rich in different types of AMPs, as it is a case with other similar insect species such as *Lucilia sericata*. These types include: members of the attacin, cecropin/cecropin-like, defensins, diptericins, and proline-rich peptide families. Attacins were first discovered in the silk moth *Hyalophora cecropia* and subsequently also in several Diptera. They are characterized by a molecular mass of 20 to 25 kDa, with a high glycine residue content (Hultmark et al. 1983). Attacins are active against some Gram-negative bacteria and were shown to inhibit the synthesis of outer bacterial membrane proteins (Carlsson et al. 1991). The cecropin family of peptides comprises linear amphipathic AMPs with an α-helical structure lacking cysteine residues. The first cecropin was discovered in the silk moth *H. cecropia* (Steiner et al. 1981). They are primarily active against Gram-negative bacteria but show moderate activities toward Gram-positive bacteria, which is consistent with results in this study. Defensins are cationic AMPs with six conserved cysteine residues that form three disulfide bonds. They comprise an amphipathic α-helix, an antiparallel β-sheet, and an N-terminal loop and form voltage-dependent channels (Bonmatin et al. 1992). The majority of the defensins are strongly expressed in the salivary glands of larvae, and most of them are active against *M. luteus* bacteria. Recently lucifensin, a novel larval defensin, has been identified as one of the antibacterial agents of medicinal maggots involved in MDT (maggot debridement therapy). Lucifensin was shown to be constitutively produced in the salivary glands of all larval stages during feeding. An infectious environment could induce its production in the fat body from where lucifensin is secreted solely into the haemolymph. Lucifensin possesses antibacterial activity against Gram-positive bacteria, most notably *Streptococcus spp.* and *Staphylococcus spp*., but it fails to inhibit the growth of *E. coli* and *P. aeruginosa* (Jasenak et al. 2013.)

Diptericins represent a multigene family of closely related inducible antibacterial peptides and are

produced in the fat body of the larvae (Reichhart et al. 1989). Proline-rich peptides are linear molecules that consist of 14 to 39 amino acid residues, of which >25% are proline, often arranged in triplets with basic residues, like arginine and histidine. Most proline-rich AMPs act selectively against Gram-negative bacteria but have little impact on Gram-positive bacteria (Bulet & Stöcklin 2005).

Since the ethyl acetate and chloroform phases also inhibited bacterial growth (Figures 3.-7.), it is strongly believed that this phases have other anti-bacterial compounds different than AMPs. It has been previously reported that the whole-body extracts and haemolymph fractions from non-sterile maggots contain low molecular weight compounds which can lyse Gram-positive and Gram-negative bacteria including *P. aeruginosa*, *K. pneumoniae* and MRSA isolated from wounds. These low molecular weight compounds were identified as p-hydroxybenzoic acid (138 Da), p-hydroxyphenylacetic acid (152 Da) and octahydro-dipyrrolo[1,2-a;1',2'-d] pyrazine-5,10-dione (194 Da), also known as the cyclic dimer of proline (or proline diketopiperazine or cyclo[Pro,Pro]) (Huberman et al. 2007).

From the biotechnological and clinical points of view, the molecules with low molecular weight are more interesting due to their easier production, thermal and chemical stability and penetration properties. Novel antibacterial molecules with low molecular weight have been identified (Nigam et al. 2010).

One of them is seraticin, which is registered as a novel antibiotic able to inhibit 12 different strains of MRSA, as well as *E. coli* and *C. difficile* (Bexfield et al. 2008).

Since it has been shown previously that seraticin kills *E. coli* bacteria it could be concluded that this compound could be present in ethyl acetate phase of *Hermetia illucens* extract which showed killing of *E. coli* (Figure 3.). Less diluted ethyl acetate extract showed complete inhibition of bacterial growth while the more diluted sample showed less but still significant inhibition. On the other hand, chloroform phase did not show such inhibiting effect on the *E. coli* so it is probably absent of seraticin compound (Figure 3.).

Furthermore, when *Hermetia illucens* organic waste fed larvae extracts and organic waste fed larvae faeces extracts were applied in dilution of 1:250 and 1:1000 respectively, they showed even greater inhibiting effect in all three phases than the normal fed larvae. Possible cause of the stronger anti-bacterial effect of these extracts is that the food, which possibly contains parasites such as bacteria, fungi or viruses could induce immunoreaction in the larvae which can lead to the greater and more

concentrated production of anti-microbial compounds within the larvae. Interesting fact is that the

feces extracts from *Hermetia illucens* organic waste fed larvae showed strong inhibiting effect in high dilutions such as 1:1000 which can lend support to the further concentration of the researches to go in that direction.

Moreover, it has been shown that septically injured larvae with a bacteria-contaminated needle after extraction shows stronger anti-bacterial effect than the non infected larvae.

Further investigation and research in the matter of getting more effective anti-microbial substances would go in direction of purification of the different phases of extracts to get pure antimicrobials. Park et al. used water-soluble fraction of the larval extract which was applied to Sep-Pak C18 cartridges, and eluted with each 20 mL of 10%, 20%, 30%, 50%, and 80% ACN. Antibacterial activities of the Sep-Pak C18 eluants were measured by inhibition zone assay against MRSA, E. coli, and *B. subtilis*. Significant anti-MRSA activity was observed at the fraction eluted with 10% ACN. Also, purification of the extracts can include different methods such as HPLC.

4.2. Cytotoxicity assay

In order to understand the characteristic of the cytotoxicity effect of *Hermetia illucens* larvae extract on human cells, two cells lines were selected to be investigated throughout this study; HepG2 and NHDF. As a class of drugs, antimicrobials are particularly troublesome with regard to cytotoxicity, as their significant role is to ultimately achieve microbial cell death (Mandell et al. 2001). For example, some antimicrobial peptides may provide benefit at lower antimicrobially active concentrations with regard to prevention of infected wounds; however, at higher concentrations they may exhibit cytotoxicity that might adversely affect wound healing (Chalekson et al. 2003).

Antiseptic agents whose main mechanism of action is the targeting of membranes pose problems for therapeutic use in that they will exert a detergent-like effect, compromising both microbial and mammalian cell membranes due to antimicrobial and cytotoxic effects (Duc et al. 2007). Cationic antimicrobial peptides have been shown to exert their antimicrobial effect by selective permeabilization of predominantly negatively charged bacterial membranes (Laverty et al. 2011). However, just like antimicrobial action, cytotoxicity may be produced by a multitude of

mechanisms. The treatment of cells at toxic concentrations can result in loss of membrane integrity and cell lysis, with resulting leakage of cytoplasmic cell contents, in a process termed necrosis. Alternatively the compound may invoke genetically programmed cell death, called apoptosis, where cells stop actively dividing and therefore growing (Wyllie et al. 1980).

Aqueous phase of the *Hermetia illucens* larvae extract showed stronger cytotoxic effect, compared to both chloroform and ethyl acetate phase, when applied to HepG2 and NHDF cells respectively (Figures 8.-11.). It suggests that the main cytotoxic effects on the cells are actually caused by AMPs since it is known that H_2O phase,compared to other two phases, should contain most of the AMPs. Cytotoxicity followed dose dependent manner so that the less diluted phase of extract showed stronger toxic effect on the cells.

4.3. Future prospects of *Hermetia illucens* extracts regarding wound healing

Next steps of the research with *Hermetia illucens* larvae extracts would include application of all of the phases of extracts to NHDF cells *in vitro* and monitoring of the changes in expression of the molecular markers of wound healing. Since it is well known that MDT improves wound healing significantly when live larvae are applied to non-healing wounds it would be fair to expect that extracts would increase expression of different proteins which drive wound healing process. Also, extracts could be tested alone with the RT-PCR to quantify if they consist growth factors or other molecules which could improve wound healing.

Since the wound healing process includes repair and restoration in the affected tissue and also scar formation and finally wound closure, keratinocyte migration could be measured after application of extracts. Most likely, extract treated keratinocytes or fibroblasts would show faster migration and scar closure compared to mock treated cells but further experiments needs to be done.

5. Conclusion

For centuries, larvae were known to have beneficial effects on wounds. One of the ‘old’ techniques in wound care is maggot debridement therapy (MDT). In MDT, live and ‘medical-grade’ fly larvae are applied to the patient's wounds to achieve debridement, disinfection, and, ultimately, wound healing. MDT is indicated for open wounds and ulcers that contain gangrenous or necrotic tissues with infection.

This study has investigated *Hermetia illucens* larvae extracts influence on the disinfection step in the wound healing process. Different phases of the extracts were applied to four bacterial species and it was shown that they had a capacity of growth inhibition. Different phases have different substances which can inhibit bacterial growth and therefore they showed varying magnitude in inhibiting bacterial growth. Aqueous phase of the extract possibly contains AMPs which can inhibit bacterial growth. Chloroform and ethyl acetate very likely have other anti-microbial substances such as large anti-bacterial proteins and divergent derivatives of benzoic acid which also posses capacity for bacterial elimination. Bacterial growth inhibition was efficient with dilutions starting from 1:100 and going up to more diluted 1:200 (most potent when applied to *P. fluorescens* bacteria in all phases), but some of the dilutions showed efficacy in very high dilutions of 1:1000 (H_2O phase organic waste fed larvae´s feces applied to *E. coli*)

Furthermore, extracts applied to human cells in different concentration showed toxicity just in highest concentrations. In addition to that, most of the extracts in two concentrations used to kill bacteria did not show cytotoxic effects on human cells. This results are consistent in both human cell lines tested.

The emerging roll out of antibiotic resistant bacteria (specifically *K. pneumoniae* and MRSA) in combination with the lack of new antibiotics means new strategies to treat resistant bacteria are urgently needed, especially in treatment of non healing wounds. Major advantage in *Hermetia illucens* extracts killing approach of bacteria, in contrast to traditional antibiotics, is the fact that several effector systems will be activated simultaneously (different AMPs, larger antimicrobial proteins and benzoic acid derivatives). This will practically make it impossible for bacteria to develop resistance and will consequently improve disinfection of wounds and the wound healing process in general.

6. References

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7. Curriculum vitae

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WORKING EXPERIENCE

2015-member of the election board on the Croatian national elections for the parliament

2012-present Occasional part-time job at Croatian Radiotelevison.

2013-present, Article writing for the National Geographic Magazine Croatia

2008-Tennis coach at Tennis club Gornji Grad

EDUCATION AND TRAINING

2013-present	Graduate university study of molecular biology (University of Zagreb, Faculty of Science)
2010-2013	Bachelor of molecular biology (univ.bacc.biol.mol) Undergraduate study of molecular biology (University of Zagreb, Faculty of Science)
2006-2010	V. Gymnasium Zagreb – general education

PROFESSIONAL EXPERIENCE

Internships Internship at AMP group at Division of Clinical Microbiology, Department of Laboratory Medicine Karolinska Institutet, Huddinge Alfred Nobels allé 8, Huddinge, Sweden, 141 52 Stockholm (Sweden) http://ki.se/en/labmed/division-of-clinical-microbiology-

Conferences International Alumni Symposium: Efficacy and Safety of Medical Plants and Dietary Supplements (Dresden, October 2015)

The Third Meeting of the Croatian Association for Cancer Research (Zagreb, November, 2014)

Workshops	Scientific publishing and intellectual property protection in life science (Zagreb, November, 2014)
Summer schools	International Summer School: Plant derived polyphenols as drug leads (Dresden, October, 2015)
Other	Undergraduate teaching assistant in Zoology (September 2011 – February 2012)

PROFESSIONAL SKILLS

Other languages	English – level C1 German – level B1