

**Biopharmaceutical investigations of the effects of immune-  
modulatory plant extracts and phytochemicals on lysozyme  
expression in human cells**

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**RACHA HELAL**  
from Syria

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**ABSTRACT**

Plants are an important source to identify clinically useful and safe natural products that can support the immune system and alleviate or prevent bacterial, viral and even tumor diseases. Lysozyme is one of the most important factors of innate immunity and a unique enzymatic in that it exerts not only antibacterial activity, but also antiviral, anti-inflammatory, anticancer and immunomodulatory activities. As bacteria become progressively more resistant to exogenously applied antibiotics, herbal medicines that affect innate immunity components, such as lysozyme, may offer new therapeutic approaches. There is only little data in the literature concerning the influence of medicinal plants and natural products on the production and release of lysozyme activity in human cells. The principal purpose of the present work was to investigate medicinal plants and natural products as potential resources for identifying immune-modulatory agents through affecting lysozyme activity levels secreted from the human cells, including several herbal extracts and its isolated phytochemicals, saponins, essential oils, flavonoids and propolis. We have investigated several methods to determine lysozyme activity of human cells. This work represents a new fluorescence-based assay, which has been proved to be highly sensitive method for detecting lysozyme activity at low levels e.g. in cell culture systems. The classical turbidimetric microplate assay has been illustrated to provide an inexpensive method with less sensitivity in comparison with the fluorometric assay. Contrary to methods such as ELISA, in which lysozyme is detected only as a protein, the sensitive fluorometric method used in our experiments can measure directly the activity of lysozyme. Because the level of active lysozyme may be more relevant for elucidating the biological properties and the defensive role of this protein, the fluorometric assay would provide more meaningful results for assessing the biological activity of the tested substances than that provided by the enzyme-linked immunosorbent assay (ELISA). Furthermore, we have identified human monocytic and epithelial cell systems suitable for lysozyme-regulation studies that can produce and release detectable amounts of lysozyme activity in cell cultures. By *utilizing* these cell systems we could find several herbal, bacterial and synthetic products as stimulators or inhibitors of lysozyme activity levels in human cells. Demonstrated ability of several herbal extracts and phytochemicals to enhance the release and/or production of lysozyme activity in monocytes and epithelial cells has presented a novel mechanism of action that contributes to explaining important biological characteristics of these natural products, including antibacterial, antiviral, anti-tumor, anti-ulcer, anti-inflammatory and immune-stimulating properties. This work has presented for the first time novel immune-stimulatory activity of tomatine, aconitine and other natural products through the stimulation of the secretion of lysozyme, the important defense molecule of the innate immune system, which is able to control the growth of susceptible bacteria and to modulate host immunity against infections and depressions of immune responses.

## ZUSAMMENFASSUNG

Pflanzen sind eine wichtige Quelle, um klinisch nützliche und sichere Naturprodukte zu identifizieren, die das Immunsystem unterstützen und bakterielle, virale und Tumor Erkrankungen abwehren oder lindern können. Lysozym ist einer der wichtigsten Faktoren der angeborenen Immunität und ein Enzym-basierendes Antibiotikum, das auch antivirale, antiinflammatorische, antitumorale und immunmodulatorische Wirkung aufweist. Da Bakterien zunehmend resistent gegen exogen applizierte Antibiotika geworden sind, können pflanzliche Arzneimittel, die wichtige Komponenten der angeborenen Immunität wie Lysozym beeinflussen, neue therapeutische Ansätze bieten. Es gibt nur sehr wenige Daten in der Literatur über den Einfluss von Heilpflanzen und Naturstoffen auf die Produktion und Freisetzung von Lysozym-Aktivität in humanen Zellen. Der Hauptzweck der vorliegenden Arbeit war es, Heilpflanzen und Naturstoffe als potenzielle Ressourcen für die Identifizierung immunmodulatorischer Substanzen durch Beeinflussung der Lysozym-Aktivität in humanen Zellen zu untersuchen, darunter mehrere Drogenextrakte und seine isolierte Pflanzenstoffe, Saponine, ätherische Öle, Flavonoide und Propolis.

Wir haben verschiedene Methoden untersucht, um den Lysozymgehalt humaner Zellen zu bestimmen. Diese Arbeit stellt einen neuen Fluoreszenz-Assay vor, der sich als hochempfindliche Methode zur Bestimmung von Lysozym-Aktivität in niedrigen Konzentrationen z.B. in den Zellkultur-Systemen erwies. Der klassische turbidimetrische Mikrotiterplatten-Assay wurde als kostengünstige Methode mit geringerer Empfindlichkeit im Vergleich zu dem fluorimetrischen Assay verwendet. Im Gegensatz zu Methoden, wie ELISA-Verfahren, in denen Lysozym nur als Protein nachgewiesen wird, kann die in unseren Untersuchungen verwendete sensitive fluorimetrische Bestimmungsmethode die Lysozym-Aktivität direkt messen. Da für die antibakteriellen und immunmodulierenden Eigenschaften des Lysozyms seine enzymatische Aktivität entscheidend ist, bietet die fluorimetrische Bestimmungsmethode eine sicherere Basis zur Beurteilung der biologischen Aktivität der eingesetzten Stoffen im Vergleich zum ELISA-Technik.

Darüber hinaus wurden für Lysozymregulationsstudien geeignete Zelllinien humaner Monozyten und Epithelzellen identifiziert, die nachweisbare Mengen von Lysozym-Aktivität in Zellkulturen produzieren und freisetzen konnten. Durch die Nutzung dieser Zellsysteme konnten mehrere pflanzliche, bakterielle und synthetische Produkte als Stimulatoren oder Inhibitoren von Lysozym Aktivität in menschlichen Zellen getestet werden.

Die nachgewiesene Fähigkeit von mehreren Drogenextrakten und Naturstoffen, die Freisetzung und/oder Produktion von Lysozym-Aktivität in Monozyten und Epithelzellen zu steigern, zeigte einen neuartigen Wirkmechanismus, der wichtige biologische Eigenschaften wie antibakterielle, antivirale, anti-inflammatorische, antitumorale, anti-Ulcus und immunstimulierende Eigenschaften der eingesetzten Stoffe erklären kann.

Diese Arbeit konnte zum ersten Mal eine neuartige immunstimulierende Wirkung von Tomatin, Aconitin und vielen anderen Naturprodukten durch Stimulation der Lysozymaktivität vorstellen.

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**Abbreviations**

ATCC	American type culture collection
BITC	Benzyl isothiocyanate
dH <sub>2</sub> O	Deionized water
DMSO	Dimethyl sulfoxide
DSMZ	German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)
<i>E. coli</i>	<i>Escherichia coli</i>
EGCG	Epigallocatechin gallate
ELISA	Enzyme linked immunosorbent assay
EtOH	Ethanol
GC	Gas chromatography
GCs	Glucocorticoids
hIFN- $\gamma$	Human Interferon-gamma
HPLC	High-performance liquid chromatography
IC <sub>50</sub>	Half maximal inhibitory concentration
ILs	Interleukins
kDa	Kilodalton
LPS	Lipopolysaccharides
NAG	N-acetylglucosamine
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
RA	Retinoic acid
rhIFN- $\gamma$	Recombinant human interferon-gamma
SD	Standard deviation
SFE-CO <sub>2</sub>	Supercritical carbon dioxide fluid extraction
TAC	N,N',N''-triacetylchitotriose
TLC	Thin layer chromatography
TNF- $\alpha$	Tumor necrosis factor - alpha
UACL	Ulcer-associated cell lineage

## 1. INTRODUCTION

### 1.1. Discovery of Lysozyme

Lysozyme (1,4-*N*-acetylmuramidase, E.C.3.2.1.17) is a small cationic protein first reported by Laschtschenko in 1909 (Lawton, 2006). Lysozyme's discovery however is attributed to Alexander Fleming, a bacteriologist in London, who was the first to report the finding to the Royal Society of London. Alexander Fleming (1881-1955), of penicillin fame, wrote in his communication to the Royal Society: '...I wish to draw attention to a substance present in the tissues and secretions of the body, which is capable of rapidly dissolving certain bacteria. As this substance has properties akin to those of ferments I have called it a Lysozyme...'  
(Fleming, 1922). Fleming was at that time suffering from a cold and he was reported to have allowed drops of his nasal secretions to have fallen onto a blood agar culture plate which was thickly colonized with bacteria. The plates were incubated at 37°C for 24 hours; bacteria grew extensively. There was complete radial inhibition of about 1cm beyond the nasal mucus on the plate. This experiment was later termed the lysoplate. Fleming concluded that the nasal secretions contained an enzyme capable of bacterial lysis; later named lysozyme.

Fleming reported the discovery of a small round bacterium, particularly vulnerable to the effects of lysozyme. This bacterium was named *Micrococcus lysodeikticus* (also referred to as *Micrococcus luteus* or *M. luteus*) due to its ability to display lysis (Fleming, 1922). With the success of his preliminary experiment, Fleming continued to work with lysozyme testing its antibacterial properties with several different bacteria species.

### 1.2. Structure of Lysozyme

Since the initial discovery of lysozyme in 1922 in tears and nasal secretions, huge literature has accumulated on its structure, function, genetics, biosynthesis, regulation, enzyme activity, and properties. Lysozymes are divided into types according to their sequence similarity and three-dimensional structure. These types are c-type (chicken-type lysozyme), including the stomach lysozyme and insect lysozyme; goose-type lysozyme (g-type); plant lysozyme; bacterial lysozyme; and T4 phage-lysozyme (phage-type). c-Type lysozymes have been isolated from many vertebrates and also from insect species (Villa & Crespo, 2010).

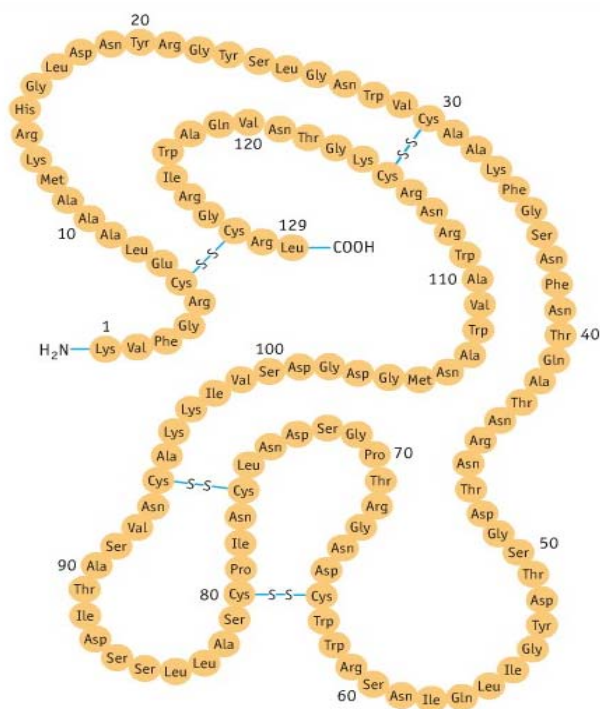
Human lysozyme has ~148 amino acids and is 3-4 times more reactive than HEW lysozyme as tested with the turbidimetric test. Hen egg white (HEW) lysozyme is very similar to human lysozyme with 129 amino acids (Jollès, 1969). However, due to its structural similarity, availability and inexpensiveness, HEW lysozyme has been used as a model for human lysozyme experiments, making it one of the most studied enzymes (Lollike *et al.*, 1995).

Lysozyme was the first protein to be sequenced and whose three-dimensional structure was completely analyzed. In 1965, the 3 dimensional structure of lysozyme was determined by David Philips and his colleagues (Blake *et al.*, 1965). Considerable physicochemical

information is available for lysozyme. Structure-function relationships are thoroughly reviewed by Imoto *et al.* (1972) and Phillips (1972). Chipman & Sharon (1969) have provided descriptions of the enzyme and its catalytic mechanism. Basic chemical and physical properties of the enzyme from hen egg white are shown in Table 1 (Huopalahti *et al.*, 2007).

Property	Value
Molecular weight	14,400
No of subunits	1
Amino acids	129
pI	10.7
Disulfide bonds	4
% Carbohydrate	0
E <sup>1%</sup> 280 nm	26.4
Thermal D at 93°C (Time in min to destroy 90% of activity)	110
Assay of enzyme activity	<i>Micrococcus lysodeikticus</i> lysis by turbidity

**Table 1.** Chemical and physical properties of the enzyme from egg white.



**Fig.1.** The primary structure of lysozyme. The cross-chain disulfide links (-S-S-) are links between cysteine amino acid residues.

Human Lysozyme is a ~148 amino acid single polypeptide chain with a low molecular weight of around 14.6kDa (Cabellero *et al.*, 1999). There are four disulfide bridges that stabilize the molecule in a compact ellipsoidal shape (Jollès & Jollès 1984; Mestecky *et al.*, 2005) (Fig.1). Its elevated isoelectric point (pI 11) means lysozyme is a cationic protein, positively charged at biological pH's (Sophianopoulos & Sassa, 1965). Lysozyme is recognized to be soluble in water and weak saline, insoluble in alcohol and ether, resistant to heat and desiccation, and stable at room temperature (Lawton, 2006), stable at acid pH and labile at alkaline pH (Jollès, 1969).

### 1.3. Determination of lysozyme

Since lysozyme has been widely applied in medicine and immunohistochemistry, as well as in gene and cell engineering, many strategies have been developed to determine it.

Quantitative lysozyme determinations are performed on two bases:

➤ **Lysozyme assay methods relied on the protein itself**, such as the electrophoretic, chromatographic, immunoenzymatic, spectrophotofluorometric, and fluorescence polarization techniques. For example, the enzyme-linked immunosorbent assay (ELISA) was reported to be sensitive, specific and convenient, especially for analysis of large number of samples (Vidal *et al.*, 2005). More recent is the resonance light-scattering technique with functionalized CdTe (cadmium telluride) nanoparticles with sensitivity at the nanogram level (Li *et al.*, 2007). Another new analytical procedure for assay of lysozyme as protein at nanogram level using immobilized reagents in flow injection chemiluminescence (CL) systems was presented in literature. These reagents, including luminol and periodate, were immobilized on the anion-exchange column in the flow injection system. Through water injection, luminol and periodate were eluted from the anion-exchange column to generate chemiluminescence, which was inhibited in the presence of lysozyme. By measuring the decrease of CL intensity, lysozyme could be analyzed quantitatively (Song & Hou, 2003).

➤ **Lysozyme assay methods relied on lysozyme lytic activity against the cell wall of *Micrococcus lysodeikticus* as substrate.** The reaction course is most often followed turbidimetrically, nephelometrically or fluorometrically in the liquid phase. The method can also be applied to the solid phase with agarose gel as reaction medium. The catalytic activity of lysozyme is dependent on the pH, ionic strength, substrate concentration and sodium and potassium concentrations of the reaction mixture. The effect of these factors is interdependent, so that it is difficult to evaluate properly the effects of any one parameter separately. Albumin enhances the reaction rate, and probably there are also more complicated interactions due to other proteins (Mörsky, 1983). In the agar diffusion method lysozyme catalyzes the hydrolysis of *Micrococcus lysodeikticus* around it while incubating together, resulting in a transparent ring. This method is simple, but it is time-consuming, sometimes more than 10 hours and easily interfered by other proteins. Besides, huge relative

error exists because it is hard to control the thickness of agar, definition of the penumbra, and it is not very accurate when measuring the diameter. Another method using agarose is agarose rocket electrophoresis, in which the bacteria could be resolved when the lysozyme moved in the electric field and reacted with the substrates in the gel, forming a transparent peak of which the value was proportional to the logarithm of lysozyme concentration. The turbidimetric method is simple and quick but with low sensitivity and narrow linear ranges. Lee and Yang improved this assay by using a microplate format. Resonance scattering spectral (RSS) assay is also a new method for the determination of lysozyme activity with high sensitivity, good selectivity, basing on the catalytic effect of lysozyme on the hydrolysis of *Micrococcus lysodeikticus* and its resonance scattering effect (Jiang & Huang, 2007).

#### **1.4. Lysozyme activity, function, origin & distribution**

Lysozyme (or muramidase) is a ubiquitous enzyme present in a wide range of biological fluids and tissues within animal and plant kingdoms. Plant lysozyme is found in ficus and papaya latex, and is chemically distinct from the egg white enzyme (Meyer *et al.*, 1946).

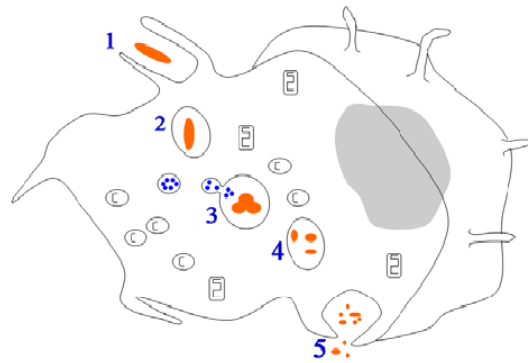
Human lysozyme is present in lysosomes of phagocytic cells, granulocytes and monocytes (Burgess *et al.*, 1994). It is released as part of the non-specific immune response, and exists among cells of the blood system, especially leukocytes. Lysozyme is found in all stages of the maturation of myelocytic series, but not in myeloblast, eosinophil or basophil (Davis, 1971). Monocytes contain large amounts of lysozyme, but none is found in lymphocytes.

Figure 2 shows the phagocytosis of bacteria (Lawton, 2006). At stage one the bacterium attaches to the pseudopodia of the phagocyte. By stage two the bacterium has been ingested inside a phagosome, a small pocket inside the phagocyte. Granules in the phagocyte act as storage for lysozyme, acid hydrolases, myeloperoxidases and complement activators (Farthman *et al.*, 1998). These granules move towards the phagosome and merge. At stage 3 the phagosome membrane fuses with the granules, releasing the lytic enzymes into the phagosome. By stage 4, digestion by the lytic enzymes begins and at stage 5 the digested bacterium is released from the cell.

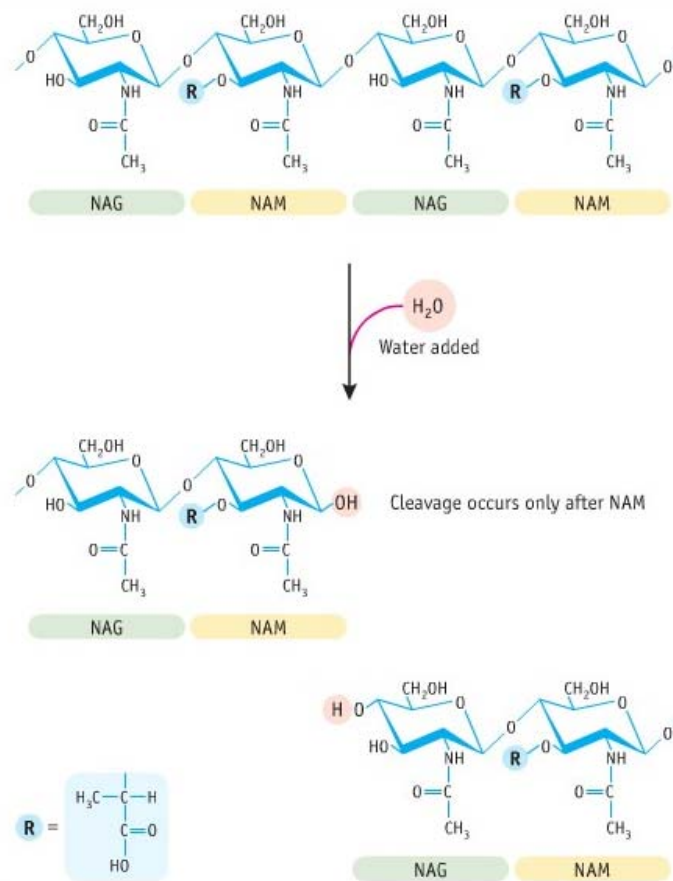
In tissues lysozyme is mainly found in bone marrow, lungs, intestines, spleen, and kidneys. Lysozyme exists here partly due to the breakdown of neutrophilic granulocytes in these organs (Hansen *et al.*, 1972). Tissue macrophages discharge lysozyme into serum, nasal and lacrimal secretions along with various other bodily secretions (Lawton, 2006).

In addition, lysozyme of lacrimal secretions is synthesized by specialized cell type in the main and accessory lacrimal glands. This enzyme is one of the most important nonspecific antibacterial factors in tears (Aho *et al.*, 1996).

In human saliva, lysozyme is produced by the salivary glands and by oral phagocytes derived from the gingival crevices (Korsrud & Brandtzaeg, 1982; Moro *et al.*, 1984).



**Fig.2.** Phagocytosis: The attachment of the bacterium to the pseudopodia (1), encapsulation in the phagosome (2), merging of phagosome and granules (3), digestion of bacterium (4) and excretion (5).



**Fig.3.** Cleavage of a bond between *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG). This reaction is accelerated by the enzyme lysozyme.

In human lung, lysozyme is secreted by serous cells of the submucosal glands and by airway epithelial cells (Dubin *et al.*, 2004; Basbaum *et al.*, 1990). Immunodepletion of lysozyme has been proved to decrease antibacterial activity in human airway and nasal secretions by about 50%, providing *in vivo* evidence that lysozyme is a major component of airway host defense (Dajani *et al.*, 2005). Mice and rats have relatively few submucosal glands but secrete



lysozyme with pulmonary surfactant from alveolar type II epithelial cells (Choi *et al.*, 2000, Singh *et al.*, 1988); in addition, macrophages also secrete lysozyme into the alveolar airspaces (Gordon *et al.*, 1974). Elevated concentration of lysozyme in the alveolar airspaces of transgenic mice conferred resistance to infection by *Pseudomonas aeruginosa* or group B *Streptococcus* and significantly enhanced survival (Akinbi *et al.*, 2000); in contrast, lysozyme deficiency increased bacterial burden and mortality following intratracheal infections (Markart *et al.*, 2004; Cole *et al.*, 2005). Thus, lysozyme plays an important role in innate host defense of the lungs (Nash *et al.*, 2006).

The principal function attributed to lysozymes in most animals is host defense (Jollès & Jollès, 1984). Lysozyme exerts its antibacterial activity by promoting the degradation of the prokaryotic cell wall of a variety of microorganisms (Francina *et al.*, 1986; Masschalck & Michiels, 2003). When the bacteria's cell wall is broken down the cell bursts due to the high internal osmotic pressure and the cell lyses. Bacterial cell walls are highly complex macromolecular structures, resistant to most solvents. The cell wall is composed of polysaccharide chains *N*-acetylglucosamine (NAG) and *N*-acetylmuramate (NAM). Muramic acid residues attached by amide bonds through the carboxyl groups to the peptide moieties act as bridges linking the polysaccharide chains, known as the "murein sacculus" (Weide & Pelzer, 1964; Jollès and Jollès, 1984). Lysozyme exerts its effects on the tetrasaccharide structure of the cell wall and hydrolyses the NAG and NAM  $\beta$ -(1→4)-glycosidic linkage (Fig.3). The result is the death of the bacterium (Sharon, 1967; Lawton, 2006).

In addition to the antibacterial activity, lysozyme exhibits antiviral, antitumor and immune modulatory activities. Soluble fragments released by lysozyme degradation of peptidoglycan may play a role in immunomodulation in both vertebrates and invertebrates (Sava *et al.*, 1989b; Sava *et al.*, 1995; Vidal *et al.*, 2005; Park *et al.*, 2007).

Moreover, lysozyme can also exert antimicrobial activity against bacteria and viruses through a mechanism independent of its muramidase activity (During *et al.*, 1999; Nash *et al.*, 2006).

### **1.5. Diagnostic uses of lysozyme**

Literature reports have indicated that determination of lysozyme levels in body fluids is helpful in the diagnosis of several diseases and infections, presenting lysozyme as an important marker for diagnosis and disease progression. Some of these diagnostic uses are given in Table 2.

Body fluid	Diagnostic use of measurement	Literature
Serum lysozyme	Peritoneal tuberculosis	Velayati <i>et al.</i> , 1994
	intrathoracic lymphadenopathy	Lodhat & Mir, 1980
	Leprosy	Near & Lefford, 1992
	Ocular sarcoidosis	Baarsma <i>et al.</i> , 1987
	Non-tubercular respiratory tract diseases	Marino <i>et al.</i> , 1982
	Pancytopenia	Iavorkovskii <i>et al.</i> , 1978
	Differential diagnosis of acute leukaemia	Jaworkowsky <i>et al.</i> , 1972
	Sepsis	Lawton, 2006
Cerebrospinal fluid lysozyme	Neurological disease	Firth <i>et al.</i> , 1985
	Central-nervous-system tumours	Di Lorenzo & Palma, 1976
	Tuberculous meningitis in children	Mishra <i>et al.</i> , 2003
	Ventriculitis	Schroeder <i>et al.</i> , 2000
	Differential diagnosis of bacterial & viral meningitis	Babich <i>et al.</i> , 1992
Urinary lysozyme	Rejection of transplanted kidney	Bastecka <i>et al.</i> , 1979 Ellis <i>et al.</i> , 1978
	Differential diagnosis of chronic glomerulonephritis & chronic pyelonephritis	Tofilo & Antonov, 1988
Tears lysozyme	Facial paralysis	Hara <i>et al.</i> , 1987
	Dry eye	Rong, 1984
Sperm lysozyme	Male infertility	Kuz'min <i>et al.</i> , 1991
Faecal lysozyme	Chronic inflammatory bowel disease	Dick, 1982

**Table 2.** Some diagnostic uses of lysozyme.

### 1.6. The therapeutic effectiveness of lysozyme

Efficacy of lysozyme as treatment of many diseases has been frequently demonstrated. Some of these therapeutic uses are shown in Table 3.

The therapeutic effectiveness of lysozyme is actually based on its ability to control growth of susceptible bacteria and to modulate host immunity against infections and depressions of immune responses. If the former is based on the first evidence of the biological activity of this enzyme, the second is a relatively recent acquisition of extreme importance for the possibilities offered in terms of regulation of host's immune system functioning (Sava, 1996).

Lysozyme preparations administered per os or parenterally are absorbed to blood circulation and distributed among various organs in an active form and maintaining their antigenic specificity. It may speak for their direct anti-inflammatory and immunomodulatory effects in respiratory, urinary, digestive and other systems (Lukaszyk *et al.*, 2001).

<b>Therapeutic use</b>	<b>Literature</b>
Treatment of chronic crural ulcerations & suppurative wounds of the soft tissues.	Gasior-Chrzan, 1988 Timofeev <i>et al.</i> , 1990
Transendoscopic treatment of gastroduodenal ulcers	Bazlov <i>et al.</i> , 2001
Local treatment of corneal ulcers	Mehra <i>et al.</i> , 1975
Treatment of acne	Villa & Crespo, 2010
Prophylaxis of infections due to skin piercing	Villa & Crespo, 2010
Treatment of some viral skin diseases as herpes zoster, viral warts, aphthous stomatitis	Cavicchini, 1984
Treatment of juvenile laryngeal papillomatosis	Altamar-Ríos, 1990
Treatment of periodontitis	Proctor & Cunningham, 1988
Treatment of exudative forms of chronic allergic sinusitis of microbial etiology	Mostovoi & Gleevoi, 1974
Treatment of tracheitis, pneumonia, amyglalitis & faucitis (aerosolized lysozyme).	Villa & Crespo, 2010
Analgesic agent & adjuvant treatment for cancer patients (immunomodulator , antimetastatic).	Proctor & Cunningham, 1988 Yamaoka & Yoshioka, 1983 Bianchi, 1983 Sava <i>et al.</i> , 1988 Sava, 1989a
Adjuvant treatment of dysentery in combination with monomycin.	Vereshchagin <i>et al.</i> , 1985
Adjuvant treatment in combination with ampicillin. (Lysozyme promoted an increase in the ampicillin concentration in the lymphatic system, blood and tissues and prolonged its antibacterial activity).	Shcherbakova <i>et al.</i> , 1990
Treatment of hair loss disorders (as dimer).	Villa & Crespo, 2010
Component of oral health products	Villa & Crespo, 2010

**Table 3.** Some of the therapeutic and prophylactic uses of lysozyme.

Based on these biological properties, in addition to the wide range of therapeutic activities for which lysozyme was exploited in the past, at present one of the most promising data concern the prevention of bacterial cariogenesis and treatment of cancer patients to improve the effectiveness of anticancer drugs or to allow the host to recover from the immune suppression caused by anticancer treatments (Sava, 1996).

Many studies have confirmed the tumor-inhibitory activity of lysozyme treatment in a number of experimental tumors. All of them emphasized tumor inhibitory effects after administration of lysozyme by various routes including admixture with tumor cells, peritumor and intratumor treatments, or indirectly by systemic injections and oral treatment. From these observations, at least two possible mechanisms of action can be derived, both involving the activation of the immune reactivity of the host. Lysozyme can directly activate immune cells or it can increase tumor cell immunogenicity. Alternatively, lysozyme can liberate substances from bacteria (peptidoglycans and/or polyribopyrimidinic acids) responsible for immunopotential and therefore antitumor activity (Sava *et al.*, 1989). In spite of reported interesting findings, lysozyme has no established place in the treatment of human tumors.

Another promising therapeutic effectiveness of lysozyme is the treatment of viral infections. For example, many studies have demonstrated the human lysozyme as highly active anti-HIV component, and this anti-HIV activity of lysozyme has been found to be independent of its muramidase activity (Lee-Huang *et al.*, 2005).

### **1.7. Enzybiotics and their potential applications in medicine**

Over the last decade, a dramatic increase in the prevalence of antibiotic resistance has been noted in several medically significant bacterial species (Hawkey, 2008). This unfavorable situation is further aggravated by a shortage of new classes of antibiotics with novel modes of action that are essential to contain the spread of antibiotic-resistant pathogens (Livermore, 2004). In fact, some infectious disease experts have expressed concerns that we are returning to the pre-antibiotic era (Larson, 2007). Therefore, there is an urgent need to develop novel antibacterial agents to eliminate multidrug-resistant bacteria (Breithaupt, 1999). A very interesting class of novel (at least in terms of their formal clinical use) antibacterials is the enzybiotics.

The term “enzybiotic” was used for the first time in a paper by Nelson *et al.* (2001) to designate bacteriophage enzymes endowed with bacterial cell wall-degrading capacity that could be used as antibacterial agents.

In view of the ever-increasing antibiotic resistance of bacteria, the most important characteristics of enzybiotics are a novel mode of antibacterial action, different from those typical of antibiotics, and the capacity to kill antibiotic-resistant bacteria (Borysowski *et al.*, 2006). Another significant feature of some lytic enzymes is the low probability of developing bacterial resistance (Kusuma *et al.*, 2007).

Well-known enzymotics are lysozymes, including hen egg white lysozyme and human lysozyme. There has been interest in lysozyme as a "natural" antibiotic (Glynn, 1968). Lysozyme is unique enzymotic in that it exerts not only antibacterial activity, but also antiviral, anti-inflammatory, anticancer, and immunomodulatory activities (Sava, 1996). Lysozyme is also the only peptidoglycan hydrolase that has been used on a larger scale in humans for the past several decades. Although the best known mode of antibacterial action of lysozyme is based on the enzymatic cleavage of peptidoglycan, in fact it can also kill bacteria by some non-enzymatic mechanisms. First, lysozyme, in view of its cationic nature, can activate bacterial autolytic enzymes (autolysins). The second nonenzymatic mechanism by which lysozyme can kill bacteria is cytoplasmic membrane destabilization resulting from the removal of divalent ions from the membrane surface (Masschalck & Michiels, 2003).

Generally, lysozyme is capable of killing only Gram-positive bacteria, while Gram-negative bacteria are resistant owing to the presence of the outer membrane. It is also worth mentioning that several modifications of the lysozyme molecule have been developed to enable the enzyme to kill Gram-negative bacteria. These are essentially based on coupling lysozyme to molecules facilitating the penetration of the outer membrane (e.g., fatty acids and hydrophobic peptides) (Ibrahim *et al.*, 2002; Masschalck & Michiels, 2003).

For the past several decades, lysozyme has been used, often combined with antibiotics, in the prophylaxis and treatment of different bacterial infections (Sava, 1996).

### **1.8. Aims & objectives of the project**

An increasing number of individuals are gradually adopting complementary and alternative medicine approach for the benefit of their personal health and need to complement conventional care system. Even with the growth in understanding of the human immune system and inflammatory mechanisms and increasing developments in antibiotics; there is a need to identify clinically useful and safe natural products from the plant kingdom which can support the immune system and alleviate or prevent bacterial, viral and even tumor diseases. Plant extracts and their phytochemical constituents have been widely investigated for their possible immunomodulatory, antibacterial, antiviral and antitumor properties. Modulation of lysozyme secretion and/or production may offer novel approaches in the treatment of a variety of diseases. One strategy in the modulation of lysozyme expression may be through the use of herbal medicines. A class of herbal medicines, known as immunomodulators, alters the activity of immune function through the dynamic regulation of an important innate immunity molecule such as lysozyme. This may offer an explanation of the effects of herbs on the immune system and other tissues.

The principal purpose of this work was to investigate several medicinal plants and phytochemicals as potential resources for identifying stimulators or inhibitors of lysozyme activity levels in human cells. Modulation of lysozyme production and/or release can present

novel mechanism of action that contributes to explaining important biological characteristics of these natural products.

To achieve this aim it was essential to investigate rapid, sensitive and accurate methods to determine lysozyme levels of human cells. We aimed to evaluate a modern and sensitive fluorescence-based assay method of lysozyme activity in comparison to the classical turbidimetric assay of lysozyme activity, and the enzyme-linked immunosorbent assay (ELISA) of human lysozyme.

Furthermore, it was important to identify human cell systems suitable for lysozyme-regulation studies. We wanted to find human cell cultures that can produce and release detectable amounts of lysozyme activity and then use these cultures for studies of lysozyme release and production.

The monocytic system as well as differentiated macrophages was of special interest for our lysozyme-regulating studies, since they constitute the primary cellular effectors of the immune response, playing a pivotal role in the detection and elimination of foreign bodies such as pathogenic microorganisms.

Another important system for our investigations was the epithelial system, considering that lysozyme is one of the most important host defense molecules in epithelial cells.

We have also aimed to identify natural or synthetic compounds as positive control for the stimulatory effect on lysozyme release and production in human cells, which was not easy due to the limited and conflicting results reported in literature.

The findings of this work may enable us to further explain the pharmacological properties of some medicinal plants through their possible positive effects on the release and production of lysozyme activity in human cells, which make them very promising natural immunostimulant agents in addition to the wide range of pharmacological activities of lysozyme, including anti-bacterial, antiviral, anti-inflammatory, antitumor and anti-ulcer effectiveness.

## 2. MATERIALS & METHODS

### 2.1. Materials

#### 2.1.1. Laboratory equipment

##### 2.1.1.1. Cell culture

- Cell counter CASY<sup>®</sup>; Schärfe System GmbH, Reutlingen, Germany.
- CO<sub>2</sub> cell culture incubator Biosafe Plus; Integra Biosciences, USA.
- CO<sub>2</sub> cell culture incubator Mini Galaxy A; Nunc Intermed GmbH, Wiesbaden, Germany.
- Gas burner Schütt flammy S; Schütt GmbH, Göttingen, Germany.
- Horizontal Laminar AirFlow cabinet Clean Air CLF 360; Clean Air Technik B.V., Woerden, The Netherlands.
- Nalgene<sup>®</sup> Cryo 1°C Freezing Container; Nalge Nunc International, UK.
- Precision hotplate; Harry Gestigkeit GmbH, Düsseldorf, Germany.
- Sterile laminar air-flow box HERAsafe<sup>®</sup>; Heraeus-Christ Instruments GmbH, Germany.
- Vacuum pump Vacusafe; Integra Biosciences, Switzerland.
- Vacuum safety suction system HLC; BioTech, Bovenden, Germany.

##### 2.1.1.2. Centrifugation

- Centrifuge Medifuge 200S; Heraeus-Christ, Hanau, Germany.
- Centrifuge Megafuge 1.0; Heraeus-Christ, Hanau, Germany.
- Centrifuge Mikro 200R (refrigerated); Hettich, Tuttlingen, Germany.

##### 2.1.1.3. Other devices

- Analytical balances; Sartorius, Göttingen, Germany.
- Inverted tissue culture microscope TMS-F; Nikon, Japan.
- Laboratory autoclave Varioklav; Thermo Scientific Inc., Germany.
- Minishaker MS2; IKA works Inc., USA.
- pH meter 766 calimatic; Knick, Berlin, Germany.
- Shaker SLT; Elmech GmbH, Celle, Germany.
- TECAN Infinite F200 filter-based multimode microplate reader; Tecan GmbH, Austria.
- TECAN SpectraFluor multifunction fluorescence and absorbance microplate reader equipped with UV-VIS fluorescence and absorbance reader; Tecan GmbH, Austria.
- Vortex Genie II; Scientific Industries, USA
- Water bath; GFL, Burgwedel, Germany.
- Water bath; VWR, Sheldon manufacturing Inc., USA.

##### 2.1.2. Kits

- EnzChek<sup>®</sup> lysozyme activity assay kit; Molecular Probes<sup>™</sup>, Invitrogen Detection Technologies, USA.

- Human lysozyme ELISA kit; AssayPro, USA.

### **2.1.3. Cell culture**

#### **2.1.3.1. Cell lines and tumor cells**

- A549: Human lung carcinoma cell line, ACC 107; DSMZ, Braunschweig, Germany.
- AGS: Human gastric adenocarcinoma cell line, CRL-1739; ATCC, USA.
- BEAS-2B: Adenovirus 12-SV40 hybrid virus-transformed human bronchial epithelial cell line, CRL-9609; ATCC, USA.
- BHY: Human oral squamous cell carcinoma cell line, ACC 404; DSMZ, Germany.
- HL-60: Human promyelocytic leukemia cell line, ACC 3; DSMZ, Germany.
- HT-29: Human colon adenocarcinoma cell line, ACC 299; DSMZ, Germany.
- THP-1: Human acute monocytic leukemia cell line, ACC 16; DSMZ, Germany.
- U-937: Human leukemic monocyte lymphoma cell line, ACC 5; DSMZ, Germany.
- Tongue derived human primary tumor cells from patient with oral tumor; Department of oral and maxillofacial surgery, Campus Benjamin Franklin, Charité-University Hospital Berlin, Germany.

#### **2.1.3.2. Reagents for cell culture**

- Casy Clean solution; Schärfe System GmbH, Reutlingen, Germany.
- Casy Ton solution; Schärfe System GmbH, Reutlingen, Germany.
- Dimethyl sulfoxide (DMSO); Merck Schuchardt, Hohenbrunn, Germany.
- Dulbecco's modified Eagle's medium with phenol red; Biochrom KG, Berlin, Germany.
- Dulbecco's phosphate buffered saline PBS (1x) without  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ; Biochrom KG, Berlin, Germany.
- Fetal bovine serum (FBS) superior; Biochrom KG, Berlin, Germany.
- L-Alanyl-L-Glutamine (200nM); Biochrom KG, Berlin, Germany.
- RPMI-1640 cell culture medium with 2.0 g/l  $\text{NaHCO}_3$ , without phenol red; Biochrom KG, Berlin, Germany.
- Triton X-100; Carl Roth GmbH, Karlsruhe, Germany.
- Trypsin / EDTA solution [0,05% / 0,02%(w/v)] in PBS without  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ; Biochrom KG, Berlin, Germany.

#### **2.1.3.3. Tissue culture lab ware & Consumables**

- Tissue culture flasks (25, 75cm<sup>2</sup>), sterile; Greiner Bio-One, Frickenhausen, Germany.
- Cryovials (2 ml), sterile; Greiner Bio-One, Frickenhausen, Germany.
- Disposable centrifuge tubes 15 & 50 ml, sterile; Corning Inc., USA.
- Disposable injection cannulas; Sterican B. Braun Melsungen AG, Melsungen, Germany.
- Disposable injection syringes 1ml/2ml/10ml; B. Braun Melsungen AG, Germany.



- Filter papers Whatman® 3MM; Whatman International Ltd., Maidstone, UK.
- Microscope slides and cover slips; Gerhard Menzel GmbH, Braunschweig, Germany.
- Multichannel disposable solutions basins, sterile; Carl Roth GmbH, Karlsruhe, Germany.
- Syringe filters Minisart®, sterile, pore size 0.2µm; Sartorius, Göttingen, Germany.
- 24/ 96 -Well Plates, sterile; Greiner Bio-One, Frickenhausen, Germany.
- Other sterile disposables for cell culture (disposable pipettes, tips, Eppendorf tubes, etc.) were purchased from Nunc (Wiesbaden), Merck, Greiner Bio-One and Carl Roth.

#### 2.1.4. Chemicals and reagents

- Buffer solutions for pH meter calibration pH 2,00 / pH 7,00 / pH 9,00 ; Carl Roth GmbH, Karlsruhe, Germany.
- Carbachol (Carbamoylcholine chloride), ≥98% (titration),  $\text{NH}_2\text{COOCH}_2\text{CH}_2\text{N}(\text{Cl})(\text{CH}_3)_3$ ; Sigma-Aldrich, Steinheim, Germany.
- Disodium hydrogen phosphate dihydrate GR ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ); Merck, Darmstadt, Germany.
- Ethanol, absolute; Merck KGaA, Darmstadt, Germany.
- N,N',N''-triacylglucosamine (N,N',N''-triacylchitotriose),  $\text{C}_{24}\text{H}_{41}\text{N}_3\text{O}_{16}$ ; Fluka BioChemika, Sigma-Aldrich, Steinheim, Germany.
- Partially deacetylated chitopentose DP5; Prof. Dr. Andreas Hensel, Institut für Pharmazeutische Biologie und Phytochemie, Universität Münster, Germany.
- Phorbol 12-myristate 13-acetate (PMA),  $\text{C}_{36}\text{H}_{56}\text{O}_8$ ; Fluka BioChemika, Sigma-Aldrich, Steinheim, Germany.
- Potassium dihydrogen phosphate GR ( $\text{KH}_2\text{PO}_4$ ); Merck, Darmstadt, Germany.
- Prednisolone-21-hydrogen-succinate, sodium salt; Prednisolut®, mibe GmbH Arzneimittel, Germany.
- Retinoic acid,  $\text{C}_{20}\text{H}_{28}\text{O}_2$ ; Sigma-Aldrich, Steinheim, Germany.
- Sodium thioglycolate,  $\text{C}_2\text{H}_3\text{O}_2\text{SNa}$ ; Sigma-Aldrich, Steinheim, Germany.

#### 2.1.5. Reagents from natural sources

- Aprotinin from bovine lung (Trypsin inhibitor), lyophilized,  $\text{C}_{284}\text{H}_{432}\text{N}_{84}\text{O}_{79}\text{S}_7$ ; Fluka BioChemika, Sigma-Aldrich, Steinheim, Germany.
- Auxoferm® YGT plus: (1,3)-(1,6)-β-D-Glucan derived from the cell wall of baker's yeast (*Saccharomyces cerevisiae*), lot no. EP 040604; Deutsche Hefewerke GmbH, Germany.
- Human Interferon-gamma (hIFN-γ), recombinant (*E. coli*), Sterile-filtered solution; Roche Applied Science, Germany.
- Human plasma fibronectin; Biochrom, Berlin, Germany.

- Leiber<sup>®</sup>-Beta S: (1,3)-(1,6)- $\beta$ -D-Glucan derived from the cell wall of baker's yeast (100% *Saccharomyces cerevisiae*), lot no. 3706; Leiber GmbH, Germany.
- Lipopolysaccharides (LPS) from *Salmonella enteritidis*, purified by phenol extraction; Sigma-Aldrich, Steinheim, Germany.
- Lysozyme (Chloride form) from chicken egg white, lyophilized powder; Sigma-Aldrich, Steinheim, Germany.
- *Micrococcus lysodeikticus* lyophilized cells; Sigma-Aldrich, Steinheim, Germany.
- Propolis originating from west central Europe region (Germany) and from Mediterranean region (Syria).

## 2.1.6. Plant extracts and isolated phytochemicals

### 2.1.6.1. Essential oils

- Anise oil; Caelo, Caesar & Loretz GmbH, Hilden, Germany.
- Chamomile oil; Carl Roth GmbH, Karlsruhe, Germany.
- Cinnamon oil; Caelo, Caesar & Loretz GmbH, Hilden, Germany.
- Clove oil; Caelo, Caesar & Loretz GmbH, Hilden, Germany.
- Eucalyptus oil; Caelo, Caesar & Loretz GmbH, Hilden, Germany.
- Lavender oil; Bombastus-Werke AG, Freital, Germany.
- *Lippia dulcis* essential oil; extracted from air dried leaves; Origin: Mexico.
- Majoran oil; Bombastus-Werke AG, Freital, Germany.
- *Melissa officinalis* oil; Caelo, Caesar & Loretz GmbH, Hilden, Germany.
- *Nigella sativa* seed oil; Allcura Naturheilmittel GmbH, Wertheim, Germany.
- Orange blossom oil; Caelo, Caesar & Loretz GmbH, Hilden, Germany.
- Pine needle oil; Caelo, Caesar & Loretz GmbH, Hilden, Germany.
- Rosemary oil; Bombastus-Werke AG, Freital, Germany.
- *Salvia officinalis* L. oil; Bombastus-Werke AG, Freital, Germany.
- Tea tree oil; Caelo, Caesar & Loretz GmbH, Hilden, Germany.
- Thyme oil; Carl Roth GmbH, Karlsruhe, Germany.

### 2.1.6.2. Saponins

- Aescin; according to Ph. Eur. standards.
- Ginsenosid-Rd; Carl Roth GmbH, Karlsruhe, Germany.
- Glycyrrhizinic acid; Carl Roth GmbH, Karlsruhe, Germany.
- Hederacosid C; Carl Roth GmbH, Karlsruhe, Germany.
- Primulic acid (isolated from *Primula officinalis* L.); Fluka AG, Germany.
- Saponins from *Gypsophila paniculata* L., saponinum album; Merck, Darmstadt, Germany.

- Saponins from *Helianthus annuus* L.; isolated from air-dried marginal flowers by Prof. Zieschang, Humboldt-Universität Berlin, Germany
- Saponins from *Hydrocotyle vulgaris* L.; isolated and identified by Prof. Hiller, Humboldt-Universität Berlin, Germany.
- Saponins from *Quillaja saponaria* bark; Carl Roth GmbH, Karlsruhe, Germany.

#### 2.1.6.3. Plant extracts

- *Aloe vera* spray-dried powder; Tom's of Maine (Colgate-Palmolive company), Lafayette center, Kennebunk, USA.
- Extract of *Echinacea purpurea* L. root; Tom's of Maine (Colgate-Palmolive company), Lafayette center, Kennebunk, USA.
- Green tea leaves extract; Tom's of Maine (Colgate-Palmolive company), Lafayette center, Kennebunk, USA.
- Hot water extract of *Scutellaria lateriflora* L. herb (skullcap); Tom's of Maine (Colgate-Palmolive company), Lafayette center, Kennebunk, USA.
- *Lycopersicon esculentum* Mill. (Tomato) tincture (70% EtOH) from fresh tomato fruits; rent r & d, Dietfurt, Germany.
- *Lycopersicon esculentum* Mill. (Tomato) extract (70% EtOH) from dried tomato fruits; rent r & d, Dietfurt, Germany.
- *Rhodiola rosea* root extract, nutriffin energy; Finzelberg GmbH & Co. KG, Andernach, Germany.
- Supercritical carbon dioxide fluid extract of *Glycyrrhiza uralensis* L. roots (Chinese licorice); Tom's of Maine (Colgate-Palmolive company), Lafayette center, Kennebunk, USA.
- Supercritical carbon dioxide fluid extract of *Humulus lupulus* L. flowers (Hop); Tom's of Maine (Colgate-Palmolive company), Lafayette center, Kennebunk, USA.
- Supercritical carbon dioxide fluid extract of *Matricaria chamomilla* L. flowers (Chamomile); Tom's of Maine (Colgate-Palmolive company), Lafayette center, Kennebunk, USA.
- Supercritical carbon dioxide fluid extract of *Rosmarinus officinalis* L. leaves (Rosemary); Tom's of Maine (Colgate-Palmolive company), Lafayette center, Kennebunk, USA.

#### 2.1.6.4. Isolated phytochemicals

- Aconitine, crystalline,  $C_{34}H_{47}NO_{11}$ ; Sigma-Aldrich, Steinheim, Germany.
- Benzyl isothiocyanate (Benzyl mustard oil),  $C_6H_5CH_2NCS$ ; Sigma-Aldrich, Steinheim, Germany.
- Curcumin, crystalline; Eastman Chemical Company, USA.

- Dodeca-2,4,8,10-tetraenoic acid isobutylamide, mixture of E,E,Z,Z and E,E,Z,E isomers, isolated from *Echinacea* sp., C<sub>16</sub>H<sub>25</sub>NO; PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany.
- Forskolin from *Coleus forskohlii* (≥98% ;HPLC); Sigma-Aldrich, Germany.
- Naringin (4',5,7-Trihydroxyflavanone 7-rhamnoglucoside), C<sub>27</sub>H<sub>32</sub>O<sub>14</sub>, from citrus fruit; Sigma Chemical Co., USA.
- Quercetin dihydrate (3,3',4',5,7-Pentahydroxyflavone dihydrate), C<sub>15</sub>H<sub>10</sub>O<sub>7</sub>.2H<sub>2</sub>O; Sigma-Aldrich, Germany.
- Rutin trihydrate (Quercetin 3β-D-rutinoside), C<sub>27</sub>H<sub>30</sub>O<sub>16</sub>.3H<sub>2</sub>O; Sigma Chemical Co., USA.
- Silymarin group, mixture of isomers; Sigma-Aldrich, Germany.
- Tomatine, C<sub>50</sub>H<sub>83</sub>NO<sub>21</sub>; Carl Roth GmbH, Karlsruhe, Germany.

## **2.2. Methods**

### **2.2.1. Cell biology methods**

Prior to the initiation of cell culture work, it was essential to ensure that all equipment was in optimal working condition. Scheduled checks and regular maintenance of the equipment was performed including the following: check to ensure that the temperature and CO<sub>2</sub> levels in the incubator were at the desired levels; check to be sure that the water pan in the incubator was full of clean water and that it contained copper sulfate to inhibit bacterial growth; check to ensure that the water bath was at the required temperature and contained adequate amounts of clean water; check to ensure that the biological safety cabinet to be used was certified and operating correctly; ascertain that the centrifuge was cleaned and decontaminated.

#### **2.2.1.1. THP-1, HL-60 and U-937 non-adherent cell lines**

THP-1 is a human acute monocytic leukemia cell line. THP-1 cells proliferate as single cells in suspension culture partly in clusters, and are characterized by a round shape, with doubling time of approximately 26 hours. HL-60 is a human promyelocytic leukemia cell line. HL-60 cells proliferate continuously in suspension culture and have doubling time of about 40 hours. U-937 is a human leukemic monocyte lymphoma cell line. U-937 cells grow in suspension and are characterized by a round shape with short microvilli, scarce cytoplasm and a large bean-shaped nucleus, with replication time of 20-48 hours.

##### **2.2.1.1.1. Cultivation of continuously growing non-adherent cell lines**

Non-adherent cell lines used in this work were cultured in disposable sterile cell culture flasks with white filter caps designed for suspension culture (CELLSTAR<sup>®</sup>). Suspension cultures were maintained in RPMI-1640 medium without phenol red, supplemented with 10% fetal bovine serum and 2% (2mM) L-glutamine without addition of antibiotics. Incubation was carried out at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/ 95% air.

##### **2.2.1.1.2. Subculturing of continuously growing non-adherent cell lines**

Maintenance of healthy, viable cells required routine medium exchanges or passage of the cells to ensure that the nutrients in the medium did not become depleted and/or that the pH of the medium did not become acidic as a result of the presence of large amounts of cellular waste. Cultures were viewed under an inverted phase-contrast microscope. Healthy growing cells should be round, bright, and refractile. Cell suspension in the flask was pipetted up and down two or three times to disperse any clumps. 500µl of the suspension was transferred to a fresh flask containing 5ml of complete growth medium. This subculture step was repeated every 2–3 days to maintain cells in an exponential growth phase.

### **2.2.1.2. HT-29, AGS, A549, BHY and BEAS-2B adherent cell lines**

HT-29 is a human colon adenocarcinoma cell line. They are adherent, epitheloid cells growing as monolayers and in large colonies, with doubling time of about 40-60 hours. AGS is a human gastric adenocarcinoma cell line. They are adherent, epitheloid cells growing as monolayers, with doubling time of about 20 hours. A-549 is a human lung carcinoma cell line. They are adherent epithelial cells growing as monolayers, with doubling time of about 40 hours. BHY is a human oral squamous cell carcinoma cell line. They are adherent epithelial-like polygonal or round and flat cells (sometimes spindle-form, very heterogenous) growing in monolayers, with doubling time of about 70. BEAS-2B is an adenovirus 12-SV40 hybrid virus-transformed human bronchial epithelial cell line. Epithelial cells were isolated from normal human bronchial epithelium, infected with an adenovirus 12-SV40 virus hybrid (Ad12SV40) and cloned.

#### **2.2.1.2.1. Cultivation of continuously growing adherent cell lines**

Adherent cell lines used in this work were cultured in disposable sterile cell culture flasks with red filter caps designed for adherent culture (CELLSTAR®). AGS and HT-29 cells were maintained in RPMI-1640 medium without phenol red, supplemented with 10% fetal bovine serum and 2% L-glutamine without addition of antibiotics. A549, BHY and BEAS-2B cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Incubation was carried out at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/ 95% air.

#### **2.2.1.2.2. Subculturing of continuously growing adherent cell lines**

Culture medium was removed from the flask and discarded. Cell layer was briefly rinsed with 5ml PBS to remove all traces of serum which contains trypsin inhibitor. 2ml of Trypsin-EDTA solution was added to the flask and the trypsin was allowed to cover the plate. Plate was incubated at 37°C for 4-9 minutes. Cells were observed every minute under an inverted microscope until cell layer was dispersed. When cells rounded up and were coming off the plate, 5ml serum-containing complete growth medium was added and cells were resuspended by gently repeated pipetting. When precise cell counts were needed or the required seeding density was known, an aliquot of the cell suspension was counted at this time. The appropriate aliquots of cell suspension were added to new culture vessels. New cultures were incubated at 37°C. Medium renewal was performed 2 to 3 times per week.

#### **2.2.1.3. Cryopreservation of continuously growing cells**

Continuous culture of cell lines can lead to the accumulation of unwanted karyotype alterations or the outgrowth of clones within the population. In addition, continuous growth increases the possibility of cell line contamination by bacteria or other unwanted organisms. The only insurance against loss of the cell line is to ensure that adequate numbers of vials (i.e., at least 10) are cryopreserved for future use. For newly acquired cell lines,

cryopreservation of stock (master cell bank) vials should be done as soon as possible after the cell line has been confirmed to be free of mycoplasma. Cellular damage induced by freezing and thawing is generally believed to be caused by intracellular ice crystals and osmotic effects. The addition of a cryoprotective agent, such as DMSO, and the selection of suitable freezing and thawing rates can minimize cellular injury (Helgason & Miller, 2004).

Cell cultures were viewed under a phase-contrast inverted microscope to assess cell density and confirm the absence of bacterial or fungal contamination. For adherent cell cultures, cells were trypsinized as mentioned previously. A small aliquot of the cells was removed for determination of cell numbers. Cells for cryopreservation should be in log growth phase with greater than 90% viability. Cryopreservation vials were prepared by indicating the name of the cell line, the number of cells per vial, the passage number, and the date on the surface of the vial using a permanent marker. The required volume of freezing medium was freshly prepared, consisting of complete growth medium supplemented with 20% serum and 5% DMSO (v/v). The desired number of cells was centrifuged at 1800 rpm for 5 min and the supernatant was then aspirated from the tube. Cells were suspended to a density of  $2 \times 10^6$  cells/ml in the freezing medium. 1ml was quickly aliquoted into each of the prepared cryovials using a pipet. Cells should be frozen slowly, and a steady decrease of one degree per minute is ideal (Mather & Roberts, 1998). Therefore, the cryovials of cells were placed in a freezing container filled with isopropanol (Cryo 1°C Freezing Container, Nalgene). The container was placed in refrigerator for 30 min, and then moved to a -80°C freezer where it remained for 2 hours. Cryovials were then transferred to liquid-nitrogen tank (-196°C) for long-term storage.

#### **2.2.1.4. Thawing cryopreserved cells**

Cells should be thawed as rapidly as possible, in contrast to the optimal slow rate of freezing. In both instances, this was to minimize ice crystal formation, which may damage cells.

A sterile tube containing 10ml of growth medium supplemented with 20% serum and warmed to at least room temperature was prepared in the biological safety cabinet. One vial of cells was removed from the storage container (liquid nitrogen), and transferred to a 37°C water bath until the suspension was just thawed. In the cell culture hood, a sterile pipet was used to transfer the contents of the vial into the tube containing the growth medium. Cells were gently centrifuged at 1800 rpm for 5min to obtain a pellet. The supernatant containing DMSO was aspirated and the cell pellet was suspended in 10ml of growth medium supplemented with 20% serum and warmed to 37°C. Cells were transferred to a tissue culture flask and incubated at 37°C, 5% CO<sub>2</sub>. Cultures were examined daily using an inverted microscope to ensure that the culture was not contaminated during the freeze–thaw process and that the cells were growing. After the first passage, most cells were fully recovered from freezing and could be handled normally. If most of the cells were lost on thawing, there was a real

possibility that the characteristics of the thawed culture would differ from those of the line frozen. Therefore, at least two passages were allowed post-thaw before using the cells in the experimental protocols.

#### **2.2.1.5. Determination of cell number and cell viability**

For the experiments in which precise cell counts were needed or the required seeding density should be known, an aliquot of the cell suspension was counted using CASY® technology system. The CASY® cell counter and analyzer is an electric field multi-channel cell counting system, which determines cell number, size distribution and viability of the cell sample. Cell viability can be assessed based on the integrity of plasma membrane.

##### **Procedure:**

For non-adherent cell cultures, cells were resuspended by gently repeated pipetting. For adherent cell cultures, flask was trypsinized as mentioned previously. 100µl aliquot of the cell suspension was transferred to a counting cuvette (polypropylene CASYcup) containing 10ml isotonic diluting solution CASYton. The cup was closed and gently swirled to resuspend the cells. The cap was removed and the cup was placed on the platform of CASY counter taking care that the electrode was immersed.

#### **2.2.1.6. Incubation of cells with the various examined factors**

The human cells were counted, viability determined and pelleted by centrifugation. Cells were seeded into 24-well plates at an initial concentration of  $1.0 \times 10^6$  cells/ml for monocytic cells and  $0.5 \times 10^6$  cells/ml for epithelial cells. Incubation was carried out at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for various incubation periods up to 72 hours in culture medium, or in preparations containing culture medium and one of the examined substances in question. Cell cultures containing DMSO or PBS (solvent of the examined substance) served as the respective controls.

##### **➤ Secreted lysozyme activity determination**

Supernatants of the examined and control cell cultures were collected at various time points according to the treatment period examined, and used to determine lysozyme activity released into culture medium.

##### **➤ Total lysozyme activity determination using cell lysis**

Cell lysis was needed to facilitate the cell membrane breakage and the release of intracellular lysozyme activity in order to determine total lysozyme activity synthesized by the cells. This procedure was performed using two freeze-thaw cycles as following: cell cultures were rapidly frozen using liquid nitrogen, then immediately thawed in 37°C water bath, and vortexed briefly to help lyse cells. This freeze-thaw cycle was repeated two times to completely extract the lysozyme from inside the cells. By lysozyme assay of the whole cell



lysates we could determine the total amount of lysozyme activity, including lysozyme released in the culture medium and the intracellular lysozyme.

#### **2.2.1.7. Differentiation of monocytic cells using PMA**

THP-1 monocytic cells were differentiated into macrophages using Phorbol 12-myristate 13-acetate (PMA) (Park *et al.*, 2007; Weng, 2009). PMA was dissolved in sterile DMSO in a 1mg/ml (1.62mM) stock solution, and then diluted with sterile PBS and culture medium to the final concentration of 20nM. 24-well plates were coated with human fibronectin (75µg/ml) and covered for 30 minutes at room temperature. Each well was then washed twice with 500µl PBS. After seeding of  $0.5 \times 10^6$  THP-1 cells per well in 450µl complete culture medium, 50 µl of PMA solution was added and incubated for 72 hours. The medium was then removed and the attached cells were gently washed with pre-warmed PBS (500µl). Subsequently, 500µl complete culture medium supplemented with the examined substance was added to each well and incubated for various treatment periods.

#### **2.2.2. Phytochemical methods**

##### **2.2.2.1. Supercritical CO<sub>2</sub> extraction & standardization of rosemary, hop, Chinese liquorice & chamomile**

###### **2.2.2.1.1. Supercritical CO<sub>2</sub> extraction**

Supercritical fluid extraction (SFE) is an extraction method using supercritical fluids as extractant instead of normal liquids. In this process, pressurized CO<sub>2</sub> gas is pumped into a chamber filled with plant matter. Pressurized CO<sub>2</sub> functions as a liquid and extracts lipophilic components from the herb (Wynn & Fougère, 2007). SFE with supercritical carbon dioxide (SFE-CO<sub>2</sub>) can be far superior to conventional methods of extraction (such as liquid extraction or Soxhlet), and ideally suitable for thermally labile-heat sensitive components (Mukhopadhyay, 2000; Kutta *et al.*, 2007).

###### **2.2.2.1.2. Standardization of supercritical CO<sub>2</sub>-extracts**

###### **2.2.2.1.2.1. Supercritical CO<sub>2</sub> fluid extract of rosemary**

- Raw material: *Rosmarinus officinalis* – Leaves; Origin: Spain.
- Production: Produced by supercritical fluid extraction with natural carbon dioxide and a small amount of ethanol as entrainer, no inorganic salts, no heavy metals, no reproducible microorganisms. The essential oil was widely removed by multistep separation. The antioxidative CO<sub>2</sub>-extract was standardized with sunflower oil (organic).
- Extract: Dark brown and at room temperature viscous liquid product with weak flavour.
- Extract standardization data: 24-26% total antioxidative phenolic diterpenes with >16% of carnosic acid; essential oil <4%, water <1%, alcohol <2%, sunflower oil (organic), cuticular waxes.

#### **2.2.2.1.2.2. Supercritical CO<sub>2</sub> fluid extract of German chamomile**

- Raw material: *Chamomilla recutita* – Flowers.
- Production: Produced by supercritical fluid extraction with natural carbon dioxide, no inorganic salts, no solvent residues, no heavy metals, no reproducible microorganisms.
- Extract: Green-brown colour, liquid at 40°C, fine chamomile-like smell.
- Extract standardization data: The extract contained 7-17% essential oil with 20-45% bisabolol and 5-20% bisabolol oxides in addition 1.0-3.0% matricine (analysed as chamazulene), cis- and trans-en-in-dicycloether, spartulenol, herniarine, waxes and non-volatile components. The labile matricine was not decomposed at the CO<sub>2</sub>-extraction to blue coloured chamazulene with inferior anti-inflammatory effect.

#### **2.2.2.1.2.3. Supercritical CO<sub>2</sub> fluid extract of Chinese liquorice**

- Raw material: *Glycyrrhiza uralensis* – Roots.
- Extract standardization data: 15% licoricidin and 5% licorisoflavan A.

#### **2.2.2.1.2.4. Supercritical CO<sub>2</sub> fluid extract of hop**

- Raw material: *Humulus lupulus* – Flowers; Origin: Germany.
- Production: Produced by supercritical CO<sub>2</sub> extraction with natural carbon dioxide, no solvent residues, no inorganic salts, no heavy metals, no reproducible microorganisms. In a second step the extract was flavour reduced by counter current column extraction with natural carbon dioxide. The extract was standardized with organic sunflower oil to a content of 49-51% sum of humulones and lupulones.
- Extract: Brown viscous extract at room temperature, weak typical smell, bitter taste.
- Extract standardization data: 49-51% sum of humulones (alpha acids consist of n-humulon, adhumulon and cohumulon) and lupulones (beta acids consist of n-lupulon, adlupulon and colupulon), residual content of essential oil <2%, fatty oil (MCT-oil).

#### **2.2.2.1.3. Treatment of human cells with the CO<sub>2</sub>-extracts**

Each of the CO<sub>2</sub>-extracts mentioned above was dissolved in sterile DMSO in a 10mg/ml stock solution soon before use, and then diluted with sterile PBS and culture medium to the final concentrations examined. Control cell cultures were supplemented with DMSO (extracts solvent) in order to obtain the respective concentration of DMSO in the various examined concentrations of the extract.

#### **2.2.2.2. Extraction & standardization of skullcap, *Echinacea*, *Aloe* & green tea**

##### **2.2.2.2.1. Extract of skullcap**

- Raw material: *Scutellaria lateriflora* - Herb.
- Production: Hot water extraction

- Extract standardization data: 23.6% flavonoids (consisting of 12.88% baicalin, 7.63% dihydrobaicalin, 0.91% scutellarin, 0.86% lateriflorin and other flavonoids in small amounts).

#### **2.2.2.2.2. Extract of *Echinacea***

- Raw material: *Echinacea purpurea* - Roots.
- Extract standardization data: Polysaccharides content: 7.84%

#### **2.2.2.2.3. Extract of green tea**

- Raw material: *Camellia sinensis* - Leaves.
- Extract standardization data: Epigallocatechin gallate (EGCG) content: >90%.

#### **2.2.2.2.4. Extract of *Aloe vera***

- Raw material: *Aloe vera* - Leaves.
- Production: Spray-dried powder.
- Extract standardization data: The powder is rich in polysaccharides, anthraquinones content: 0%.

#### **2.2.2.2.5. Treatment of human cells with the extracts**

Each of the extracts mentioned above was dissolved in sterile PBS in a 0.5mg/ml stock solution soon before use, and then diluted with culture medium to the final concentrations.

#### **2.2.2.3. Extraction and quantification of the essential oil of *Lippia dulcis***

The essential oil was extracted from the air dried leaves of *Lippia dulcis* (origin: Mexico) using continuous water distillation method. 10-50 g of dried leaves were crushed and distilled in a steam distiller apparatus for 4 hours, according to the method recommended in the European pharmacopoeia. The distillation yielded an amount of  $1.8 \pm 0.05\%$  (v/w) of clear yellow oil, which was very aromatic with a very sweet taste. The essential oil was analyzed and quantified in our laboratory using GC analysis (Nayal, 2009; Souto-Bachiller *et al.*, 1997). The essential oil contains as main constituents  $11.4 \pm 0.5\%$  (w/v) of hernandulcin and  $34.1 \pm 0.6\%$  (w/v) of camphor.

#### **Treatment of human cells with the essential oil**

Each of the examined essential oils, including the oil of *Lippia dulcis*, was diluted with sterile DMSO in a 100 $\mu$ l/ml stock solution soon before use, and then further diluted with sterile PBS and culture medium to the final treatment concentrations.

#### **2.2.2.4. Extraction and standardization of tomato fruits**

##### **2.2.2.4.1.1. Tincture of fresh-tomato fruits**

- Raw material: *Lycopersicon esculentum* Mill. – Fresh fruits.

- Production: Extracting solvent: 70% EtOH
- Extract standardization data: Tomatine content 0.26%.

#### **2.2.2.4.1.2. Tincture of dried-tomato fruits**

- Raw material: *Lycopersicon esculentum* Mill. – Dried fruits.
- Production: Extracting solvent: 70% EtOH
- Extract standardization data: Tomatine content 3.51%.

#### **2.2.2.5. Extraction of crude propolis**

Ethanollic extracts of propolis were prepared as previously described by Gekker *et al.* (2005). Propolis was ground and 20% ethanolic extracts of propolis were prepared (20g of propolis completing the volume to 100ml of 95% ethanol), protected from light, with moderate shaking at room temperature. After 1 week, extracts were filtered and the dry weight of the extracts was calculated (German propolis: 141mg/ml, yield: 70.6%; Syrian propolis: 77mg/ml, yield: 38.5%). Propolis extracts were then diluted in PBS. Specific dilutions of this solution were prepared using culture medium for each assay in order to achieve different propolis concentrations. Control cell cultures were supplemented with ethanol (propolis solvent), in order to obtain the respective concentrations of alcohol in propolis concentrations.

### **2.2.3. Lysozyme assay methods**

#### **2.2.3.1. Photometric assays**

##### **2.2.3.1.1. Turbidimetric assay**

##### **2.2.3.1.1.1. Principal of the Assay**

The enzyme lysozyme has the function of hydrolyzing bacterial cell walls and is conveniently assayed by observing the change in turbidity that occurs when it is added to a suspension of dried bacterial cells. This decrease in turbidity is clearly the result of a complex process of progressive random hydrolysis and it is therefore not possible to express the rate in molar terms. The unit is defined in terms of the rate of decrease in turbidity. The wavelength chosen for these turbidity measurements is arbitrarily set at 450 nm and one unit of activity is defined as that which produces an initial rate of change in 'absorbance' of 0.001 per min when the volume in the cuvette is 2.6 ml (other conditions being pH 6.24 and 25°C) (Eisenthal & Danson, 2002). In this work, some conditions in the unit definition were adjusted to fit the use of a microplate instead of a cuvette.

##### **2.2.3.1.1.2. Experimental protocol**

##### **2.2.3.1.1.2.1. Cell culture supernatants collection**

Cell culture media in question were centrifuged at 2000 x g for 10 minutes to remove debris. Fresh supernatants were collected and used for lysozyme activity assay.

#### **2.2.3.1.1.2.2. Preparation of reagents**

➤ Lysozyme from chicken egg white (45200 Units/mg solid, 50800 Units/mg proteins) in the form of lysozyme chloride (Sigma, Germany) was used as lysozyme standard. One unit (1 U) of lysozyme activity is defined by the manufacturer as decreasing a 0.001 absorption value at 450 nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* under the conditions of pH 6.2 and 25°C. 4000 U/ml lysozyme stock solution was prepared with a phosphate buffer solution (0.1 M; pH 6.24) soon before use. The working solutions were obtained by diluting the stock solution to the appropriated concentrations with the phosphate buffer solution (0.1 M; pH 6.24).

➤ Lyophilized *Micrococcus lysodeikticus* cells suspension was made soon before use. A 0.36mg/ml *Micrococcus lysodeikticus* working suspension was prepared with a phosphate buffer solution (0.1 M; pH 6.24), and mixed for 10 min in vortex mixer.

#### **2.2.3.1.1.2.3. Preparation of lysozyme standard curve:**

Using 96-well transparent microplate, 8 wells were filled with 50µl of the buffer (0.1 M; pH 6.24). 50µl of the 4000U/ml stock solution of lysozyme was added to the first well, mixed by pipetting then 50µl was transferred to the second well. This process was repeated from one well to the next, except 50µl from the mixture in the seventh well was discarded and nothing was added to the eighth well. Thus, lysozyme concentration will range from 2000U/ml to 0U/ml in the 50µl volumes, for a range of 400U/ml to 0U/ml in the final 250µl volumes. Additional well was filled with 50µl of 400U/ml lysozyme solution to achieve additional final concentration of 80U/ml in the final 250µl volume (The units here are under the conditions stated from the manufacturer). Standard concentration series were repeated in triplicates.

#### **2.2.3.1.1.2.4. Starting the reaction**

The reaction was started by addition of 200µl of the 0.36mg/ml *Micrococcus lysodeikticus* working suspension to each microplate well containing the experimental or the standard curve sample.

#### **2.2.3.1.1.2.5. Absorption measurement**

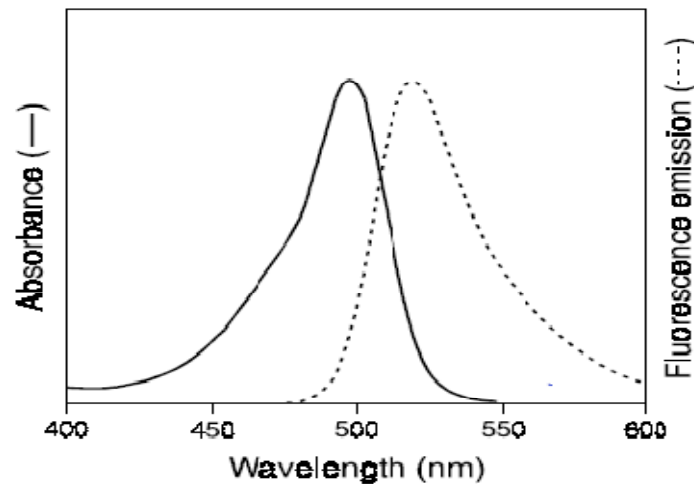
The absorption was read at a wavelength of 450nm every minute to follow the kinetics of the reaction for 10 min at 37°C. The absorbance values derived from the no-enzyme controls were subtracted. The chicken egg white lysozyme standard (Sigma) was calibrated by measuring the decrease in absorption per minute under the used conditions.

#### **2.2.3.1.2. Fluorescence-based assay**

##### **2.2.3.1.2.1. EnzChek<sup>®</sup> lysozyme assay kit**

The EnzChek<sup>®</sup> lysozyme assay kit provides a sensitive fluorescence-based assay to measure levels of lysozyme activity in solution. The assay measures lysozyme activity on

*Micrococcus lysodeikticus* cell walls, which are labeled to such a degree that the fluorescence is quenched. Lysozyme action can relieve this quenching, yielding a dramatic increase in fluorescence that is proportional to lysozyme activity. The fluorescence increase can be measured using any spectrofluorometer or fluorescence microplate reader that can detect fluorescein (Fig.4).



**Fig.4.** Normalized absorption and fluorescence emission spectra of fluorescein.

#### **2.2.3.1.2.2. Experimental protocol**

##### **2.2.3.1.2.2.1. Cell culture supernatants collection**

Cell culture media in question were centrifuged at 2000xg for 10 minutes to remove debris. Fresh supernatants were collected and used for lysozyme activity assay.

##### **2.2.3.1.2.2.2. Kit Contents**

- DQ™ lysozyme substrate, fluorescein conjugate (Component A), 1mg of *Micrococcus lysodeikticus*, labeled with fluorescein.
- 1X Reaction buffer (Component B), 50mL of 0.1M sodium phosphate, 0.1M NaCl, pH 7.5, containing 2mM sodium azide as a preservative.
- Lysozyme from chicken egg white (Component C), 1000U. One unit is defined as the amount of enzyme required to produce a change in the absorbance at 450nm of 0.001 units per minute at pH 6.24 and 25°C, using a suspension of *Micrococcus lysodeikticus* as the substrate.

##### **2.2.3.1.2.2.3. Preparation of kit reagents**

- DQ lysozyme substrate stock suspension: A 1mg/ml stock suspension of the DQ lysozyme substrate was prepared by suspending the contents of the vial (Component A) in 1ml of deionized water (dH<sub>2</sub>O). The DQ lysozyme substrate suspension was stored at -20°C in single-use aliquots.

- 1000U/ml lysozyme stock solution: The content of the vial (Component C) was dissolved in 1ml of dH<sub>2</sub>O.

#### **2.2.3.1.2.2.4. Preparation of DQ lysozyme substrate working suspension**

The DQ lysozyme substrate working suspension was prepared by diluting the 1mg/ml stock suspension 20-fold in reaction buffer. A 50µl volume will be used for each reaction.

For example, to prepare enough working solution for 20 assays including the standard curve, 50 µl of DQ lysozyme substrate stock suspension was added to 950µl of reaction buffer.

The final concentration of the DQ lysozyme working suspension will be twofold lower in the final reaction buffer.

#### **2.2.3.1.2.2.5. Preparation of lysozyme standard curve**

Using 96-well black microplate, 8 wells were filled with 50µl of reaction buffer. 50µl of the 1000U/ml stock solution of lysozyme was added to the first well, mixed by pipetting, then 50µl was transferred to the second well. This process was repeated from one well to the next, except 50µl from the mixture in the seventh well was discarded and nothing was added to the eighth well. Thus, the lysozyme concentration will range from 500U/ml to 0U/ml in the 50µl volumes, for a range of 250U/ml to 0U/ml in the final 100µl volumes (The units here are under the conditions stated from the manufacturer). Standard concentration series were repeated in triplicates.

#### **2.2.3.1.2.2.6. Starting the reaction**

The reaction was started by addition of 50µl DQ lysozyme substrate working suspension to each microplate well containing the experimental or the standard curve samples.

#### **2.2.3.1.2.2.7. Fluorescence measurement**

After beginning the reaction, fluorescence was measured every 5 minutes to follow the kinetic of the reaction at 37°C for 60 min. Digestion products from the DQ lysozyme substrate have absorption maximum at ~494nm and fluorescence emission maximum at ~518nm.

Correction for background fluorescence was done by subtracting the value derived from the no-enzyme control. Lysozyme activity of the experimental samples was determined from the standard curve.

#### **EnzChek<sup>®</sup> lysozyme standard calibration**

The chicken egg white lysozyme standard (EnzChek<sup>®</sup>) was calibrated spectrophotometrically by measuring the decrease in absorption per minute, using the same dilutions and conditions used in the turbidimetric assay mentioned previously.

### **2.2.3.2. Enzyme linked immunosorbent assay (ELISA)**

#### **2.2.3.2.1. Human lysozyme ELISA kit**

The human lysozyme ELISA kit used in our experiments is a non-competitive indirect ELISA assay designed for detection of lysozyme in human plasma, serum, urine, saliva, milk, other body fluids and cell culture supernatant. This assay employs a quantitative sandwich enzyme immunoassay technique that measures lysozyme in 4 hours. A polyclonal antibody specific for lysozyme has been pre-coated onto a 96-well microplate. Lysozyme in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for lysozyme, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured. Color intensity is directly proportional to the amount of lysozyme in the sample.

#### **2.2.3.2.2. Experimental protocol**

##### **2.2.3.2.2.1. Preparation of kit reagents**

- All reagents were freshly diluted and brought to room temperature before use. Concentrates were gently mixed to dissolve the formed crystals completely.
- MIX diluent concentrate (10x): MIX diluent concentrate was diluted 1:10 with reagent grade water, and stored at 2-8°C.
- Lysozyme standard: The 50ng of human lysozyme standard was reconstituted with 5ml of MIX diluent to generate a standard solution of 10ng/ml. The standard was allowed to sit for 10 minutes with gentle agitation prior to making dilutions. Triplicate standard points were prepared by serially diluting the standard solution (10ng/ml) twofold with equal volume of MIX diluent to produce 5, 2.5, 1.25, 0.625, 0.313 and 0.156 ng/ml. MIX diluent served as the zero standard (0ng/ml). Any remaining solution was frozen at -20°C.
- Biotinylated lysozyme antibody (100x): Biotinylated antibody was briefly spun down and the desired amount of the antibody was diluted 1:100 with MIX diluent. Any remaining solution was frozen at -20°C.
- Wash buffer concentrate (20x): wash buffer concentrate was diluted 1:20 with reagent grade water.
- Streptavidin-peroxidase conjugate (100x): SP conjugate was briefly spun down and the desired amount of the conjugate was diluted 1:100 with MIX diluent. Any remaining solution was frozen at -20°C.

##### **2.2.3.2.2.2. Cell culture supernatants collection and storage**

Cell culture media were centrifuged at 2000xg for 10 minutes to remove debris. Supernatants were collected for assay. The remaining samples were stored at -80°C. Repeated freeze-thaw cycles were avoided.



#### 2.2.3.2.2.3. Assay Procedure

- All reagents, working standards and samples were prepared as mentioned and brought to room temperature before use. The assay was performed at room temperature (20-30°C).
- 50µl of standard or sample was added per well. Wells were covered with a sealing tape and incubated for two hours. The timer was started after the last sample addition.
- Wells were washed five times with 200µl of wash buffer. The plate was inverted to decant the contents, and hit 4-5 times on absorbent paper towel to completely remove liquid at each step.
- 50µl of biotinylated lysozyme antibody was added to each well and incubated for 1 hour.
- Wells were washed five times with 200µl of wash buffer.
- 50µl of streptavidin-peroxidase conjugate was added per well and incubated for 30 min.
- Wells were washed five times with 200µl of Wash buffer.
- 50µl of chromogen substrate was added per well and incubated for about 8 minutes till the optimal blue color density developed.
- 50µl of stop solution was added to each well. The color changed from blue to yellow.
- Absorbance was measured on a microplate reader at a wavelength of 450nm immediately.

#### 2.2.3.2.2.4. Data Analysis

The mean value of the triplicate readings was calculated for each standard and sample. To generate a standard curve, the graph was plotted using the standard concentrations on the x-axis and the corresponding mean 450nm absorbance on the y-axis. The best-fit line was determined by regression analysis. The unknown sample concentration was determined from the standard curve.

#### 2.2.4. Inhibition of lysozyme activity

The enzymatic activity of lysozyme can be blocked by the specific competitive inhibitor N,N',N''-triacetylglucosamine, also referred to as N,N',N''-triacetylchitotriose (TAC) or chitotriose. TAC is the trisaccharide of NAG that has three NAG residues linked by  $\beta(1-4)$ -glycosidic linkages. TAC inhibits competitively the enzymatic activity of lysozyme, and it was proved to be quite specific (Mink *et al.*, 2004).

#### Experimental protocol

1000units/ml stock solution of Sigma<sup>®</sup> lysozyme standard was prepared with phosphate buffer solution (0.1M; pH 6.24), and then diluted to achieve a final concentration of 80 units/ml. 10mg/ml stock solution of TAC was prepared with phosphate buffer solution (0.1M; pH 6.24), and then diluted to the final tested concentrations. TAC solution was added to the

lysozyme solution in different amounts to reach final concentrations of TAC of 0.2, 0.5 and 0.8 mg/ml. After TAC addition, lysozyme activity was immediately determined using the turbidimetric assay by incubation with 0.36mg/ml *Micrococcus lysodeikticus* suspension for 10 minutes at 37°C. The absorbance was measured kinetically using microplate reader with a 450 nm filter. Details of assay protocol were mentioned previously. The measured kinetic curve of 80 units/ml lysozyme solution without addition of TAC served as control, and was considered to have 100% enzymatic activity.

#### **2.2.5. Human saliva collection and sample preparation**

Participants represent a random sample of younger and older individuals (20-58 years).

Non-stimulated whole saliva was collected from 33 healthy non-medicated volunteers using expectoration method in sterile bottles in the amount of 3-5ml. Saliva samples were immediately centrifuged at 8000 rpm for 15 min at 4°C to remove cellular debris.

After centrifugation, saliva supernatant of each sample, indicated as saliva, was immediately diluted to the appropriated dilution with the phosphate buffer solution and vortex mixed for 30s. Series of saliva dilutions were done to determine the optimal dilution, which was 1:500. In few samples, where low lysozyme levels were detected, a dilution of 1:400 was necessary. The whole process of the assay must not exceed 2 hours after collecting the samples, since the salivary lysozyme has been proved in our experiments to start losing activity after 2 hours. Lysozyme activity in each sample was measured immediately after collection in triplicate using the turbidimetric assay as mentioned above. The slope of the kinetic curve of each saliva sample was used to determine the lysozyme concentration in comparison with the standard using the regression equation of the standard.

#### **2.2.6. Human tears collection and sample preparation**

Non-stimulated tear sample was collected from healthy non-medicated non-smoking 27 years old woman in a sterile bottle. She had no ocular or systemic disease. Tear sample was diluted to the appropriated dilution with the phosphate buffer solution and vortex mixed for 30s. Lysozyme activity in the sample was measured in triplicate using the turbidimetric assay as mentioned previously.

#### **2.2.7. Statistical analysis**

Data in tables and figures were presented as mean  $\pm$  standard deviation (SD). Differences between groups were assessed by Mann-Whitney U test. A probability of  $P < 0.05$  was considered significantly different.

### 3. RESULTS

#### 3.1. Lysozyme assay methods

##### 3.1.1. Turbidimetric lysozyme activity assay

Chicken egg white lysozyme standard curve using the microtiter plate turbidity assay was linear with a correlation coefficient of  $R^2=0.9857$  (Fig.5.A).

The rate of lysis of *Micrococcus lysodeikticus* suspension induced by lysozyme standard at different concentrations, represented in the slope values of the kinetic curve of each concentration, was linear in the range of 12.5-100 units/ml. The linear regression equation of this standard curve was:  $y = 0.0003x - 0.0017$  (Fig.6). Lysozyme activity levels presented in the cell culture supernatants in question were determined directly from this standard curve. Units of activity were calculated according to manufacturer's data. Sigma<sup>®</sup> egg white lysozyme standard was stated by the manufacturer to have an activity of 45200 units/mg. Expressing lysozyme as micrograms of enzyme per millilitre, lysozyme standard curve was linear in the range of 0.28-2.2µg/ml. The linear regression equation and correlation coefficient of this standard curve were:  $y = 0.0115x - 0.0017$  and  $R^2=0.9831$ , respectively (Fig.5.B). The rate of the reaction was measured for 10 minutes as absorbance change per minute using spectrophotometer with a 450 nm filter. The average change in absorbance per minute for each concentration of lysozyme activity, as illustrated in Fig.7, was proportional to lysozyme concentration.

##### Sigma<sup>®</sup> lysozyme standard calibration

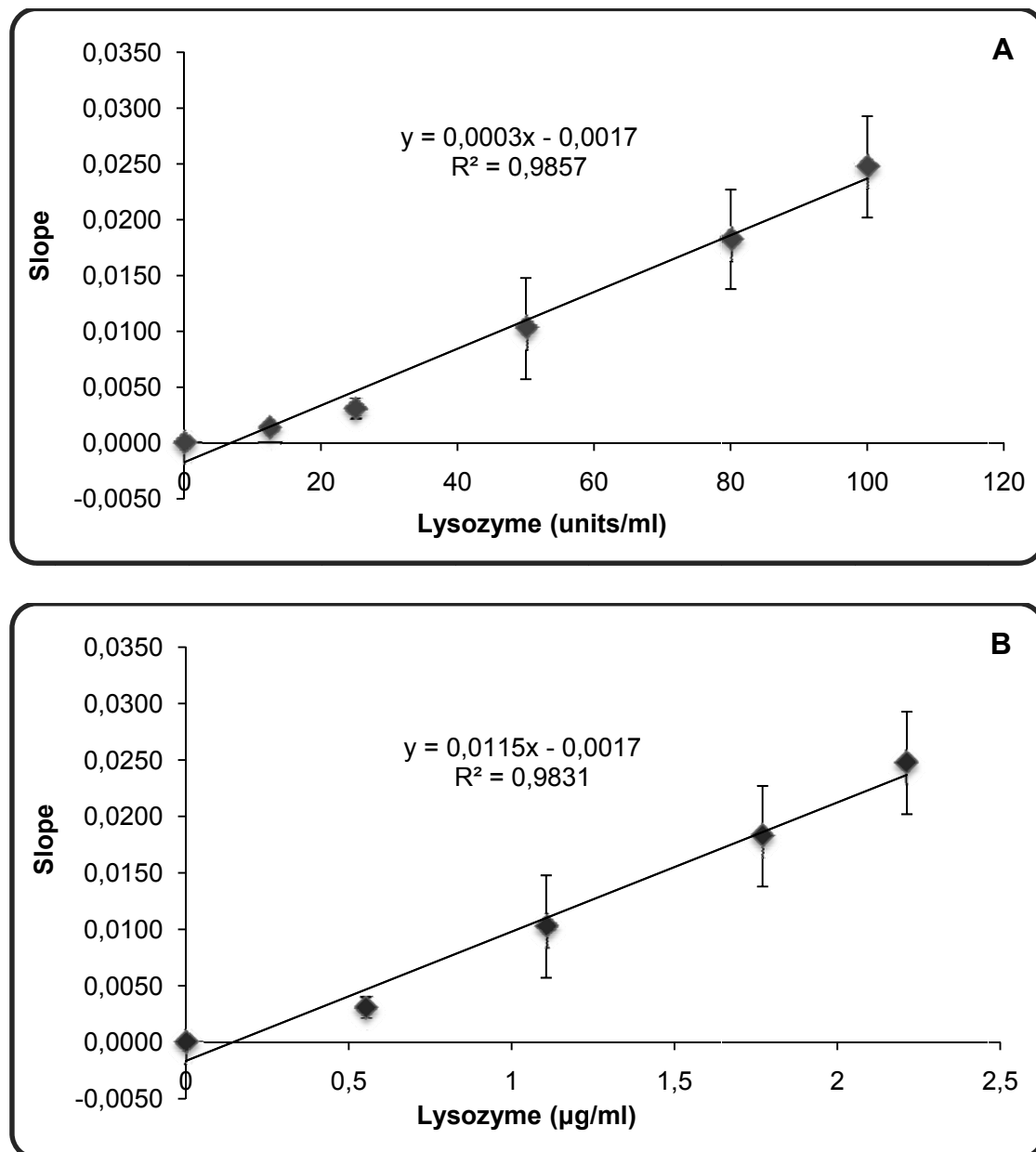
It was necessary for the chicken egg white lysozyme standard, which was used in the turbidimetric lysozyme assay, to be calibrated in the laboratory under our conditions.

One unit (1 U) of lysozyme activity was defined by the manufacturer (Sigma) as decreasing a 0.001 absorption value at 450nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* suspension as substrate, under the conditions of pH 6.24 and 25°C, in a 2.6ml reaction mixture (1cm light path). In our study, we have defined the unit of lysozyme activity under the used conditions as decreasing a 0.001 absorption value at 450 nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* suspension as substrate, under the conditions of pH 6.24 and 37°C, in a 250µl reaction mixture using 96-well-microtiter plate.

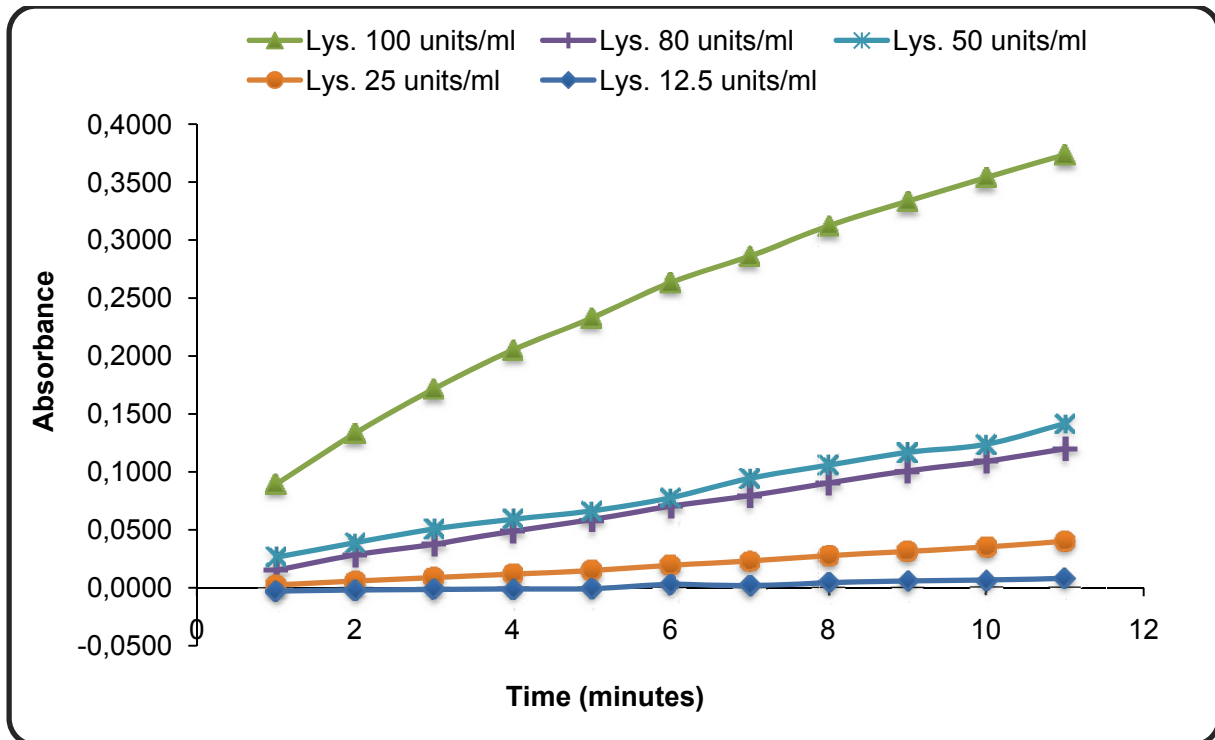
The graph in Fig.8 indicates a linear relationship between lysozyme as measured calibrated units of activity per ml and as units of activity stated by the manufacturer per ml, while the graph in Fig.9 indicates a linear relationship between lysozyme as measured calibrated units of activity per ml and as micrograms of lysozyme per ml.

Sigma<sup>®</sup> egg white lysozyme standard has been declared to have an activity of 45200 units/mg under the conditions stated by Sigma<sup>®</sup>. In comparison with our calibration diagram, however, an activity of approximately 10932 units/mg was recorded under our conditions (Fig.8; Fig.9).

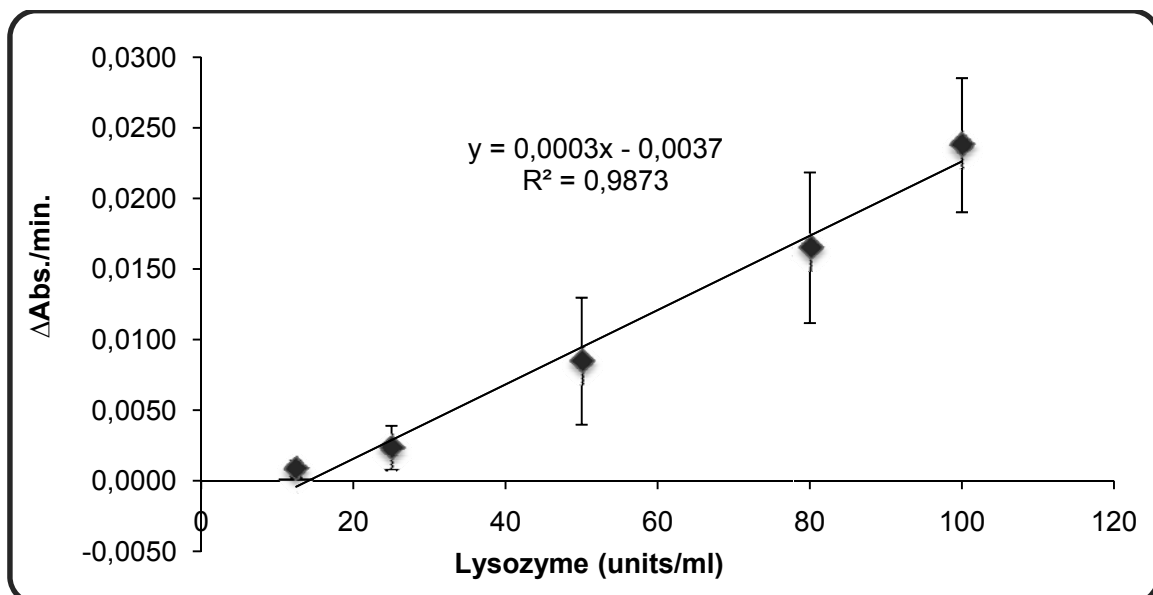
The rate of lysis of *Micrococcus lysodeikticus* suspension induced by lysozyme standard at different concentrations, represented in the slope values of the kinetic curves of each concentration, was linear in the range of 0.8-23.8 calibrated units/ml (0.28-2.2 $\mu$ g/ml), according to our definition of the activity unit of lysozyme (Fig.10). The linear regression equation and the correlation coefficient of this standard curve were:  $y = 0.001x + 0.0006$  and  $R^2 = 0.9967$ , respectively.



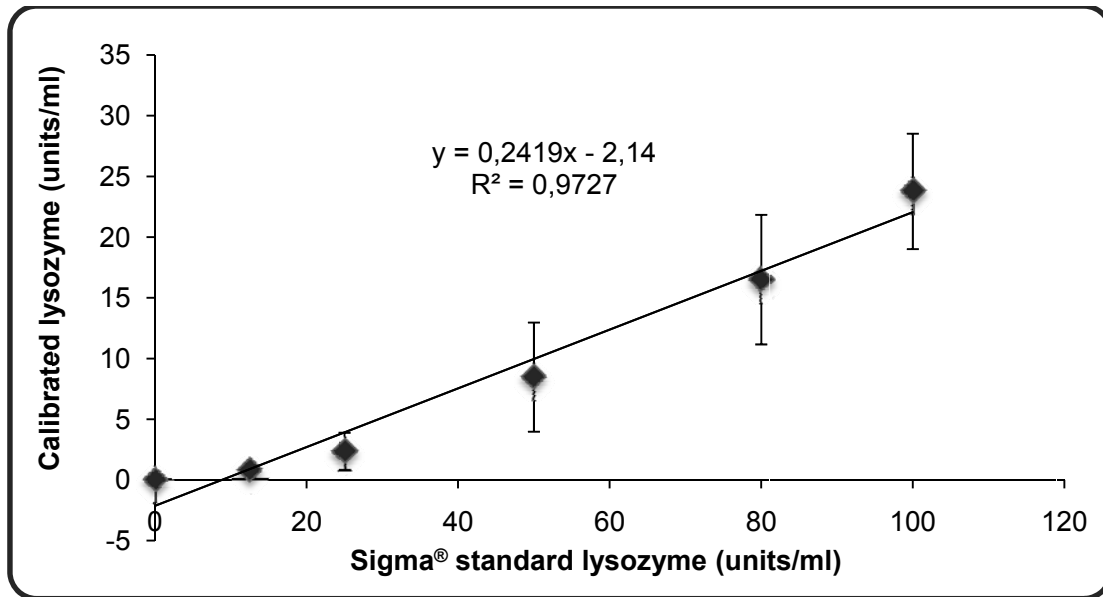
**Fig.5.** Chicken egg white lysozyme standard curve using the microtiter plate turbidity assay. Lysozyme was expressed as units of activity per ml in diagram A, and as micrograms of lysozyme per ml in diagram B. All points were measured in triplicate, and the curve is representative of six separate experiments.



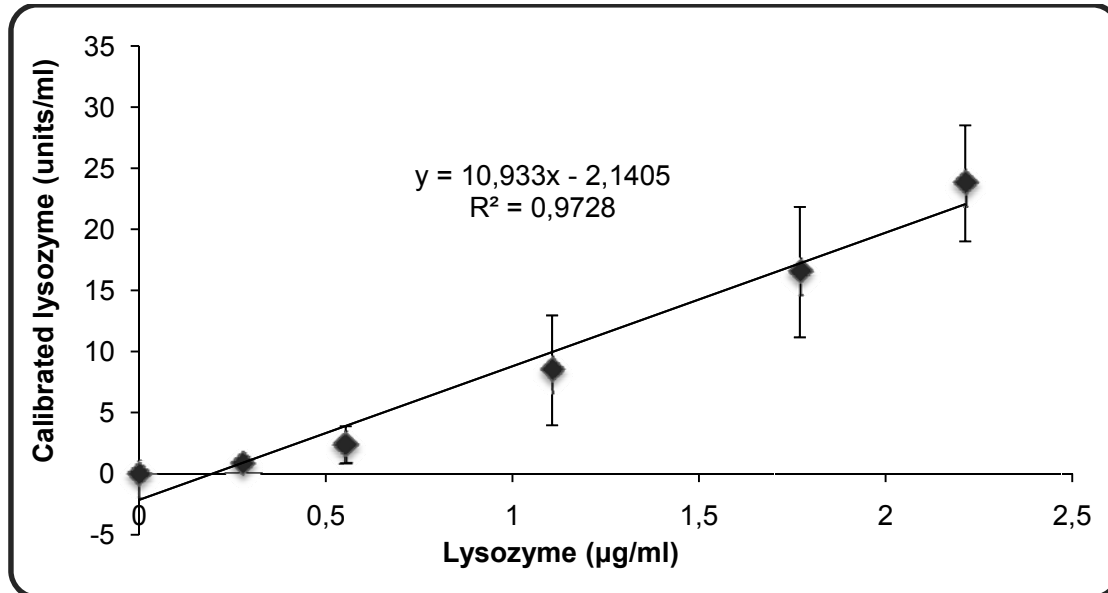
**Fig.6.** Chicken egg white lysozyme standard kinetic curve using the microtiter plate turbidity assay. Increasing concentrations of lysozyme were incubated with 0.36mg/ml *Micrococcus lysodeikticus* suspension for 10 minutes at 37°C. The absorbance was measured kinetically using microplate reader with a 450 nm filter. A background absorbance was subtracted from each value. All points were measured in triplicate.



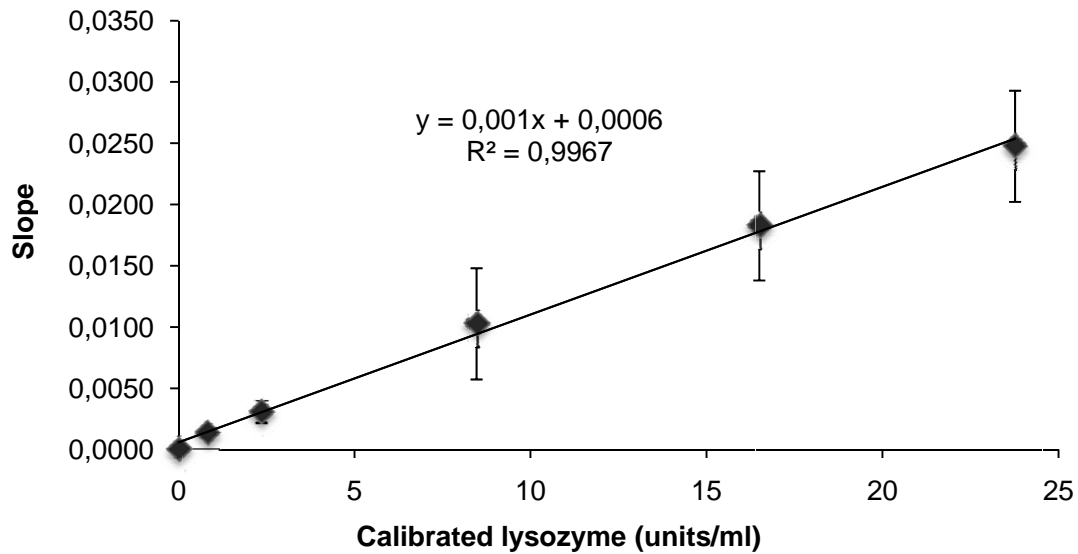
**Fig.7.** Increasing concentrations of lysozyme activity were incubated with 0.36mg/ml *Micrococcus lysodeikticus* suspension at 37°C. The rate of the reaction was measured kinetically for 10 minutes as absorbance change per minute using spectrophotometer with a 450nm filter. Average changes in absorbance per minute for each concentration of lysozyme activity are illustrated. All points were measured in triplicate, and the curve is representative of three separate experiments.



**Fig.8.** Calibration diagram of Sigma® chicken egg white lysozyme standard. Calibrated unit of lysozyme activity was defined as decreasing a 0.001 absorption value at 450nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* suspension as substrate, under the conditions of pH 6.24 and 37°C, in a 250µl reaction mixture using 96-well-microtiter plate. All points were measured in triplicate, and the curve is representative of three separate experiments.



**Fig.9.** Calibration diagram of Sigma® chicken egg white lysozyme standard. The graph indicates a linear relationship between lysozyme as measured calibrated units of activity per ml and as micrograms of lysozyme per ml. All points were measured in triplicate, and the curve is representative of six separate experiments.



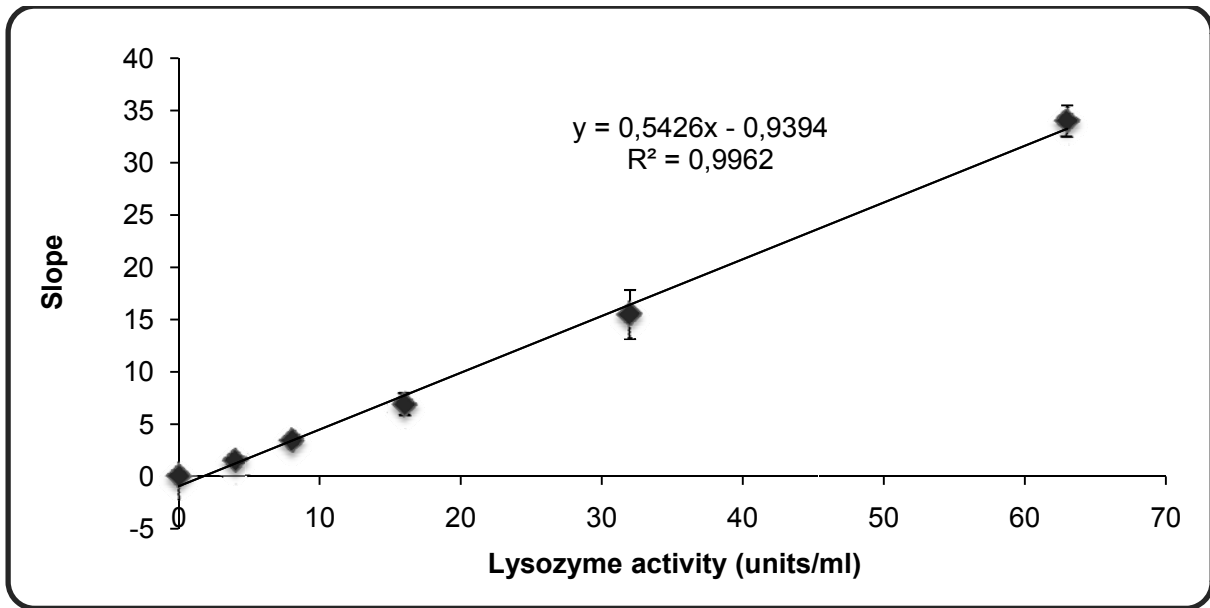
**Fig.10.** Chicken egg white lysozyme standard curve using the microtiter plate turbidity assay. Lysozyme is expressed as calibrated units of activity per ml. All points were measured in triplicate, and the curve is representative of three separate experiments.

### 3.1.2. Fluorescence-based lysozyme activity assay using EnzChek<sup>®</sup> kit

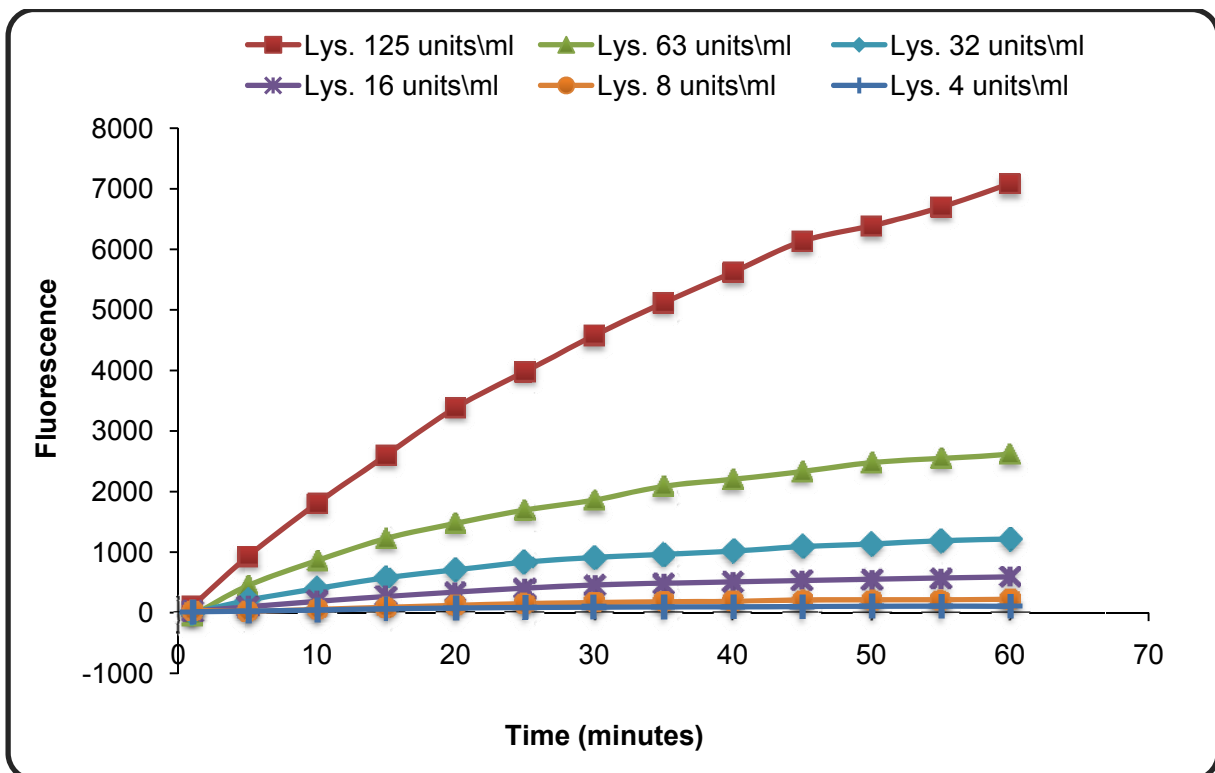
Lysozyme standard curve using EnzChek<sup>®</sup> fluorescence-based assay was linear with a correlation coefficient mean of  $R^2 = 0.9962$ . The linear regression equation of a selected standard curve is illustrated in Fig.11. Several EnzChek<sup>®</sup> assay kits with different lot numbers were used to determine lysozyme activity in our experiments. Therefore, it was necessary to create lysozyme standard curve for each kit lot number used in lysozyme assay in order to avoid any variation between the different lot numbers. The rate of lysis of fluorescein labeled *Micrococcus lysodeikticus* suspension induced by lysozyme standard at different concentrations, represented in the slope values of the kinetic curve of each concentration, was linear in the range of 4-63 units/ml (Fig.12). Lysozyme activity levels presented in the cell culture supernatants in question were determined directly from the respective standard curve, according to the lot number of the kit used in the assay.

#### EnzChek<sup>®</sup> lysozyme standard calibration

Lysozyme standard of the EnzChek<sup>®</sup> assay kit was turbidimetrically calibrated in the laboratory under our conditions, considering the definition of calibrated unit of lysozyme activity mentioned above. The line in Fig.13 indicates a linear relationship between lysozyme as measured calibrated units of activity per ml and as units of activity stated by the manufacturer per ml. The rate of lysis of *Micrococcus lysodeikticus* suspension induced by lysozyme standard at different concentrations, represented in the slope values of the kinetic curves of each concentration, was linear in the range of 0.47-10.6 calibrated units/ml, according to our definition of the activity unit of lysozyme (Fig.14).

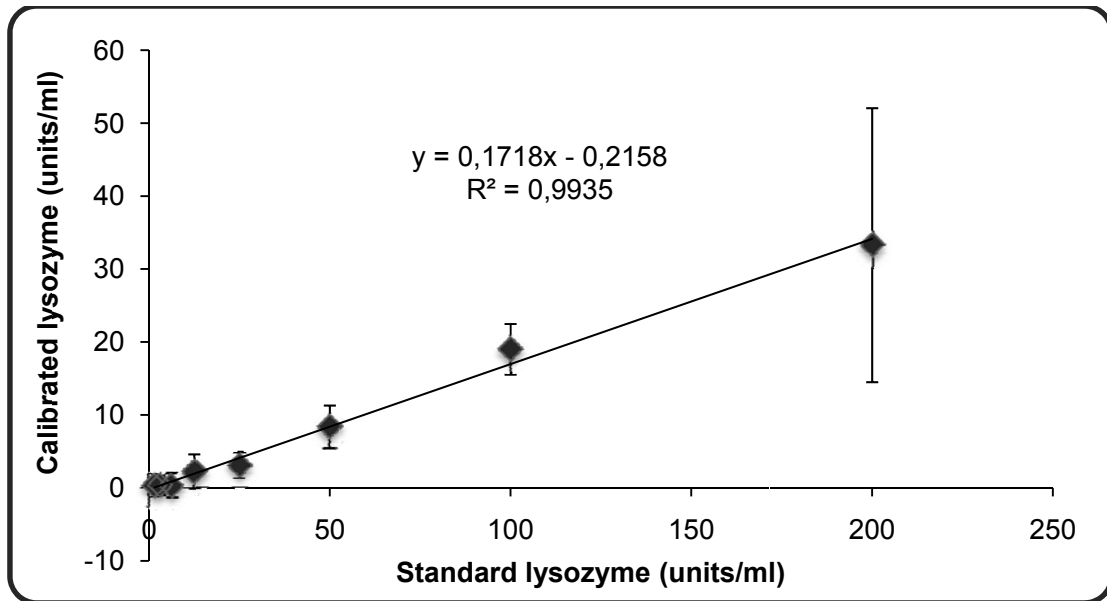


**Fig.11.** Lysozyme standard curve using EnzChek<sup>®</sup> fluorescence-based lysozyme assay kit. Lysozyme was expressed as units of activity per ml. All points were measured in triplicate, and the curve is representative of six separate experiments.

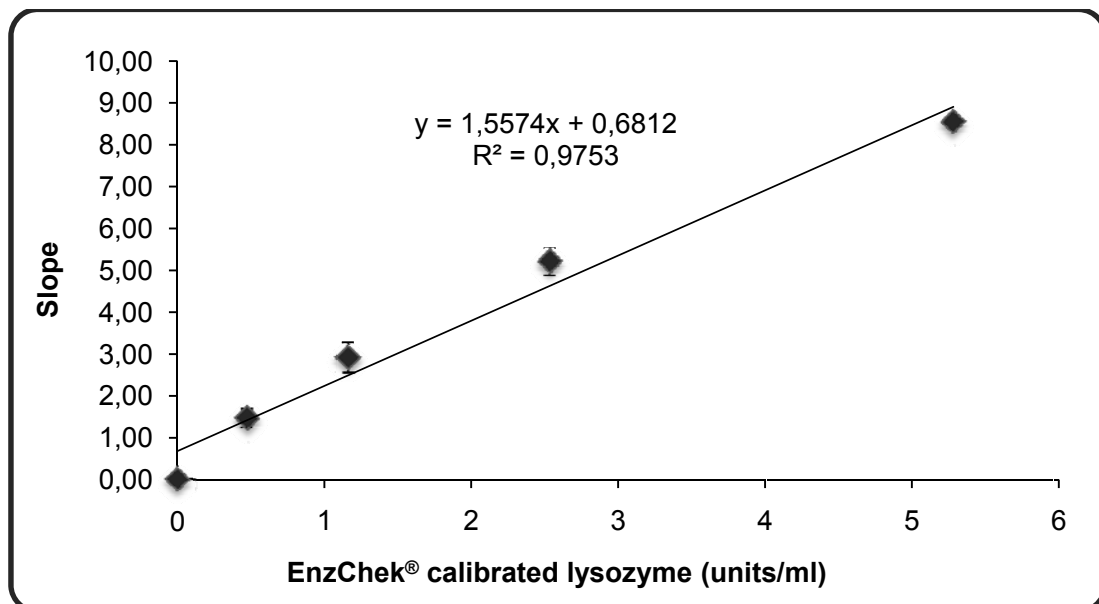


**Fig.12.** Lysozyme standard kinetic curves using EnzChek<sup>®</sup> lysozyme assay kit. Increasing concentrations of lysozyme were incubated with the DQ lysozyme substrate for 60 minutes at 37°C. The fluorescence was measured kinetically in a fluorescence microplate reader using excitation/emission of ~485/530 nm. The background fluorescence was subtracted from each value. All points were measured in triplicate.





**Fig.13.** Calibration diagram of EnzChek® chicken egg white lysozyme standard. Calibrated unit of lysozyme activity is defined as decreasing a 0.001 absorption value at 450 nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* suspension as substrate, under the conditions of pH 6.24 and 37°C, in a 250 µl reaction mixture using 96-well-microtiter plate. All points were measured in triplicate, and the curve is representative of three separate experiments.



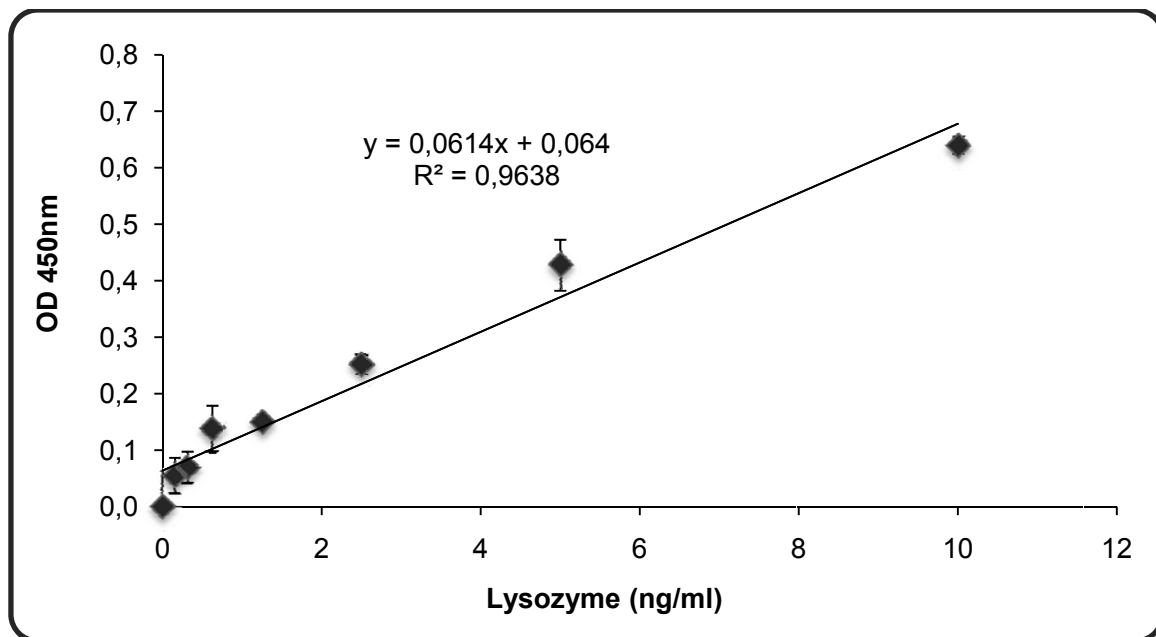
**Fig.14.** EnzChek® lysozyme standard curve using the microtiter plate turbidity assay. Lysozyme is expressed as calibrated units of activity per ml. All points were measured in triplicate, and the curve is representative of three separate experiments.

### 3.1.3. Enzyme-linked immunosorbent assay (ELISA) of lysozyme

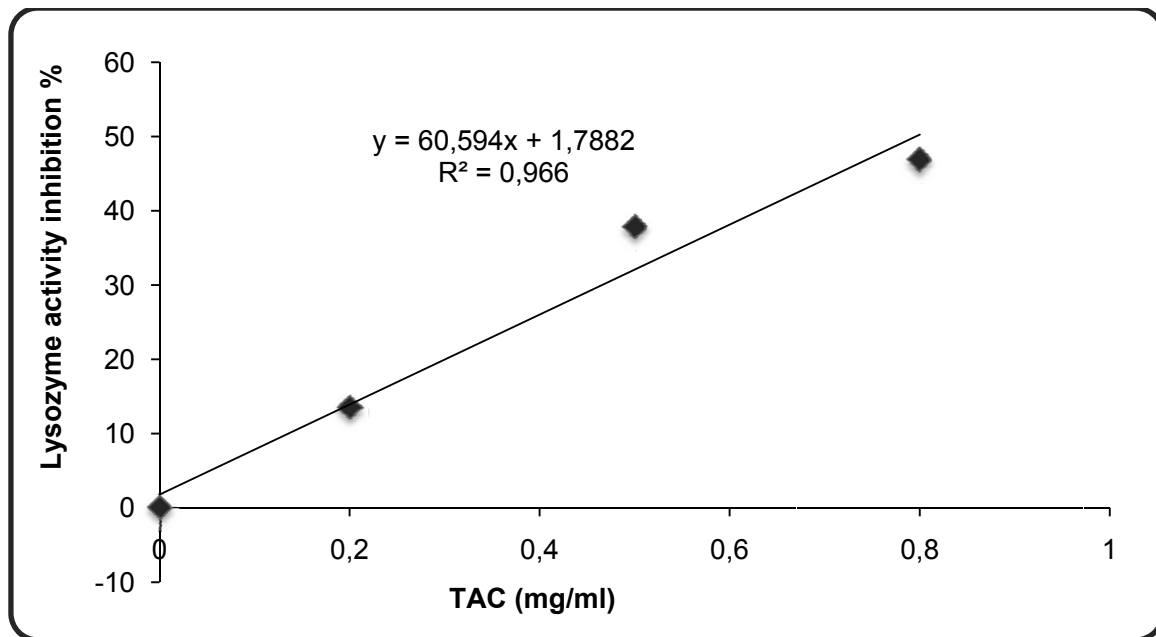
Human lysozyme standard curve using ELISA assay was linear with a correlation coefficient of  $R^2 = 0.9638$  (Fig.15). The 450nm absorbance was proportional to standard lysozyme concentration. The linear regression equation of this standard curve was:  $y = 0,0614x + 0,064$  (Fig.15). Expressing lysozyme as nanograms of protein per millilitre, lysozyme standard curve was linear in the range of 0.156-10 ng/ml. Lysozyme activity levels presented in the cell culture supernatants in question were determined directly from this standard curve.

### 3.1.4. Lysozyme activity inhibition

The enzymic activity of lysozyme can be blocked by a competitive inhibitor of lysozyme N,N',N''-triacetylglucosamine, also referred to as N,N',N''-triacetylchitotriose (TAC) or chitotriose. TAC is the trisaccharide of NAG that has three NAG residues linked by  $\beta$  (1– 4) glycosidic linkages and competitively inhibits the enzymatic activity of lysozyme, and this inhibitor was found to be quite specific (Mink *et al.*, 2004). The inhibitory activity of TAC for lysozyme activity was examined to determine the IC<sub>50</sub> of this inhibitor using the turbidimetric assay as means to detect the accuracy and the specificity of this assay in measuring lysozyme activity. Dose-dependent inhibition of lysozyme activity by triacetylchitotriose could be proved, which was proportional to TAC concentration with a linear correlation coefficient of  $R^2=0.966$ . The IC<sub>50</sub> of N,N',N''-triacetylchitotriose (TAC) was determined to be approximately 0.8mg/ml (1.28mM) (Fig.16). Concentrations lower than 0.2 mg/ml were tested and did not show significant inhibition of lysozyme activity.



**Fig.15.** Human lysozyme standard curve using ELISA assay. Lysozyme was measured as nanogram per ml. The absorbance was measured using microplate reader with a 450nm filter. All points were measured in triplicate.



**Fig.16.** Dose-dependent curve of lysozyme activity inhibition using increasing concentrations of N,N',N''-triacetylchitotriose (TAC). Lysozyme activity was measured using the turbidimetric assay. 80units/ml lysozyme solution without addition of TAC served as control, and was considered to have 100% enzymatic activity. All points were measured in triplicate.

### 3.2. Expression and release of lysozyme in human cell cultures

#### 3.2.1. Human monocytic cell lines

##### 3.2.1.1. Human acute monocytic leukemia cell line THP-1

Detectable amounts of lysozyme activity could be determined intracellularly as well as extracellularly in the supernatants of THP-1 cell cultures. Lysozyme activity levels synthesized (total lysozyme) and released (secreted lysozyme) in THP-1 monocytes cultures at various incubation periods of up to 72 hours are illustrated in Fig.17. Lysozyme was determined using EnzChek<sup>®</sup> fluorescence-based lysozyme activity assay and expressed in units per ml (Fig.17.A), or using ELISA lysozyme protein-assay and expressed in nanogram per ml (Fig.17.B).

Our data suggested that after one hour incubation, only 30% of the lysozyme activity synthesized by THP-1 cells, equivalent to 10 units/ml, was secreted in the medium of the cell cultures; whereas 70% non-secreted lysozyme activity, equivalent to 23 units/ml, was detected inside the cells.

After incubation of 24 hours, approx. 70% of the lysozyme activity synthesized by the cells, equivalent to 32 units/ml, was secreted in the medium of cell cultures, while only 30% equivalent to 13 units/ml of the lysozyme activity was detected non-secreted inside the cells.

After further incubation of 48 hours, about 80% of the lysozyme activity synthesized by the cells was secreted in the culture medium, whereas 95% of the lysozyme activity was secreted after 72 hours incubation (Fig.17.A).

Based on these results, we found increases in the lysozyme activity levels with the time course. Total lysozyme activity synthesized by THP-1 cells cultured at initial concentration of  $1 \times 10^6$  cells/ml increased by 42% after 24 hours incubation, whereas an increase of 96% was detected after 48 hours, and of 105% after 72 hours incubation in culture. This rise of lysozyme activity levels with the increasing incubation periods was logical due to the continuous lysozyme expression by cells with the time, as well as the multiplication of cells in the cell culture.

### **3.2.1.2. Human promyelocytic leukemia cell line HL-60**

Detectable levels of lysozyme activity in the supernatants of HL-60 cell cultures could be determined (Fig.18). We could also find increases in the lysozyme activity with the time course. Higher lysozyme activity could be determined in the cell lysate.

Our measurements illustrated that after 1 hour incubation, only small amount of lysozyme activity of 2units/ml was found secreted in the medium of the cell cultures; whereas approx. 90% of the lysozyme activity, equivalent to 15.7units/ml, was detected non-secreted inside the cells. After 24 hours incubation, approx. 87% of the lysozyme activity, equivalent to 48units/ml, was secreted in the medium of the cell cultures; whereas little non-secreted lysozyme activity, equivalent to 7units/ml, was detected inside the cells.

Compared with THP-1 monocytes, total lysozyme activity synthesized by  $1 \times 10^6$  cells/ml culture of HL-60 monocytes after 1 hour incubation in culture medium was less by 45%, while with further 24 hours incubation total lysozyme activity synthesized by HL-60 cells was higher by 20% than that synthesized by THP-1 cultures with the same initial cell density.

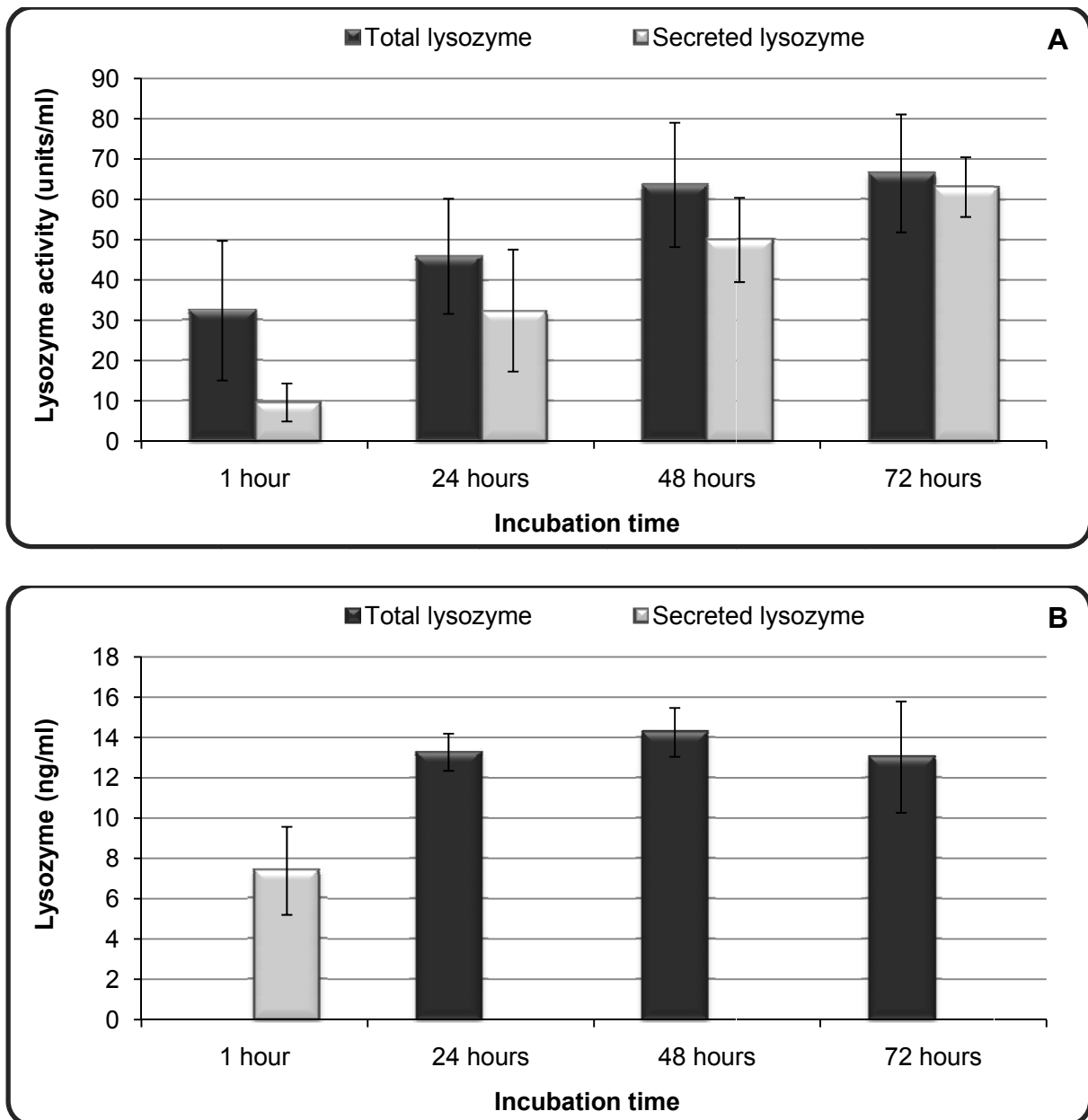
### **3.2.1.3. Human leukemic monocyte lymphoma cell line U937**

No lysozyme activity could be detected in the cultured U937 cells either with or without addition of the inducing factors PMA and hIFN- $\gamma$  at an initial concentration of  $1.0 \times 10^6$  cells/ml and for incubation periods up to 72 hours (n=6). Moreover, no lysozyme activity was found in the whole cells lysates. Contrary to previous reports, we couldn't find any evidence that U937 cell line was able to produce and secrete detectable levels of active lysozyme, neither with nor without addition of PMA and hIFN- $\gamma$ .

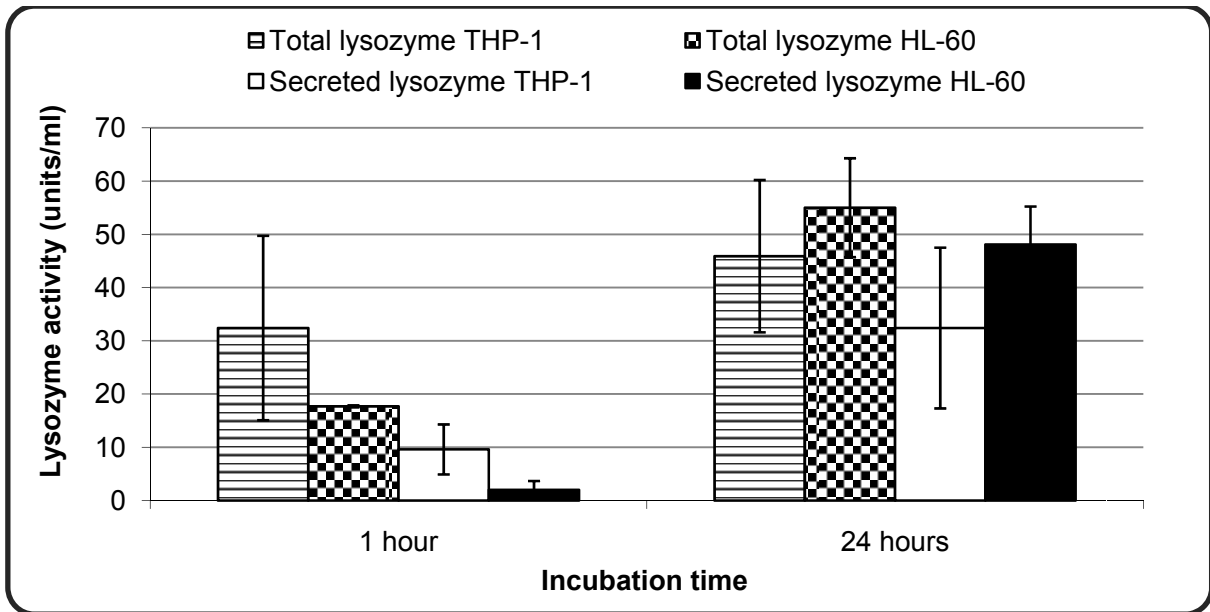
## **3.2.2. Human epithelial cell lines & tumor cells**

### **3.2.2.1. Human colon adenocarcinoma cell line HT-29**

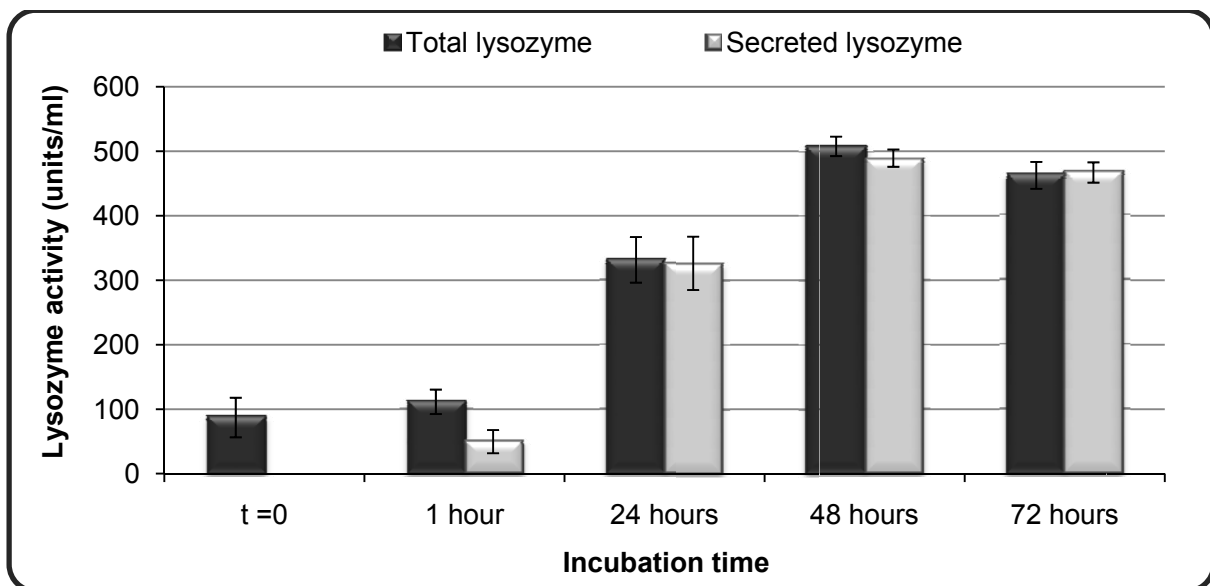
Detectable amounts of lysozyme activity could be determined intracellularly as well as extracellularly in the supernatants of HT-29 cell cultures. We could also find increases in the lysozyme activity with the time course. Lysozyme activity synthesized by  $0.5 \times 10^6$  HT-29 cells measured directly after culture medium change (t=0) was 87 units, which represented the non-secreted lysozyme activity inside the cells.



**Fig.17.** Total and secreted lysozyme activity produced by  $1 \times 10^6$  cells/ml THP-1 cell cultures at various incubation periods of up to 72 hours. Lysozyme was determined using EnzChek<sup>®</sup> fluorescence-based lysozyme activity assay (A) or ELISA lysozyme protein-assay (B). All values were measured in triplicate. The columns depict the combined averaged results of at least 20 independent experiments.



**Fig.18.** Total and secreted lysozyme activity in  $1 \times 10^6$  cells/ml THP-1 and HL-60 cell cultures after 1 hour and after 24 hours incubation using sensitive fluorescence-based lysozyme assay. All values were measured in triplicate. The figure depicts the combined, averaged results of six independent experiments for HL-60 cell line, and of at least 20 independent experiments for THP-1 cell line.



**Fig.19.** Total and secreted lysozyme activity produced by  $0.5 \times 10^6$  cells/ml HT-29 cell cultures at various incubation periods of up to 72 hours using sensitive fluorescence-based lysozyme assay. All values were measured in triplicate. The columns depict the combined averaged results of at least 20 independent experiments.

Our results suggested that after one hour incubation, about half of the lysozyme activity synthesized by HT-29 cells, equivalent to 50 units/ml, was secreted in the medium of the cell cultures; whereas the second half was detected non-secreted inside the cells.

After incubation of 24 hours, the majority of lysozyme activity synthesized by the cells, equivalent to 326 units/ml, was secreted in the cell cultures medium, while only 2% equivalent to 5 units/ml of the lysozyme activity was detected non-secreted inside the cells.

After further incubation of 48 hours, about 96% of the lysozyme activity synthesized by the cells was secreted in the culture medium equivalent to 490 units/ml, whereas only 18 units/ml of the lysozyme activity remained inside the cells

After 72 hours incubation, a decrease in the expression of lysozyme activity was determined. The total lysozyme activity synthesized at this time point was 467 units/ml.

Moreover, no detectable amount of lysozyme activity could be assayed inside the cells, which indicated that the whole amount of lysozyme activity synthesized by cells after 72 hours incubation was secreted into the culture medium (Fig.19).

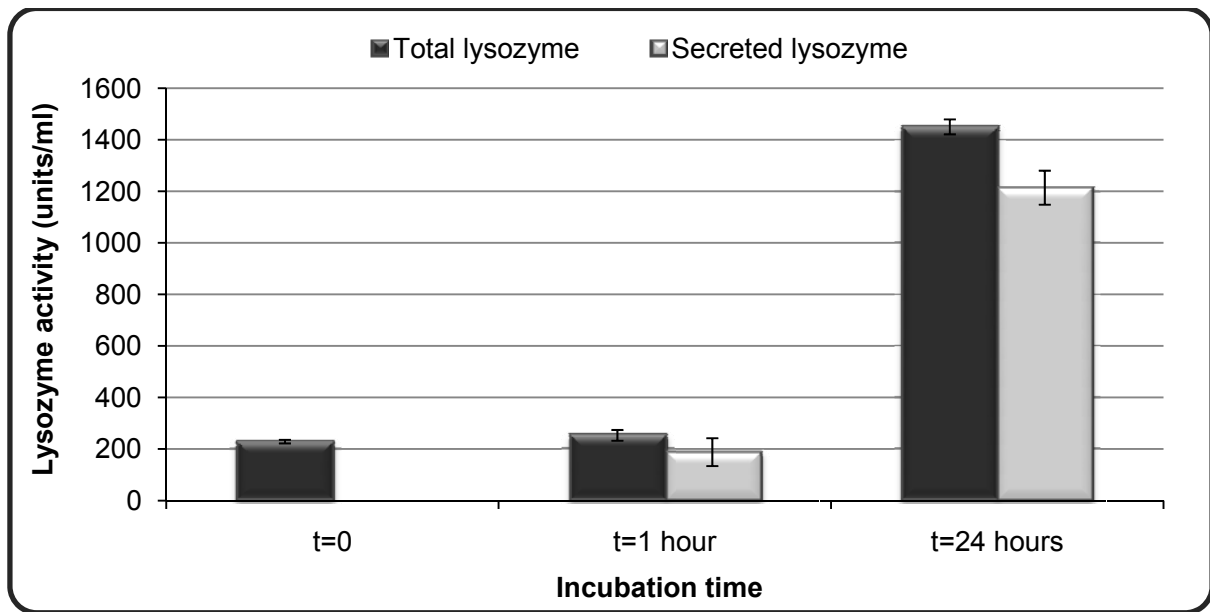
Based on these results, increases in the lysozyme activity levels expressed by HT-29 cells with the time course were proved. Total lysozyme activity synthesized by HT-29 cells cultured at initial concentration of  $0.5 \times 10^6$  cells/ml increased by 28% after 1 hour incubation, whereas an increase of 280% was detected after 24 hours, and of 480% after 48 hours incubation in culture. This rise of lysozyme activity levels with the increased incubation period could be explained by the continuous lysozyme expression of the cells with the time, as well as the multiplication of cells in culture.

On the other hand, a drop in the expression of lysozyme activity started to appear after 72 hours incubation. This drop could be justified in virtue of the lack of sufficient nutrients owing to the excessive cell count after such a long incubation period without renewing culture medium. This lack could lead to decrease in cell viability and ability to express enzymes.

#### **3.2.2.2. Human gastric adenocarcinoma cell line AGS**

Detectable amounts of lysozyme activity could be determined in the supernatants of AGS cell culture (secreted lysozyme) and inside the cells (intracellular lysozyme). Total lysozyme activity synthesized by  $0.5 \times 10^6$  AGS cells measured directly after culture medium change ( $t=0$ ) was 229 units, which represented the non-secreted lysozyme activity inside the cells. After one hour incubation, about three-quarters of the lysozyme activity synthesized by AGS cells, equivalent to 188 units/ml, was secreted in the medium of the cell cultures; whereas less non-secreted lysozyme activity, equivalent to 65 units/ml, was detected inside the cells. After further incubation of 24 hours, approx. 84% of the lysozyme activity synthesized by the cells, equivalent to 1214 units/ml, was secreted in the culture medium, while only 16% equivalent to 236 units/ml of the lysozyme activity was detected non-secreted inside the cells (Fig.20).

Based on these results, we found increases in the lysozyme activity levels with the time course. Total lysozyme activity synthesized by AGS cells cultivated in  $0.5 \times 10^6$  cells/ml cultures increased by only 10% after one hour incubation, whereas an increase of 533% was detected after 24 hours incubation in culture. This rise of lysozyme activity levels with the increased incubation period was logical due to the continuous lysozyme expression by the cells with the time, as well as the multiplication of cells in culture.



**Fig.20.** Total and secreted lysozyme activity produced by  $0.5 \times 10^6$  cells/ml AGS cell cultures at various incubation time points of up to 24 hours using sensitive fluorescence-based lysozyme assay. All values were measured in triplicate. The columns depict the combined averaged results of at least six independent experiments.

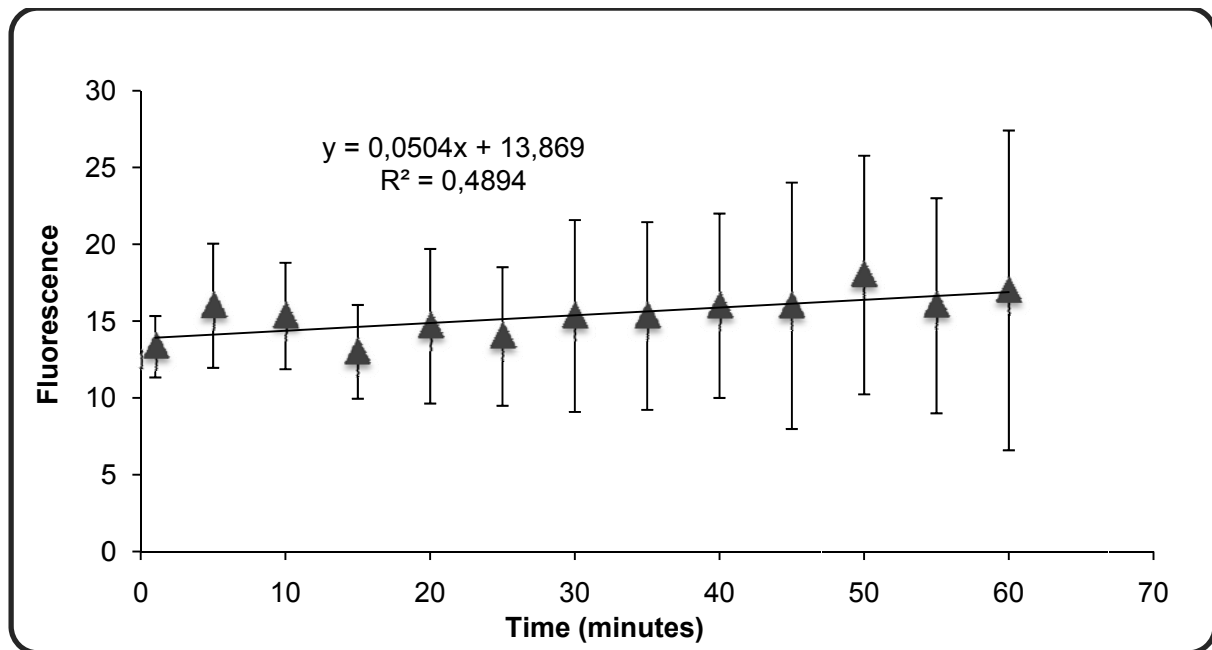
### 3.2.2.3. Human lung carcinoma cell line A549

Lysozyme activity levels of A549 cells after 4 days' cultivation were measured using highly sensitive fluorescence-based EnzChek lysozyme assay (Helal & Melzig, 2008). No measurable levels of lysozyme activity could be detected in the supernatants of A549 cell cultures after 4 days incubation (Fig.21).

### 3.2.2.4. Transformed human bronchial epithelial cell line BEAS-2B

Lysozyme activity of BEAS-2B cell cultures was measured using highly sensitive fluorescence-based EnzChek lysozyme assay (Helal & Melzig, 2008). Lysozyme activity levels in the supernatants of the cell cultures were determined after 1, 2, 3 and 4 days incubation of the cells in culture medium. No secreted lysozyme activity could be detected in the supernatants of BEAS-2B epithelial cell cultures at all incubation periods tested up to 4 days.





**Fig.21.** Assay kinetic curve of lysozyme activity in A549 cell cultures after 4 days incubation using EnzChek® fluorescence-based assay. The background fluorescence was subtracted from each value. All points were measured in triplicate.

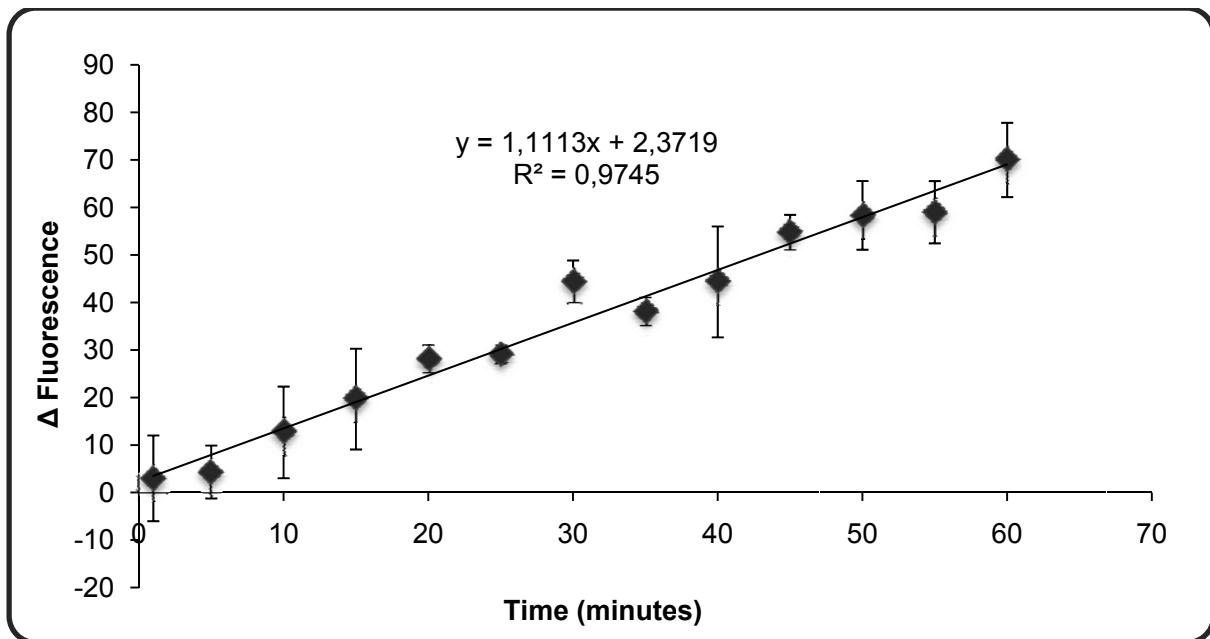
### 3.2.2.5. Human oral squamous cell carcinoma cell line BHY

Lysozyme activity of BHY cell cultures was measured using highly sensitive fluorescence-based EnzChek lysozyme assay (Helal & Melzig, 2008). Lysozyme activity levels in the supernatants of the cell cultures were determined after 24 and 48 hours incubation of the cells in the culture medium. No secreted lysozyme activity could be detected in the supernatants of BHY epithelial cell cultures at all incubation periods tested.

Our findings suggest that BHY oral squamous epithelial cells do not secrete active lysozyme when cultured for periods up to 2 days.

### 3.2.2.6. Tongue-derived human tumor cells

Detectable amounts of lysozyme activity could be determined in the primary culture of tumor cells derived from patient with oral tumor. Lysozyme activity secreted in the medium of six days old culture measured using sensitive fluorescence-based lysozyme assay was equivalent to  $5 \pm 0.3$  units/ml (Fig.22).



**Fig.22.** Assay kinetic curve of secreted lysozyme activity of 6 days old primary cell culture of tongue derived tumor cells from patient with oral tumor. Lysozyme activity was assayed according to sensitive fluorescence-based lysozyme assay. The background fluorescence was subtracted from each value. All points were measured in triplicate.

### 3.3. In vitro evaluation of the effects of various compounds and natural products on the expression and release of lysozyme in human cells

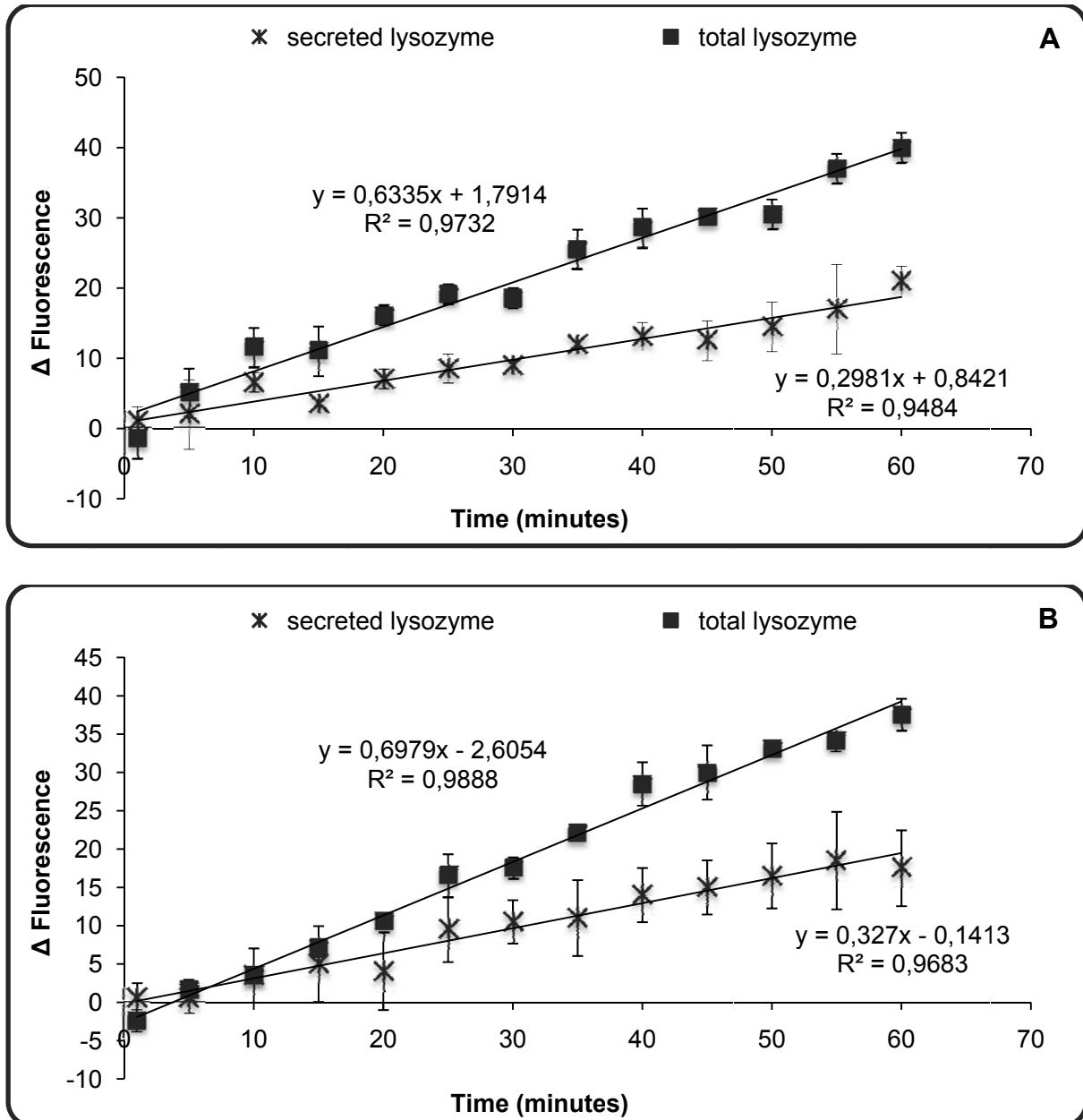
#### 3.3.1. Effects of solvents and helping agents on lysozyme activity

##### 3.3.1.1. The serine protease inhibitor aprotinin

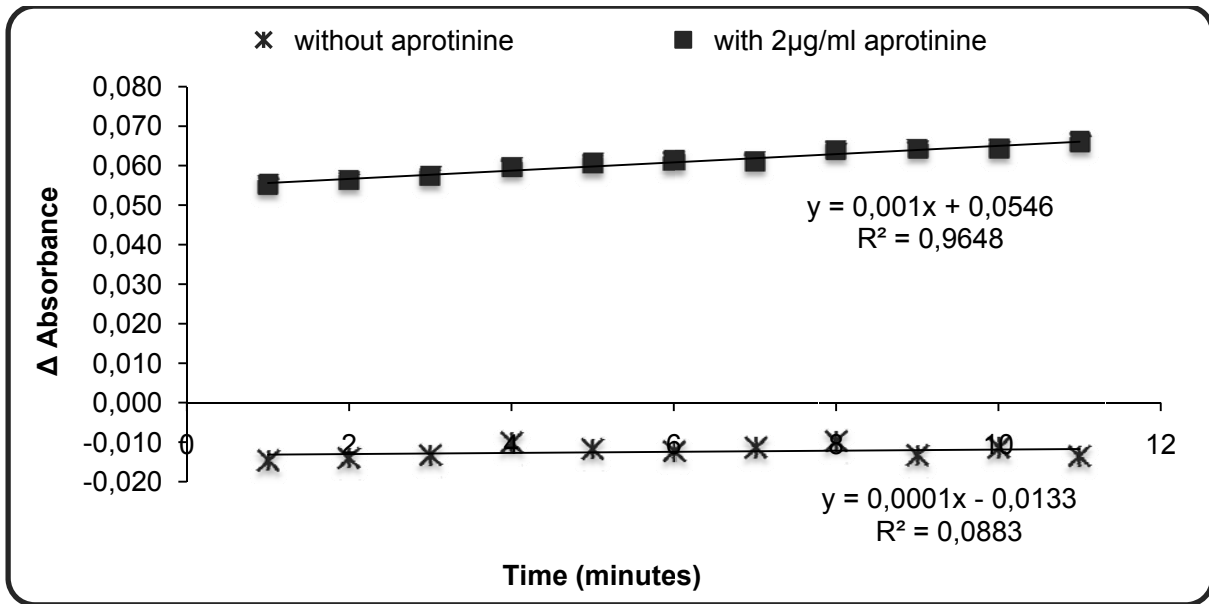
Effect of aprotinin, a competitive serine protease inhibitor, on lysozyme activity secreted in culture medium of monocytic cell lines was studied. Aprotinin was added to the cell culture media with a final concentration of 2 $\mu$ g/ml (0.3 $\mu$ M), and lysozyme activity in these cell cultures was measured fluorometrically and turbidimetrically after 1 hour incubation. Cell cultures incubated for one hour without addition of aprotinin were used as controls.

Higher levels of secreted and total lysozyme activity in THP-1 cultures supplemented with 2 $\mu$ g/ml aprotinin after one hour incubation could be measured in comparison to control cultures using both lysozyme activity assay methods. Kinetic curves of lysozyme activity measured fluorometrically illustrated in Fig.23 showed that addition of aprotinin caused an increase in the slope values as well as linearity enhancement. This slope increase was even more significant when lysozyme activity was measured using the turbidimetric assay (Fig.24). On the other hand, addition of aprotinin to HL-60 cultures led to decrease in lysozyme activity secreted in culture medium after one hour incubation when compared to control cultures (Fig.25). Furthermore, culture medium supplemented with aprotinin was examined with U-937 cultures, which was not able to enhance lysozyme secretion in this cell line.

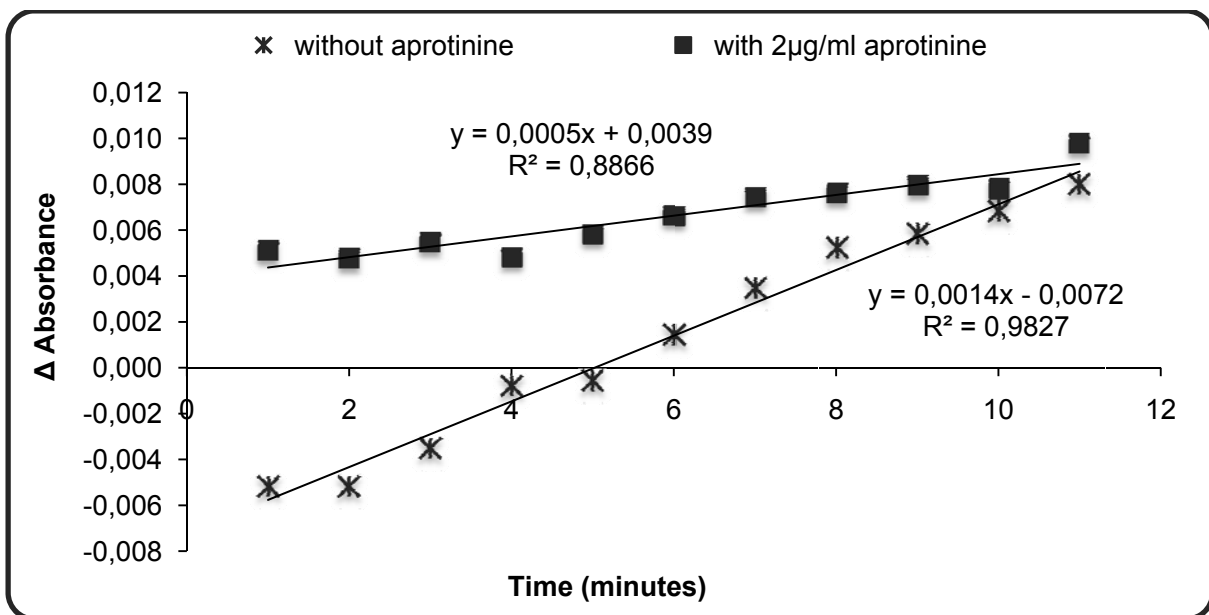
Based on these results, aprotinin was added to the incubation culture medium of THP-1 at a final concentration of 2 $\mu$ g/ml (0.3  $\mu$ M), in order to achieve best lysozyme determination results. Aprotinin addition to HL-60 and U-937 cell culture media was not necessary, because it had no enhancing effects on the results of lysozyme activity assay.



**Fig.23.** Total and secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after 1 hour incubation in culture medium only (A), and in culture medium supplemented with 2 $\mu$ g/ml aprotinine (B) using EnzChek lysozyme assay. The background fluorescence was subtracted from each value. All points were measured in triplicate, and the curves are representative of three separate experiments.



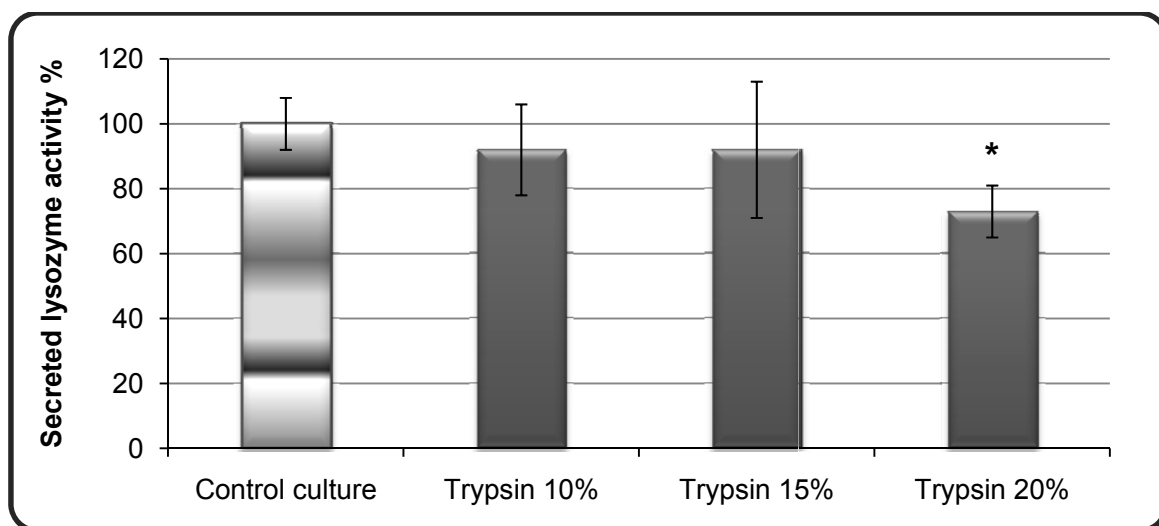
**Fig.24.** Secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after 1 hour incubation in culture medium only, and in culture medium supplemented with  $2 \mu\text{g/ml}$  aprotinine using the microtiter plate turbidity assay. The background absorbance was subtracted from each value. All points were measured in triplicate, and the curves are representative of three separate experiments.



**Fig.25.** Secreted lysozyme activity of  $1 \times 10^6$  cells/ml HL-60 cell cultures after 1 hour incubation in culture medium only, and in culture medium supplemented with  $2 \mu\text{g/ml}$  aprotinine using the microtiter plate turbidity assay. The background absorbance was subtracted from each value. All points were measured in triplicate, and the curves are representative of three separate experiments.

### 3.3.1.2. Trypsin

Trypsin-EDTA solution was used in the subculturing of adherent cell lines to dissociate the cell layer adherent to culture flask. Influence of trypsin solution on the enzymatic activity of lysozyme in HT-29 epithelial cell cultures was analyzed. Trypsin solution was added to HT-29 cell culture medium at final concentrations of 10%, 15% and 20% (v/v). Lysozyme activity levels in these cell cultures were measured after one hour incubation using the EnzChek<sup>®</sup> fluorescence-based assay. Cell cultures incubated without addition of trypsin served as controls. Trypsin solution concentrations equal to or less than 15% had no significant effect on lysozyme activity secretion in HT-29 cell cultures. A significant inhibition of 27% in the secreted lysozyme activity of HT-29 cells could be detected at trypsin concentration as high as 20% (v/v) (Fig.26). However, trypsin solution concentrations used in epithelial cell cultures examined in this work were less than 10%.

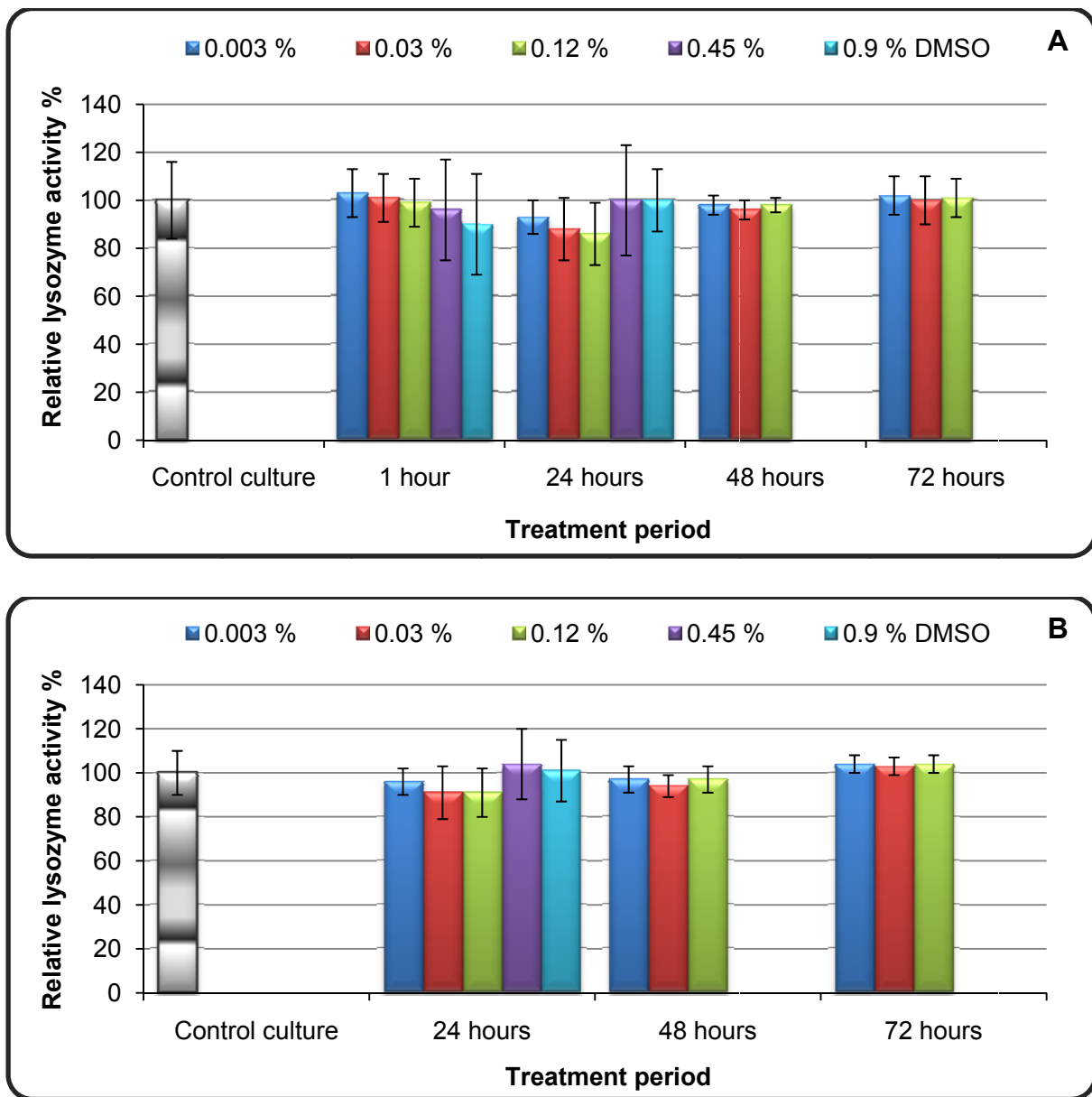


**Fig.26.** Percent change of lysozyme activity secretion in  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after 1 hour treatment with 10%, 15% and 20% (v/v) final concentration of trypsin using sensitive fluorescence-based lysozyme assay. Values are presented as mean of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures without trypsin addition.

### 3.3.1.3. Dimethylsulphoxide (DMSO)

Water-insoluble solid test substances and essential oils should be dissolved or suspended in an appropriate solvent or vehicle and diluted prior to treatment of the cells. The selected organic solvent to be used in our experiments was dimethylsulphoxide (DMSO). We investigated the influence of DMSO on the expression and release of lysozyme activity of monocytes and epithelial cells. Our data suggested that the amounts of DMSO used in our experiments did not significantly affect lysozyme activity of the cultivated monocytes and epithelial cells taking into consideration the incubation time (Fig.27). Nevertheless, negative controls containing the solvent DMSO in the culture medium and treated in the same way as

the tested cultures were included for every harvest time. Negative control cultures with DMSO were considered to have 100% lysozyme activity and utilized to make comparisons with cell cultures treated with DMSO-soluble investigated substances.



**Fig.27.** Percent changes in the secreted lysozyme activity (A) and in the total lysozyme activity (B) synthesized by  $0.5 \times 10^6$  cells/ml HT-29 cell cultures treated with DMSO at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without DMSO addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of at least six separate experiments.

### 3.3.1.4. Triton X-100 (polyethylene glycol octylphenol ether)

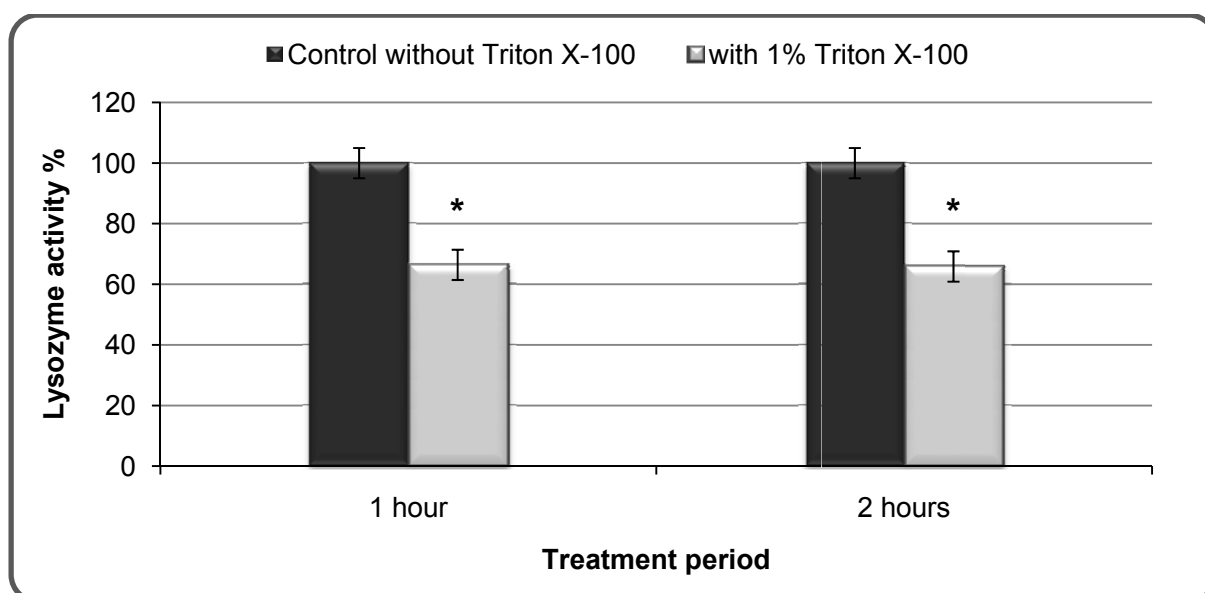
Cell lysis was an essential procedure to determine the remainder lysozyme activity inside the cells, in order to estimate the total lysozyme activity produced by the cells in the tested cell cultures. By lysozyme assay of the cell lysates we could determine the whole amount of lysozyme activity, including the extracellular lysozyme released in the culture medium and the intracellular lysozyme retained inside the cells.

Triton X-100 is a nonionic surfactant utilized to lyse cells due to its ability to permeabilize eukaryotic cell membranes. Before using Triton X-100 in cell lysis, it was necessary to examine whether it can affect the enzymatic activity of lysozyme. Triton X-100 was diluted with distilled water and culture medium to produce a final concentration of 1% (v/v).

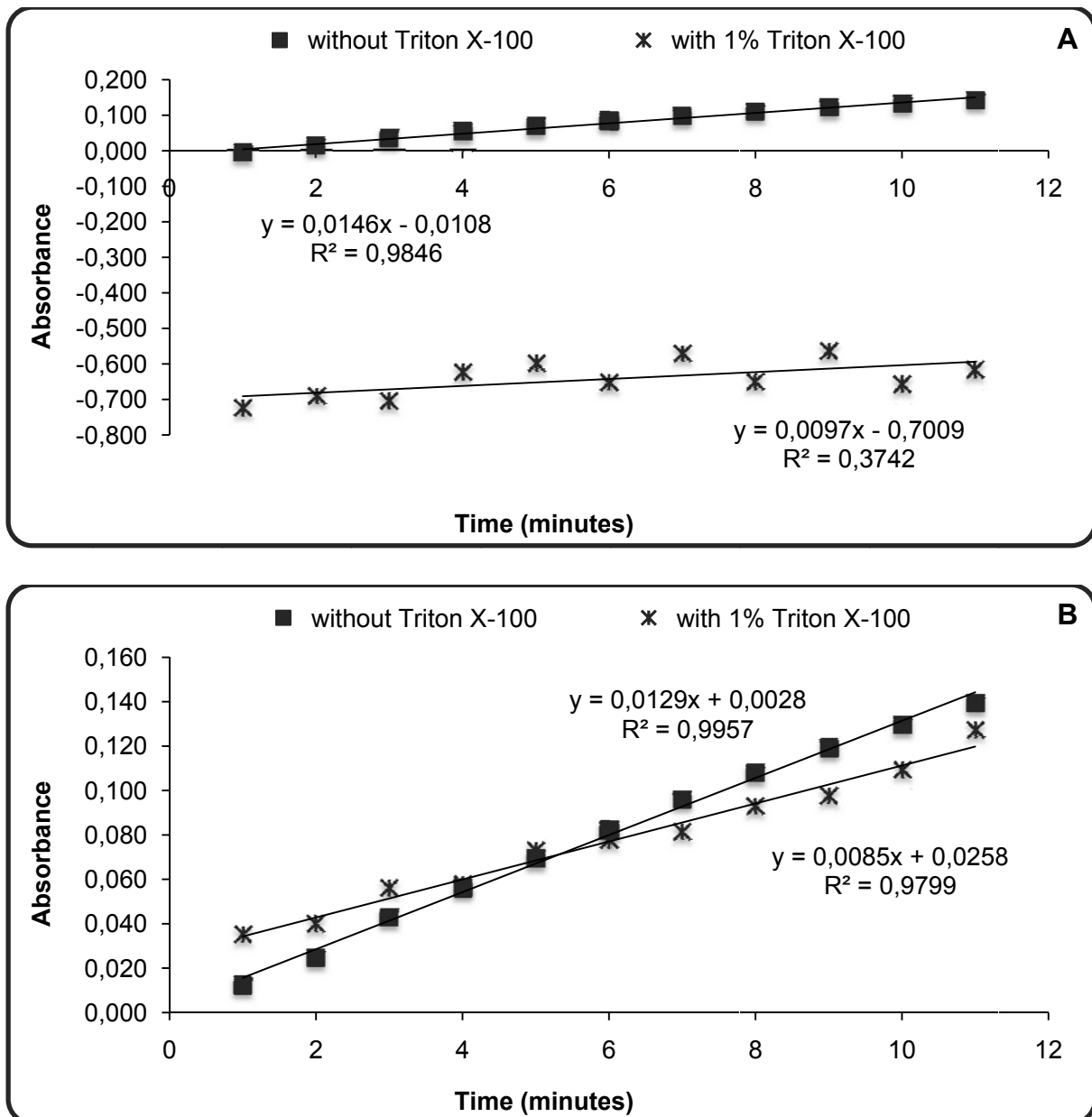
#### 3.3.1.4.1. Effect of Triton X-100 on the enzymatic activity of Sigma<sup>®</sup> standard lysozyme

Triton X-100 solution was added to Sigma<sup>®</sup> standard lysozyme solution (125 units/ml), and then lysozyme activity was measured turbidimetrically after 1 hour and after 2 hours treatment period. Standard lysozyme solution without addition of Triton X-100 was used as control. Triton X-100 caused a significant inhibition of about 34% in the lysozyme activity compared to control (Fig.28).

Kinetic curves of lysozyme activity measured turbidimetrically illustrated in Fig.29 show that addition of Triton X-100 led to drop in the slope values and linearity.



**Fig.28.** Percent decrease in standard lysozyme activity after treatment with 1% Triton X-100 in comparison to standard lysozyme control without Triton X-100 addition using the microtiter plate turbidity assay. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control.



**Fig.29.** Standard lysozyme activity after 1 hour (A) and after 2 hours (B) treatment with 1% Triton X-100 in comparison to standard lysozyme control without Triton X-100 addition using the microtiter plate turbidity assay. The background absorbance was subtracted from each value. All points were measured in triplicate, and the curves are representative of three separate experiments.

### 3.3.1.4.2. Effect of Triton X-100 on lysozyme activity produced by human monocytes

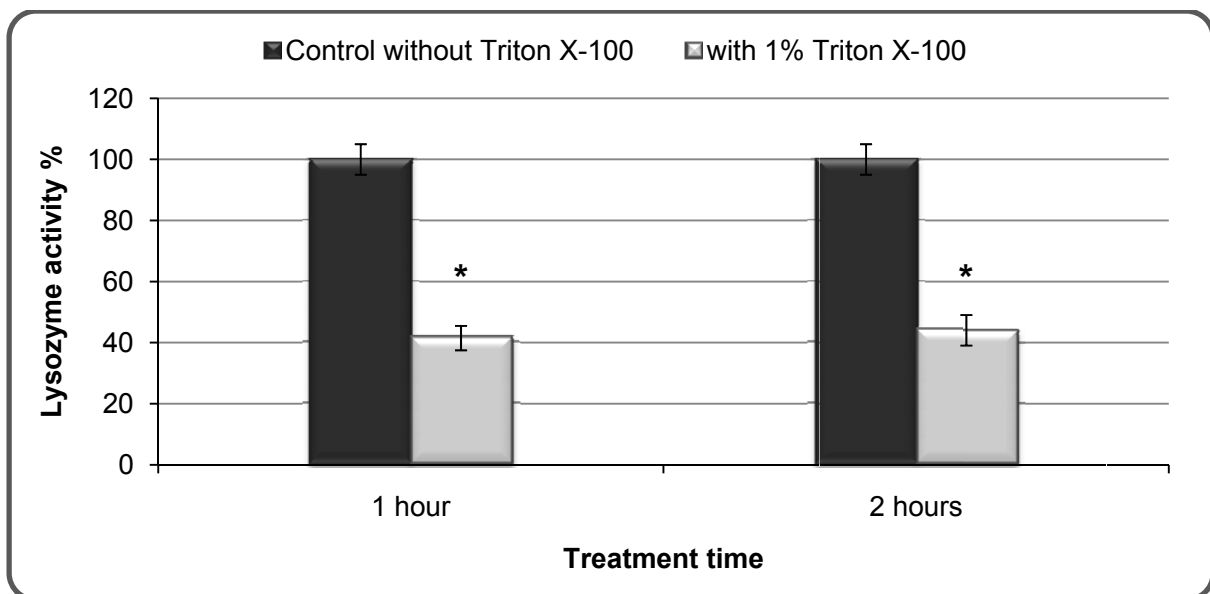
Triton X-100 was added to THP-1 cell culture medium at a final concentration of 1% (v/v). Lysozyme activity in these cell cultures was measured turbidimetrically after 1 hour and after 2 hours treatment period. Cell cultures incubated without addition of Triton X-100 were used as control. Triton X-100 resulted in a significant inhibition of about 58% in the lysozyme activity compared to control (Fig.30). Important point to be considered that higher lysozyme activity levels were expected to be measured after Triton X-100 addition to the cell culture due to the release of intracellular lysozyme activity inside the cells after lysis, in comparison



to control culture in which only secreted lysozyme activity was assayed. Kinetic curves of lysozyme activity measured turbidimetrically illustrated in Fig.31 show that addition of Triton X-100 caused decrease in the slope values and linearity.

Based on the previous results, Triton X-100 has not been found to be suitable for lysis of cell cultures subjected to studies concerning lysozyme synthesis because of its inhibitory effect on the enzymatic activity of lysozyme. These findings confirm previous reports of inhibitory effect of surface-active reagents on lysozyme (Smith & Stoker 1949).

Hence, cell lysis in our work was performed using two freeze-thaw cycles of cell cultures, which were able to cause damage to the integrity of the cell membrane in the cell culture and accordingly led to the release of intracellular lysozyme activity from inside the cells. Two freeze-thaw cycles had no significant effect on lysozyme activity produced by cell cultures, as proved by our experiments (Data not shown).

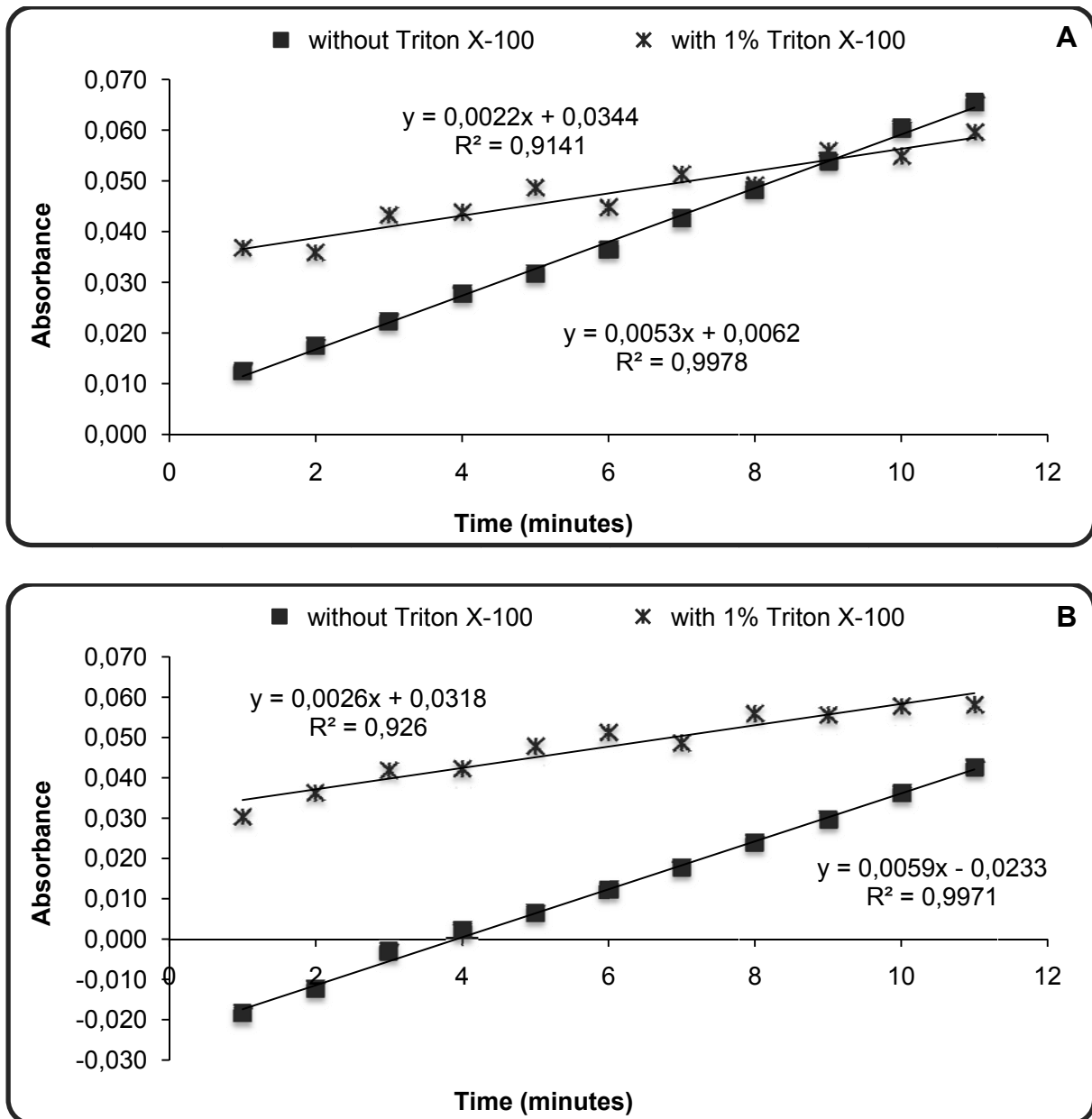


**Fig.30.** Percent decrease in lysozyme activity produced by THP-1 cell culture after 1 hour and after 2 hours treatment with 1% Triton X-100 in comparison to control cell culture without Triton X-100 addition using the microtiter plate turbidity assay. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.1.5. Lysozyme activity levels in the culture medium

In order to exclude the possible presence of active lysozyme in the medium of cell cultures which may interfere with lysozyme activity levels expressed by the cells, we measured lysozyme activity in the serum-containing medium used for culturing the cell lines. Measurement results showed that serum contained small amounts of lysozyme activity. However, lysozyme activity of serum was no more detectable when serum was diluted with the culture medium (Data not shown). Our findings proved that no detectable lysozyme

activity levels could be measured in the culture medium supplemented with 10% serum, which excluded any interference of the culture medium with the detection of lysozyme activity produced by the cultivated cells examined.



**Fig.31.** Lysozyme activity of THP-1 cell culture after 1 hour (A) and after 2 hours (B) treatment with 1% Triton X-100 in comparison to control cell culture without Triton X-100 addition using the microtiter plate turbidity assay. The background absorbance was subtracted from each value. All points were measured in triplicate, and the curves are representative of three separate experiments.

### **3.3.2. Effects of differentiation-inducing agents**

#### **3.3.2.1. Human monocytic cells THP-1**

##### **3.3.2.1.1. Phorbol Myristate Acetate (PMA) and Human Interferon-gamma (hIFN- $\gamma$ )**

Slight but statistically significant increases in the secretion of lysozyme activity were observed when phorbol 12-myristate 13-acetate (PMA) was incubated with THP-1 monocytes for one hour (Fig.32.A).

When PMA was incubated with THP-1 cells for further 24 hours at different concentrations, a tendency to increase in the secreted lysozyme was detected, which was not stable in the repeated experiments. PMA concentration of 200nM caused an increase of 72% in the released lysozyme activity of monocytes after 24 hours incubation, which was statistically significant (Fig.32.A). On the other hand, PMA had no significant effects on the total lysozyme activity expressed by THP-1 cells after 24 hours incubation regardless of the concentration used (Fig.32.A). Moreover, no significant effect on the release and synthesis of lysozyme activity in THP-1 monocytes was detected upon exposure to PMA for longer periods up to 72 hours without regard to the concentrations tested (Data not shown).

Regarding human interferon gamma, slight but statistically significant increase of about 15% in the release of lysozyme activity was found after one hour incubation with 500 and 1000 U/ml of hIFN- $\gamma$ . Higher concentration of 2000 U/ml of hIFN- $\gamma$  caused to the contrary a significant drop of 31% in the release of lysozyme activity after one hour incubation with THP-1 monocytes (Fig.32.B). With longer incubation period of 24 hours, hIFN- $\gamma$  resulted in no significant effects on the expression and release of lysozyme activity of THP-1 cells without regard to the concentration used (Fig.32.B).

#### **Effect of PMA on lysozyme of THP-1 monocytes measured using ELISA lysozyme protein assay compared to enzymatic activity assay**

A tendency to increase in lysozyme levels measured as protein was detected after 48 hours treatment of monocytes with PMA, which was associated with an opposite drop in the activity levels of lysozyme under the same conditions.

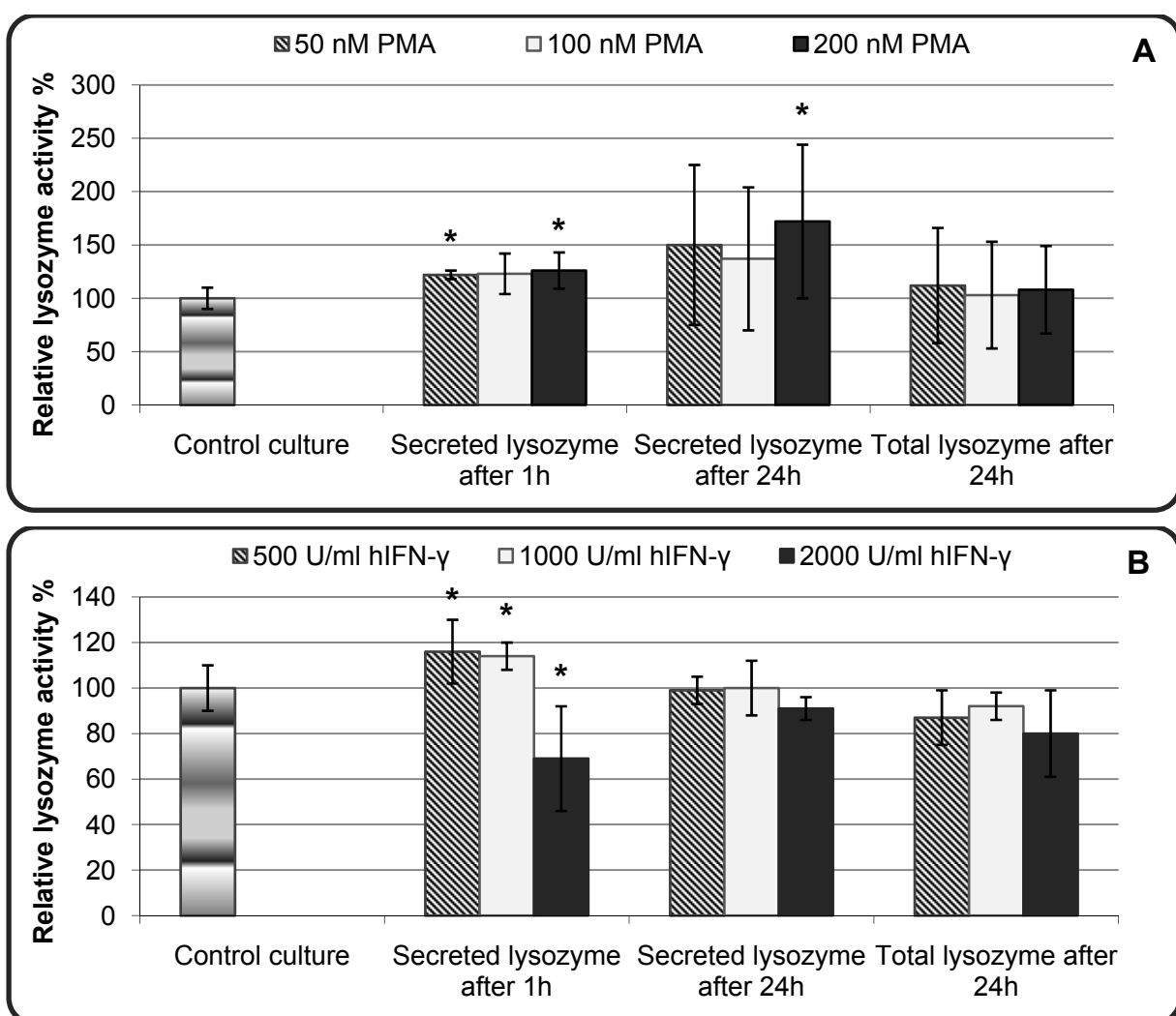
On the other hand, no statistically significant differences were found between the effects of PMA on the expression of lysozyme as a protein or as an active enzyme after 24 hours or 72 hours incubation with THP-1 cells (Fig.33).

##### **3.3.2.1.2. Retinoic acid and Forskolin**

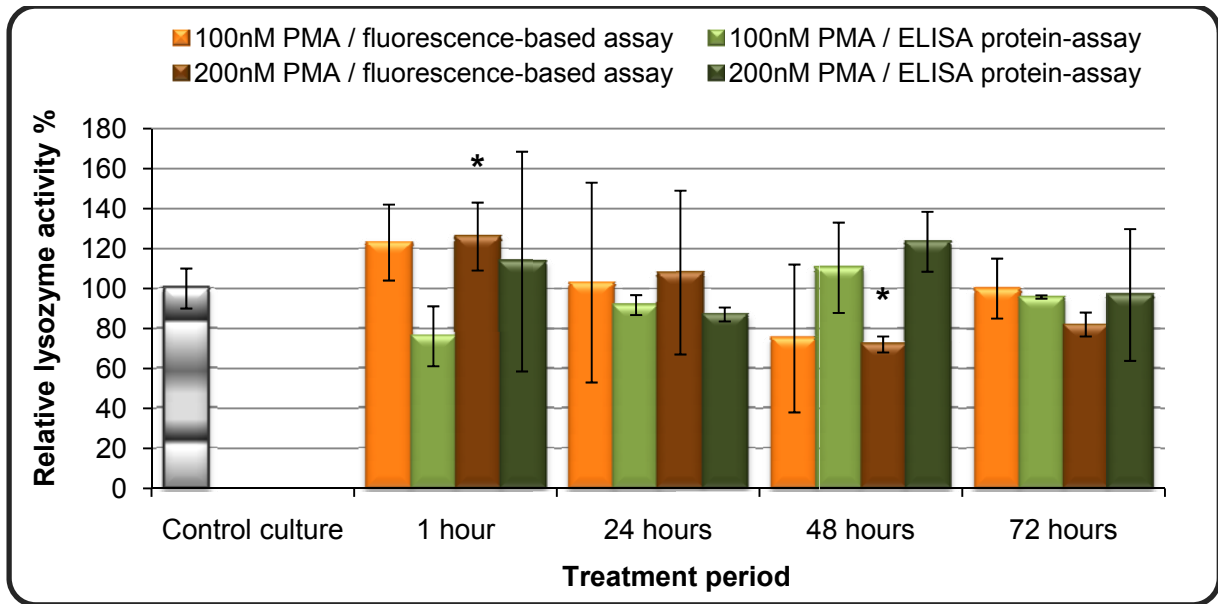
Retinoic acid incubated with THP-1 monocytes inhibited significantly the expression and release of lysozyme activity, which was observed with the short-term as well as long-term incubation periods (Fig.34). Time-dependent inhibitory effect of 1 $\mu$ M retinoic acid on the release of lysozyme activity was demonstrated with incubation periods up to 48 hours.

Waning of this inhibitory effect began to appear after further 72 hours incubation, although levels of secreted lysozyme activity of monocytes treated with retinoic acid at this time point were still significantly less than that of the control cultures. Retinoic acid was also proved to inhibit lysozyme expression, which was statistically significant with all incubation periods up to 72 hours. A significant inhibition of 36% in lysozyme expression was detected after 24 hours incubation with 1 $\mu$ M retinoic acid. With longer incubation periods, this inhibitory effect weakened to 31% inhibition after 48 hours, and to 28% after 72 hours.

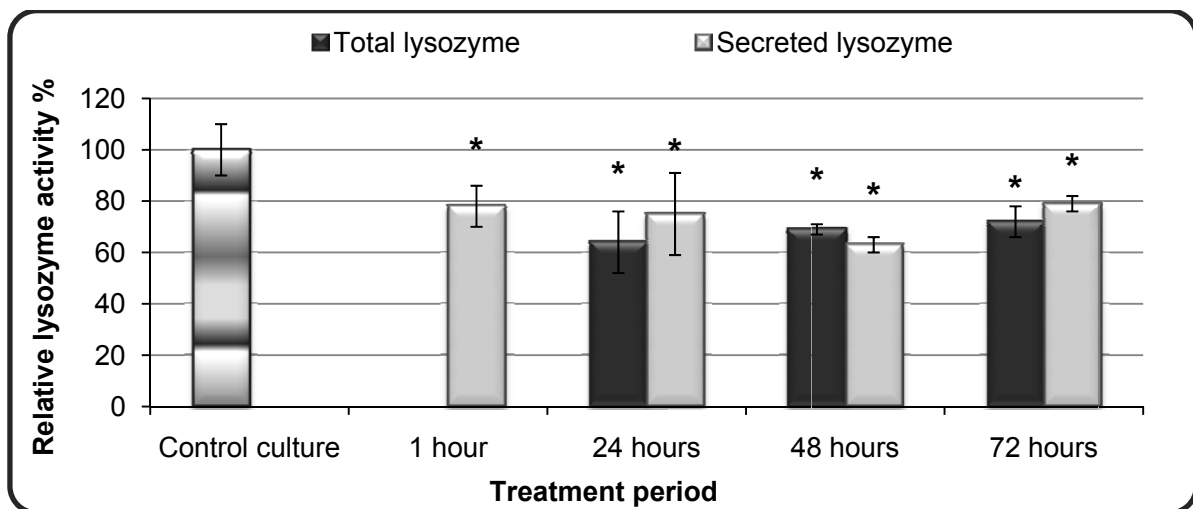
On the contrary, forskolin (10 $\mu$ M final concentration) had no significant effects on the expression and release of lysozyme activity of THP-1 monocytes, which was observed with the short-term as well as long-term exposure periods up to 72 hours (data not shown).



**Fig.32.** Total and secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after 1 hour and after 24 hours treatment with PMA (A) or with hIFN- $\gamma$  (B) using sensitive fluorescence-based lysozyme assay. All points were measured in triplicate, and the columns are representative of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures without substance addition.



**Fig.33.** Percent changes in the secreted lysozyme activity (after 1 hour incubation) and in the total lysozyme activity (after 24, 48 and 72 hours incubation) synthesized by  $1 \times 10^6$  cells/ml THP-1 cell cultures treated with PMA using ELISA lysozyme protein assay compared to lysozyme activity assay. Control cultures without PMA addition were considered to have 100% lysozyme activity. All points were measured in triplicate. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



**Fig.34.** Percent changes in the secreted and total lysozyme activity synthesized by  $1 \times 10^6$  cells/ml THP-1 cell cultures treated with  $1 \mu\text{M}$  of retinoic acid using sensitive fluorescence-based lysozyme assay. Control cultures without retinoic acid addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.2.2. Human monocytic cells HL-60

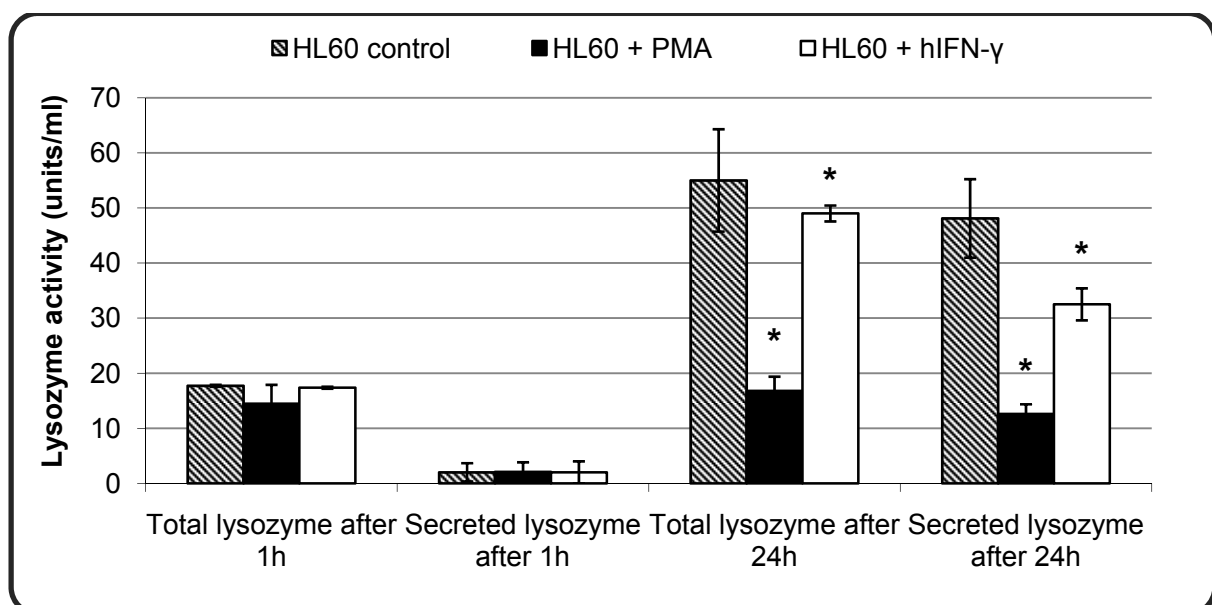
#### 3.3.2.2.1. Phorbol Myristate Acetate (PMA) and Human Interferon-gamma (hIFN- $\gamma$ )

Contrary to previous studies, PMA and hIFN- $\gamma$  had no inducing effect on the production or the release of lysozyme in HL-60 cells. Furthermore, our present findings suggested even inhibiting effect of PMA and hIFN- $\gamma$  on lysozyme activity. After one hour treatment of HL-60 cells with PMA, a slight decrease in the total lysozyme activity synthesized was detected, whereas after 24 hours treatment with PMA, a significant decrease in the total and secreted lysozyme was detected (Fig.35). Regarding human interferon gamma, no changes were found in either the total or the secreted lysozyme activity after one hour treatment of HL-60 cell cultures with hIFN- $\gamma$ , while after 24 hours, a significant tendency to decrease in the total and secreted lysozyme activity was detected (Fig.35).

### 3.3.2.3. Human colon epithelial cells HT-29

#### 3.3.2.3.1. PMA, Retinoic acid and Forskolin

In order to compare the influence of differentiation-inducing agents of monocytes on lysozyme activity of monocytes with epithelial cells, PMA, retinoic acid and forskolin were incubated with the epithelial cells to investigate their effects for periods up to 72 hours. PMA, retinoic acid and forskolin had no significant effects on the expression and release of lysozyme activity of epithelial cells regardless of the concentration or the incubation period used (Table 4).



**Fig.35.** Total and secreted lysozyme activity of  $1 \times 10^6$  cells/ml HL-60 cell cultures after 1 hour and after 24 hours treatment with PMA and hIFN- $\gamma$  using sensitive fluorescence-based lysozyme assay. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures without substance addition.

Conc.	1 hour treatment	24 hours treatment		48 hours treatment		72 hours treatment	
	Secreted lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
<b>Phorbol 12-myristate 13-acetate (PMA):</b>							
50nM	+ 12 % (±25)	+ 11 % (±7)	+ 7 % (±3)	+ 2 % (±4)	+ 2 % (±14)	- 1 % (±2)	- 4 % (±2)
100nM	- 3 % (±16)	+ 6 % (±4)	+ 2 % (±4)	- 5 % (±4)	- 5 % (±13)	- 3 % (±3)	- 6 % (±1)
200nM	+ 13 % (±22)	+ 9 % (±6)	+ 4 % (±4)	- 8 % (±5)	- 10 % (±7)	- 4 % (±2)	- 7 % (±1)
<b>Retinoic acid:</b>							
1 µM	0 % (±19)	+ 4 % (±7)	0 % (±6)	- 4 % (±2)	- 7 % (±4)	- 2 % (±3)	- 5 % (±1)
3 µM	- 8 % (±16)	+ 1 % (±7)	- 1 % (±5)	- 4 % (±3)	- 15 % (±17)	- 3 % (±4)	- 6 % (±2)
<b>Forskolin:</b>							
10 µM	- 2 % (±13)	+ 10 % (±4)	+ 3 % (±6)	- 5 % (±3)	- 17 % (±24)	- 3 % (±1)	- 6 % (±1)

**Table 4.** Percent changes (to control) in the secreted and total lysozyme activity synthesized by  $0.5 \times 10^6$  cells/ml HT-29 cells after incubation in culture medium supplemented with phorbol 12-myristate 13-acetate (PMA), with retinoic acid or with forskolin. Control cultures without tested substance addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and values are representative of average value of six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.3. Effects of the cholinergic agonist carbachol

We investigated the influence of carbachol at two non-toxic concentrations on the expression and release of lysozyme in THP-1 monocytes and HT-29 epithelial cells.

Carbachol had no significant effect on lysozyme expression or release in both monocytes and epithelial cells when incubated for short periods up to 24 hours. On the contrary, a tendency to decrease in lysozyme activity was detected when carbachol was incubated with THP-1 and HT-29 cells for incubation periods longer than 24 hours, while no clear dose-dependent relationship of this inhibitory effect was found (Table 5).

Carb. conc.	1 hour treatment	24 hours treatment		48 hours treatment		72 hours treatment	
	Secreted lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
<b>THP-1 monocytes:</b>							
10 <sup>-3</sup> M	+ 1 % (±18)	- 15 % (±20)	+ 11 % (±16)	- 1 % (±7)	- 8 % (±15)	- 9 % (±14)	- 13 %* (±9)
10 <sup>-5</sup> M	+ 5 % (±17)	- 8 % (±15)	+ 9 % (±28)	- 28 %* (±23)	- 3 % (±5)	- 2 % (±5)	- 5 % (±12)
<b>HT-29 epithelial cells:</b>							
10 <sup>-3</sup> M	+ 4 % (±17)	- 2 % (±3)	- 2 % (±3)	- 6 % (±5)	- 8 %* (±3)	- 10 %* (±3)	- 12 %* (±2)
10 <sup>-5</sup> M	- 13 % (±15)	- 7 %* (±3)	- 3 % (±5)	- 4 % (±5)	- 13 %* (±10)	- 14 %* (±3)	- 11 %* (±2)

**Table 5.** Percent change (to control) in the secreted and total lysozyme activity synthesized by THP-1 and HT-29 cells after incubation in culture medium supplemented with carbachol. Control cultures without carbachol addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and values are representative of average value of six separate experiments (±SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.4. Effects of the immunosuppressant corticosteroids

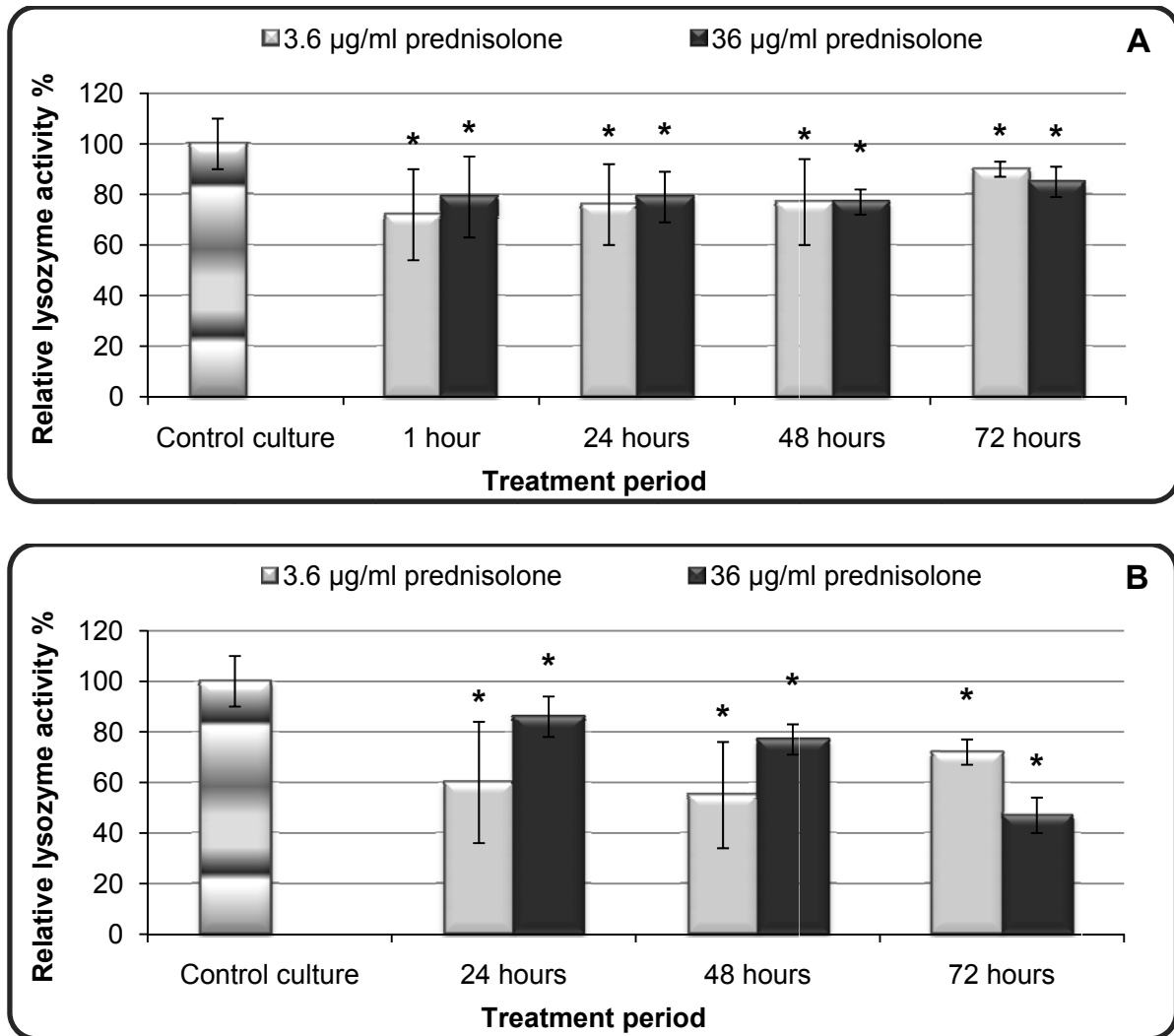
#### 3.3.4.1. Human monocytic cells THP-1

Prednisolone incubated with monocytes inhibited significantly the expression and release of lysozyme activity, which was observed with the short-term as well as long-term treatment (Fig.36). No clear dose-dependent relationship was found in the inhibitory effects of prednisolone on the release of lysozyme activity in monocytes. Both prednisolone concentrations of 3.6 and 36µg/ml caused significant decreases in the secreted lysozyme activity of about 25% after one hour, 23% after 24 hours, 23% after 48 hours and 13% after 72 hours incubation with THP-1 cells (Fig.36.A).

On the contrary, significant differences were detected between the inhibitory effects of prednisolone at different concentrations on the expression of lysozyme activity in the monocytes (Fig.36.B). With incubation periods up to 48 hours, reverse dose-dependent inhibitory effect of prednisolone on lysozyme activity synthesis of monocytes was demonstrated. The low prednisolone concentration of 3.6µg/ml caused significant decreases in the total lysozyme activity of 40% after 24 hours and 45% after 48 hours incubation with



the cells, whereas the higher concentration of 36 $\mu$ g/ml resulted in less significant decreases in the total lysozyme of 14% after 24 hours and 23% after 48 hours incubation (Fig.36.B). A direct dose-dependent inhibitory effect of prednisolone started to appear only after 72 hours incubation with THP-1 cells. Prednisolone non-toxic concentration of 36 $\mu$ g/ml led to significant decrease in the total lysozyme of 53% after 72 hours incubation with the monocytes, while lower concentration of 3.6 $\mu$ g/ml caused less significant decrease in the total lysozyme of 28% (Fig.36.B).



**Fig.36.** Percent changes in the secreted lysozyme activity (A) and in the total lysozyme activity (B) synthesized by  $1 \times 10^6$  cells/ml THP-1 cell cultures treated with prednisolone at two concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without prednisolone addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.4.2. Human colon epithelial cells HT-29

Prednisolone incubated with epithelial cells for periods up to 48 hours had no influence on the release and expression of lysozyme activity synthesized by the cells regardless of the concentration used (Table 6). A slight but statistically significant inhibitory effect of prednisolone on both secreted and total lysozyme activity was detected only after 72 hours incubation with the epithelial cells. This inhibitory effect was dose-dependent, since it was observed only at a concentration of 36 $\mu$ g/ml of prednisolone while it was non-significant with the lower concentration of 3.6 $\mu$ g/ml (Table 6).

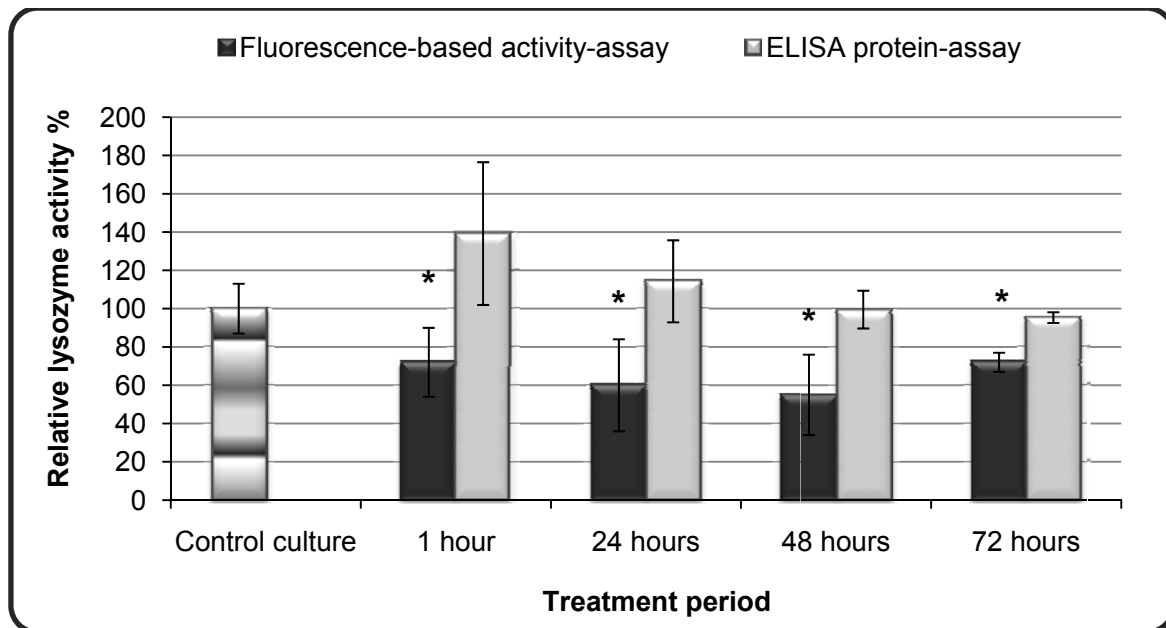
Predn. conc. ( $\mu$ g/ml)	1 hour treatment	24 hours treatment		48 hours treatment		72 hours treatment	
	Secreted lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
3.6	+1 $\pm$ 15%	0 $\pm$ 4 %	+ 1 $\pm$ 4 %	- 1 $\pm$ 3 %	+ 1 $\pm$ 4 %	+ 4 $\pm$ 2 %	+ 1 $\pm$ 4 %
36	+1 $\pm$ 12%	- 1 $\pm$ 4 %	+ 1 $\pm$ 4 %	- 4 $\pm$ 5 %	- 3 $\pm$ 6 %	- 9 $\pm$ 3 %*	-11 $\pm$ 3 %*

**Table 6.** Percent change (to control) in the secreted and total lysozyme activity synthesized by  $0.5 \times 10^6$  cells/ml HT-29 cells after incubation in culture medium supplemented with prednisolone. Control cultures without prednisolone addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### Effect of prednisolone on lysozyme expression of monocytes measured using ELISA lysozyme protein assay compared to enzymatic activity assay

The significant decreases in the release and synthesis of active lysozyme of THP-1 cells found after exposure time up to 72 hours were not detected when lysozyme was measured as a protein using ELISA lysozyme assay under the same conditions (Fig.37).

On the contrary, a clear tendency to increase in the release of lysozyme measured as a protein was detected particularly after one hour incubation with monocytes, which was enzymatically inactive (Fig.37).



**Fig.37.** Percent changes in the secreted lysozyme activity after 1 hour treatment and in the total lysozyme activity after 24, 48 and 72 hours treatment synthesized by THP-1 monocytes cultures supplemented with 10 $\mu$ M prednisolone using ELISA lysozyme protein assay compared to lysozyme activity assay. Control cultures without prednisolone addition were considered to have 100% lysozyme activity. All points were measured in triplicate. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.5. Effects of inflammatory response inducers

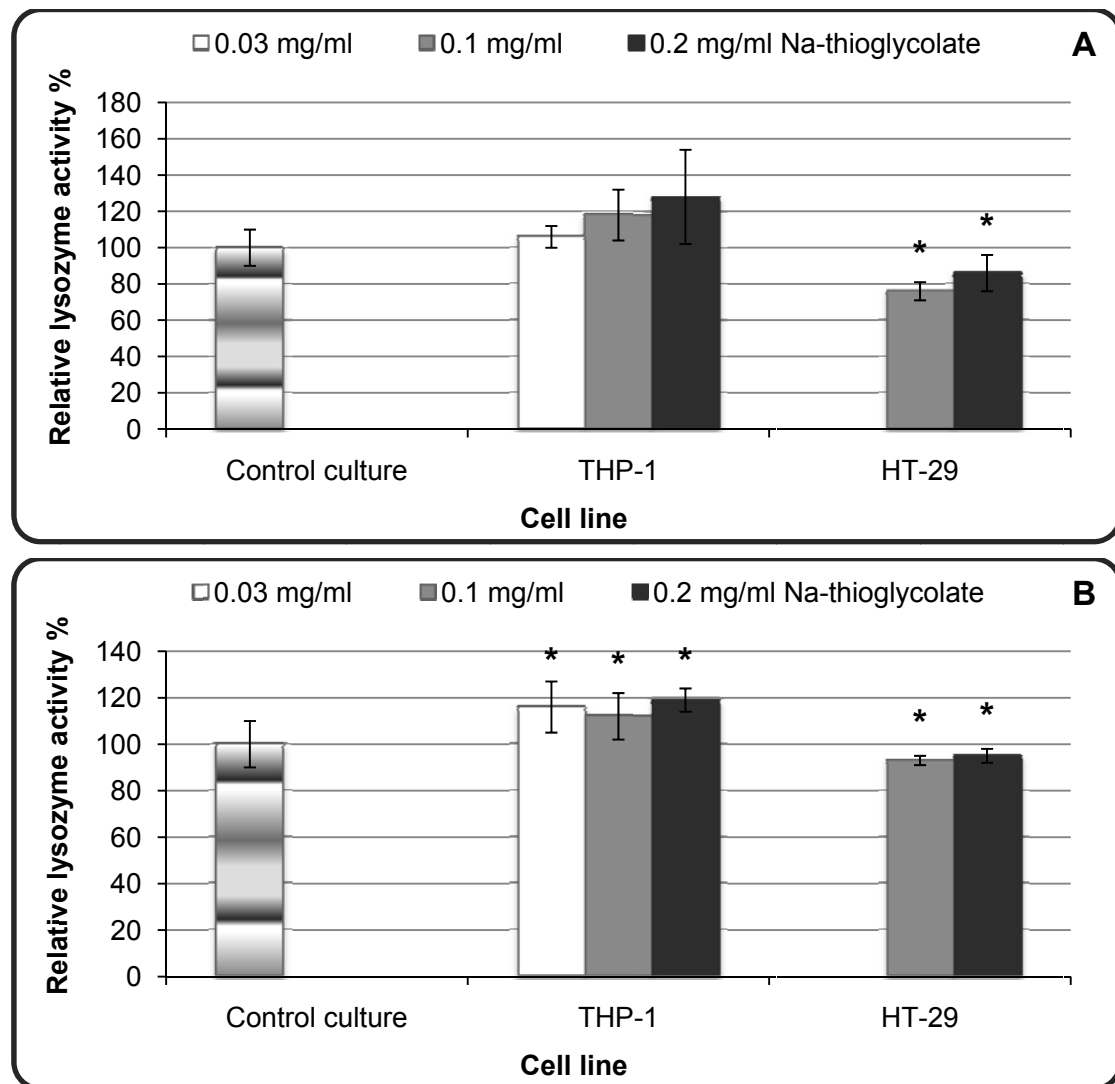
#### 3.3.5.1. Sodium Thioglycolate

Influence of sodium thioglycolate on lysozyme activity of HT-29 and THP-1 cells was investigated at non-toxic concentrations after exposure periods up to 72 hours.

A tendency to increase in the secreted lysozyme was detected with sodium thioglycolate after one hour incubation with THP-1 monocytes that was statistically not significant, however (Fig.38.A). This stimulatory effect started to be significant after 72 hours incubation with Na- thioglycolate, which caused a slight but significant increase of 16% in average with all examined concentrations (Fig.38.B). Nevertheless, an inhibitory effect of Na-thioglycolate was observed at the high non-toxic concentration of 0.2mg/ml only after 24 hours incubation with monocytes, which resulted in a significant decrease of 28% in the expression of lysozyme activity. Nevertheless, this inhibitory effect of Na-thioglycolate disappeared when incubated with monocytes for periods more than 24 hours (Table 7).

On the contrary to monocytes, Na-thioglycolate exhibited reverse dose-dependent inhibitory effect even after one hour incubation with epithelial cells. It caused significant decreases of 14% and 24% in the released lysozyme activity after one hour incubation with 0.2mg/ml and 0.1mg/ml of Na- thioglycolate respectively (Fig.38.A).

Furthermore, Na-thioglycolate continued to inhibit lysozyme activity of epithelial cells with the long-term treatment periods up to 72 hours, which was less significant compared to the effects of one-hour treatment (Fig.38.A) (Table 7).



**Fig.38.** Percent changes in the secreted lysozyme activity after 1 hour (A) and in the total lysozyme activity after 72 hours (B) synthesized by various cell lines cultures supplemented with sodium thioglycolate at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without Na- thioglycolate addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.5.2. Bacterial Components

#### 3.3.5.2.1. Secretion of lysozyme activity in cell cultures after one hour treatment with bacterial polysaccharides

Lipopolysaccharides from *Salmonella enteritidis* (LPS) caused significant increase in the lysozyme activity secreted in the medium of  $1 \times 10^{+6}$  cells/ml THP-1 cell cultures after 1 hour

treatment. The most significant induction of lysozyme secretion was at LPS concentration of 1µg/ml (Fig.39.A). Moreover, dose-dependent effect of LPS on the secretion of lysozyme activity was observed with differentiated THP-1 cells. At a concentration of 1µg/ml, LPS led to significant increase of 23% in the secreted lysozyme activity after one hour treatment, while further increase of 42% was detected at 10µg/ml of LPS (Fig.39.A).

On the contrary, LPS had no effect on the release of lysozyme activity when incubated with HT-29 epithelial cells for one hour regardless of LPS concentration (Fig.39.A) (Table 8).

Leiber-Beta S and Auxoferm YGT Plus did not have the same effects produced by LPS from *Salmonella enteritidis* on the release of lysozyme activity in the cell cultures after one hour treatment. Leiber-Beta S had no significant influence on the secretion of lysozyme activity after one hour incubation with THP-1 monocytes and HT-29 epithelial cells without regard to their concentrations (Table 9). Auxoferm YGT Plus had also no influence on monocytes, while it was able to affect epithelial cells. 50µg/ml of Auxoferm caused significant decrease of 25% in the secreted lysozyme activity of HT-29 cells after one hour treatment, whereas lower concentrations had no significant effects (Table 9).

Na-thioglyc. conc.	Cell Type	24 hours treatment		48 hours treatment		72 hours treatment	
		Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
0.03 mg/ml	THP-1	- 18 % (±21)	- 17 % (±19)	- 7 % (±12)	- 6 % (±20)	+ 9 % (±5)	+ 16 %* (±11)
0.1 mg/ml	HT-29	- 12 %* (±3)	- 4 % (±1)	- 2 % (±10)	- 4 % (±3)	- 3 %* (±3)	- 7 %* (±2)
	THP-1	- 11 % (±5)	- 7 % (±3)	- 4 % (±7)	- 1 % (±6)	- 9 % (±12)	+ 12 %* (±10)
0.2 mg/ml	HT-29	- 10 %* (±3)	- 4 % (±3)	- 13 %* (±3)	- 4 % (±2)	- 3 %* (±2)	- 5 %* (±3)
	THP-1	- 11 % (±5)	- 28 %* (±16)	- 1 % (±5)	- 1 % (±5)	+ 6 % (±4)	+ 19 %* (±5)

**Table 7.** Percent changes (to control) in the secreted and total lysozyme activity of monocytes and epithelial cells after incubation in culture medium supplemented with sodium thioglycolate. Control cultures without Na-thioglycolate addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments (±SD). Values with asterisk are significantly different (P<0.05, Mann-Whitney U test) from values of control cultures.

### **3.3.5.2.2. Synthesis and secretion of lysozyme activity in cell cultures after long-term treatment with bacterial polysaccharides**

Significant effects of LPS on lysozyme activity were observed after 72 hours incubation with THP-1 cells. A significant increase of 24% in the total lysozyme activity expressed by THP-1 monocytes could be detected after 72 hours incubation with LPS at 0.1µg/ml, whereas further rise of 31% was found at a higher concentration of 1µg/ml of LPS.

10µg/ml of LPS prompted to the contrary a slight but significant drop of 12% in the total lysozyme activity after 72 hours incubation (Fig.39.B) (Table 8).

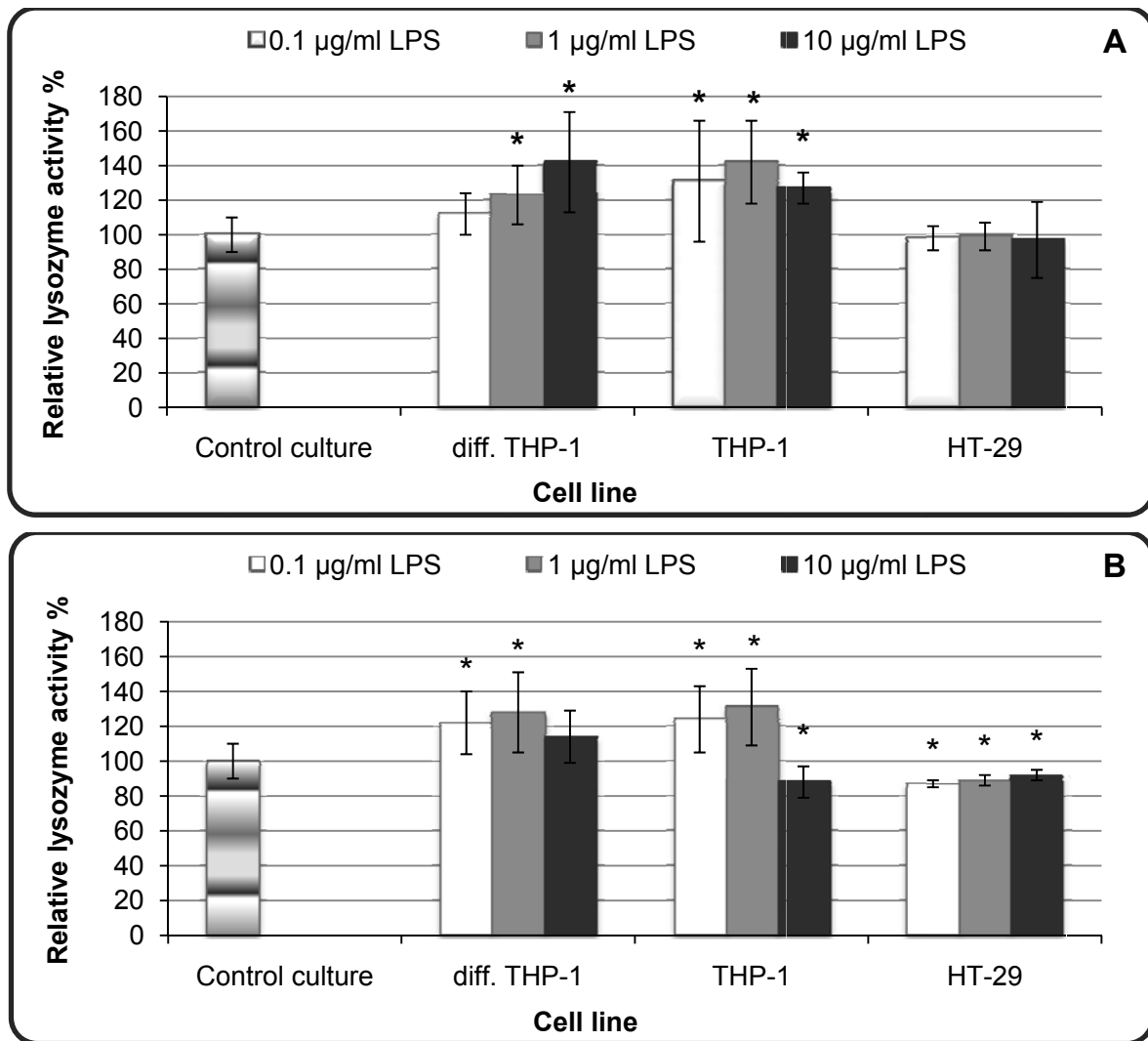
Furthermore, LPS stimulated PMA-differentiated THP-1 monocytes to express and secrete higher lysozyme activity levels after 72 hours incubation. This increase of secreted and total lysozyme activity was statistically significant particularly with LPS concentrations of 0.1µg/ml and 1µg/ml (Fig.39.B) (Table 8).

Concerning shorter incubation periods of 24 and 48 hours with PMA-differentiated THP-1 cells, LPS had no significant outcome on lysozyme activity levels, with the exception of significant drop after 24 hours incubation in the total and secreted lysozyme caused by LPS at the concentration of 10µg/ml. This inhibitory effect disappeared after 48 hours incubation (Table 8).

On the other hand, total and secreted lysozyme activity of HT-29 epithelial cells tended to decrease slightly but significantly when incubated with LPS for 72 hours, which was independent of LPS concentration (Fig.39.B). With incubation periods shorter than 72 hours LPS had no significant influence on lysozyme activity of epithelial cells (Table 8).

Leiber-Beta S and Auxoferm YGT Plus tended to cause slight but significant decreases in the expression and release of lysozyme activity when incubated with THP-1 cells for more than 24 hours (Table 9).

For HT-29 epithelial cells, no significant changes in the lysozyme activity could be detected after incubation with Auxoferm YGT Plus or Leiber-Beta S for periods more than 24 hours, irrespective of the used concentrations (Table 9).



**Fig.39.** Percent changes in the secreted lysozyme activity after 1 hour (A) and in the total lysozyme activity after 72 hours (B) synthesized by various cell lines cultures supplemented with lipopolysaccharides from *Salmonella enteritidis* (LPS) at three concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without LPS addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

LPS conc.	Cell Type	1 hour treatment	24 hours treatment		48 hours treatment		72 hours treatment	
		Secreted lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
0.1 µg/ml	HT-29	- 2 ±7 %	- 2 ±4 %	+ 2 ±3 %	- 2 ±9 %	- 1 ±9 %	- 8 ±3 %*	- 13 ±2 %*
	THP-1	+ 31 ±35 %*	+ 6 ±15 %	+ 18 ±36 %	+ 3 ±12 %	+ 21 ±52 %	+ 3 ±7 %	+ 24 ±19 %*
	Diff. THP-1	+ 12 ±12 %	- 2 ±30 %	- 20 ±17 %	+ 11 ±27 %	- 2 ±22 %	+ 39 ±14 %*	+ 22 ±18 %*
1 µg/ml	HT-29	- 1 ±8 %	- 4 ±3 %	+ 1 ±6 %	0 ±8 %	0 ±9 %	- 7 ±3 %*	- 11 ±3 %*
	THP-1	+ 42 ±24 %*	+ 15 ±28 %	- 3 ±31 %	+ 17 ±8 %*	+ 8 ±10 %	+ 6 ±13 %	+ 31 ±22 %*
	Diff. THP-1	+ 23 ±17 %*	- 12 ±33 %	- 24 ±31 %	+ 17 ±53 %	+ 24 ±57 %	+ 38 ±25 %*	+ 28 ±23 %*
10 µg/ml	HT-29	- 3 ±22 %	- 3 ±4 %	- 1 ±3 %	- 3 ±6 %	- 5 ±3 %	- 6 ±2 %*	- 8 ±3 %*
	THP-1	+ 27 ±9 %*	- 6 ±17 %	+ 23 ±16 %	- 2 ±11 %	+ 7 ±17 %	+ 7 ±1 %*	- 12 ±9 %*
	Diff. THP-1	+ 42 ±29 %*	- 56 ±30 %*	- 68 ±9 %*	- 10 ±28 %	- 23 ±19 %	+ 16 ±30 %	+ 14 ±15 %

**Table 8.** Percent changes (to control) in the secreted and total lysozyme activity of monocytic and epithelial cell cultures after incubation in culture medium supplemented with lipopolysaccharides from *Salmonella enteritidis* (LPS). Control cultures without LPS addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments (±SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control culture.



Bacterial component	Conc.	Cell Type	1 hour treatment	24 hours treatment		48 hours treatment		72 hours treatment	
			Secreted lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
Leiber-Beta S	10 µg/ml	HT-29	+ 1 ±15 %	- 1 ±4 %	- 2 ±5 %	- 5 ±4 %	- 6 ±2 %	- 3 ±5 %	+ 2 ±18 %
		THP-1	+ 5 ±14 %	- 5 ±9 %	-20 ±10%*	- 6 ±5 %*	- 9 ±6 %*	- 9 ±11 %	- 14 ±4 %*
	50 µg/ml	HT-29	- 12 ±10 %	- 2 ±5 %	- 2 ±2 %	- 8 ±8 %	- 6 ±4 %	- 1 ±4 %	0 ±6 %
		THP-1	+ 9 ±14 %	+ 5 ±5 %	- 15 ±3 %*	- 11 ±6 %*	-21 ±10%*	- 10 ±7 %*	- 18 ±4 %*
Auxoferm YGT Plus	10 µg/ml	HT-29	- 2 ±23 %	0 ±3 %	- 3 ±4 %	- 4 ±3 %	- 5 ±4 %	- 2 ±3 %	- 6 ±3 %
		THP-1	- 12 ±8 %	- 3 ±4 %	- 7 ±3 %*	- 6 ±7 %*	- 12 ±2 %*	- 13 ±9 %*	- 15 ±4 %*
	50 µg/ml	HT-29	- 25 ±17 %*	+ 1 ±8 %	- 2 ±3 %	- 6 ±16 %	- 9 ±8 %	+ 1 ±4 %	- 2 ±5 %
		THP-1	+ 7 ±17 %	+ 2 ±6 %	- 16 ±8 %*	- 9 ±11 %	-15 ±10%*	- 8 ±9 %	- 8 ±1 %*

**Table 9.** Percent changes (to control) in the secreted and total lysozyme activity of monocytic and epithelial cell cultures after incubation in culture medium supplemented with Leiber-Beta S and Auxoferm YGT Plus. Control cultures without bacterial components addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments (±SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.5.2.3. Effect of LPS on lysozyme of monocytes measured using ELISA lysozyme protein assay compared to enzymatic activity assay

Lipopolysaccharides from *Salmonella enteritidis* (LPS) at 0.1µg/ml induced significant increase of 110% in the lysozyme secreted in the medium of THP-1 cell cultures after 1 hour treatment and detected using ELISA protein assay. This increase of released lysozyme measured as a protein regardless of its enzymatic activity was significantly higher than the 31% increase of released lysozyme measured as an enzymatically active enzyme (Fig.40).

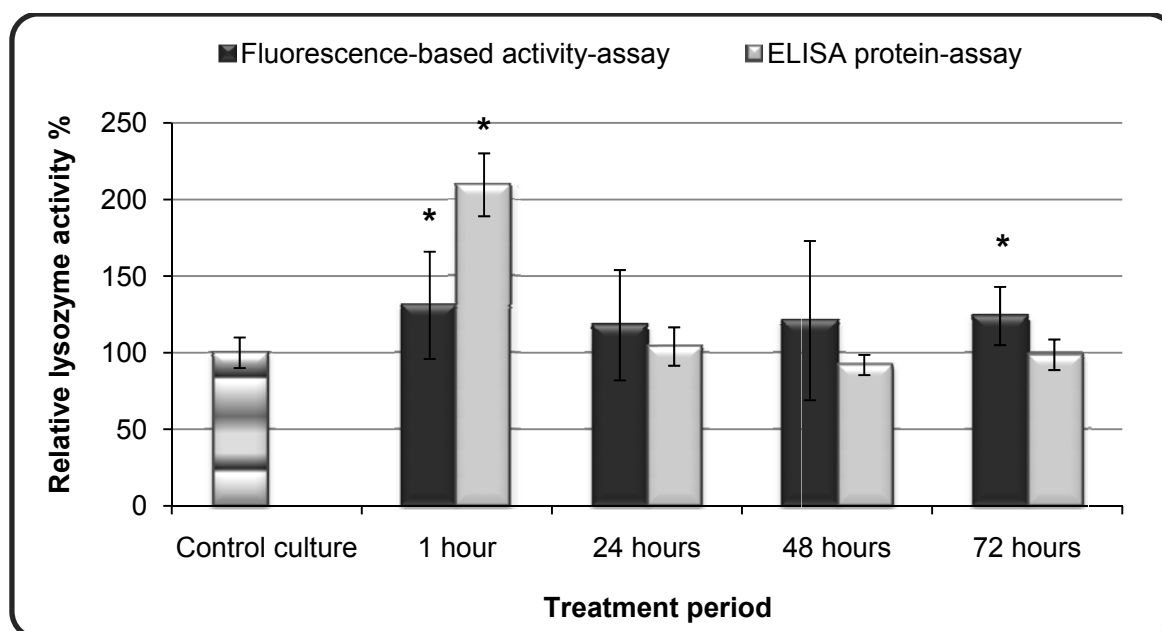
On the other hand, no statistically significant differences were found between the effects of LPS on the expression of lysozyme as a protein or as an active enzyme after long-term incubation with THP-1 cells (Fig.40).

### 3.3.6. Effects of antibacterial Chito-Oligosaccharide

Partially deacetylated chito-pentose DP5 is a chito-oligosaccharide investigated in our work concerning its possible influence on lysozyme activity of epithelial cells.

No strong effects on the synthesis or secretion of lysozyme activity were found when partially deacetylated chito-pentose was incubated with HT-29 epithelial cells.

Nevertheless, slight but statistically significant decreases of secreted and total lysozyme activity were detected at the high chito-pentose concentration of 60µg/ml, whereas no clear time-dependent relationship of this inhibitory effect was found (Table 10).



**Fig.40.** Percent change in the secreted lysozyme activity after 1 hour treatment, and in the total lysozyme activity after 24, 48 and 72 hours treatment synthesized by  $1 \times 10^6$  cells/ml THP-1 cell cultures supplemented with 0.1µg/ml LPS using ELISA lysozyme protein assay compared to fluorometric lysozyme activity assay. Control cultures without LPS addition were considered to have 100% lysozyme activity. All points were measured in triplicate. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Chitop. conc.	1 hour treatment	24 hours treatment		48 hours treatment		72 hours treatment	
	Secreted lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
20µg/ml	+ 4 % (±27)	- 4 % (±7)	+ 3 % (±3)	+ 2 % (±3)	+ 5 % (±2)	- 5 % (±18)	+ 5 % (±3)
60µg/ml	- 11 %* (±5)	- 4 % (±2)	- 10 % (±1)	- 5 %* (±1)	- 9 %* (±3)	- 3 % (±4)	- 4 % (±4)

**Table 10.** Percent change (to control) in the secreted and total lysozyme activity synthesized by  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after incubation in medium supplemented with partially deacetylated chito-pentose DP5. Control cultures without chito-oligosaccharide addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7. Effects of medicinal plants & phytochemicals

#### 3.3.7.1. Selected herbal extracts & isolated phytochemicals with immune-modulating, antibacterial, antiviral and/or anti-inflammatory activities

##### 3.3.7.1.1. Human monocytic cells THP-1 and epithelial cells HT-29

##### 3.3.7.1.1.1. Secretion of lysozyme activity in cell cultures after one hour treatment

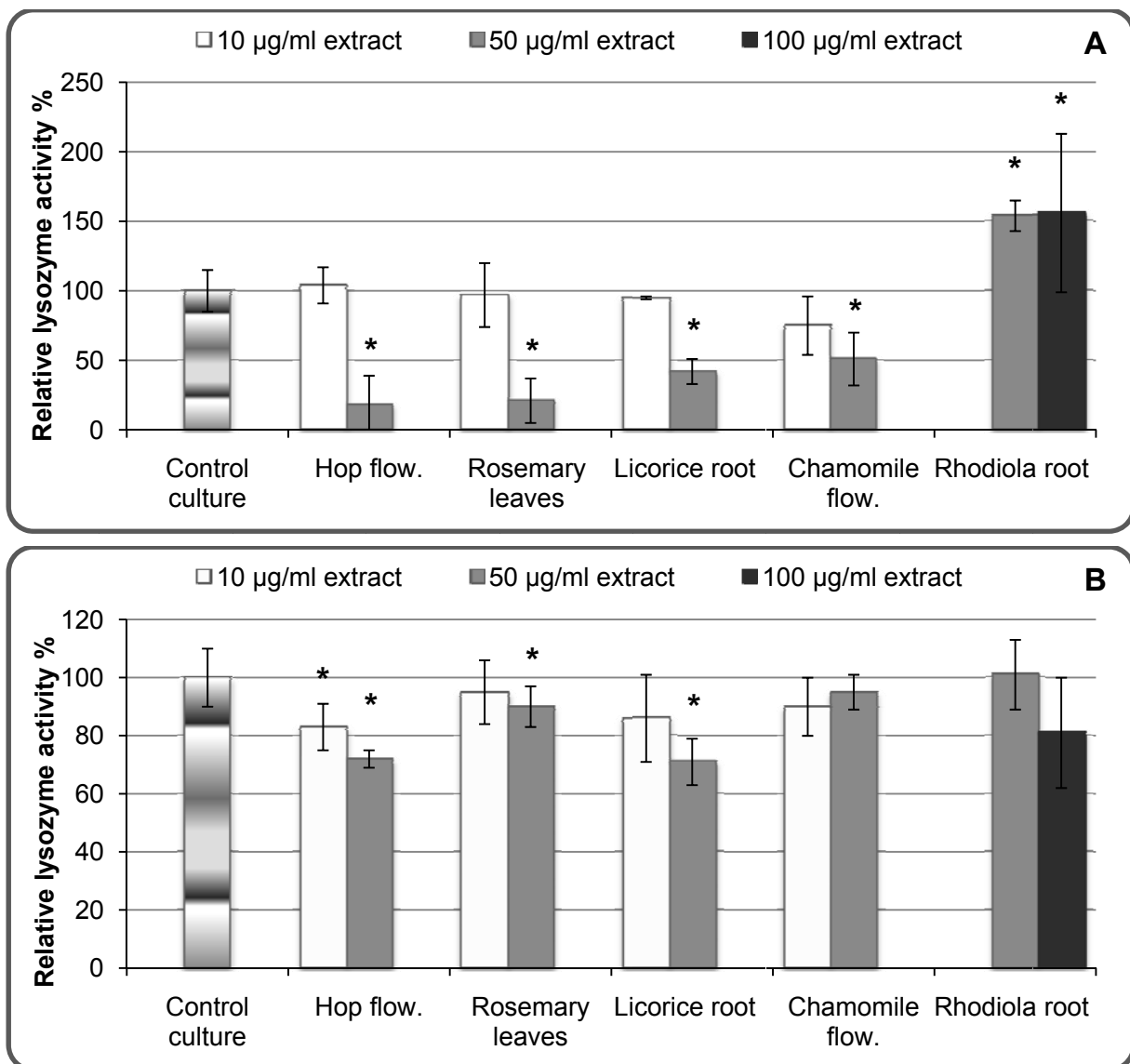
Various herbal extracts with immune-stimulating, antibacterial and/or antiviral properties were selected to be investigated.

Our results illustrated that *Rhodiola rosea* root extract induced enhanced lysozyme release of monocytes after one hour treatment. Significant increase of about 55% in the secreted lysozyme activity of monocytes was found at both concentrations of 50µg/ml and 100µg/ml, which were non-toxic to the cells after one hour treatment (Fig.41.A). This stimulatory effect of *Rhodiola rosea* extract was not demonstrated with HT-29 epithelial cells regardless of the concentration used (Fig.41.B).

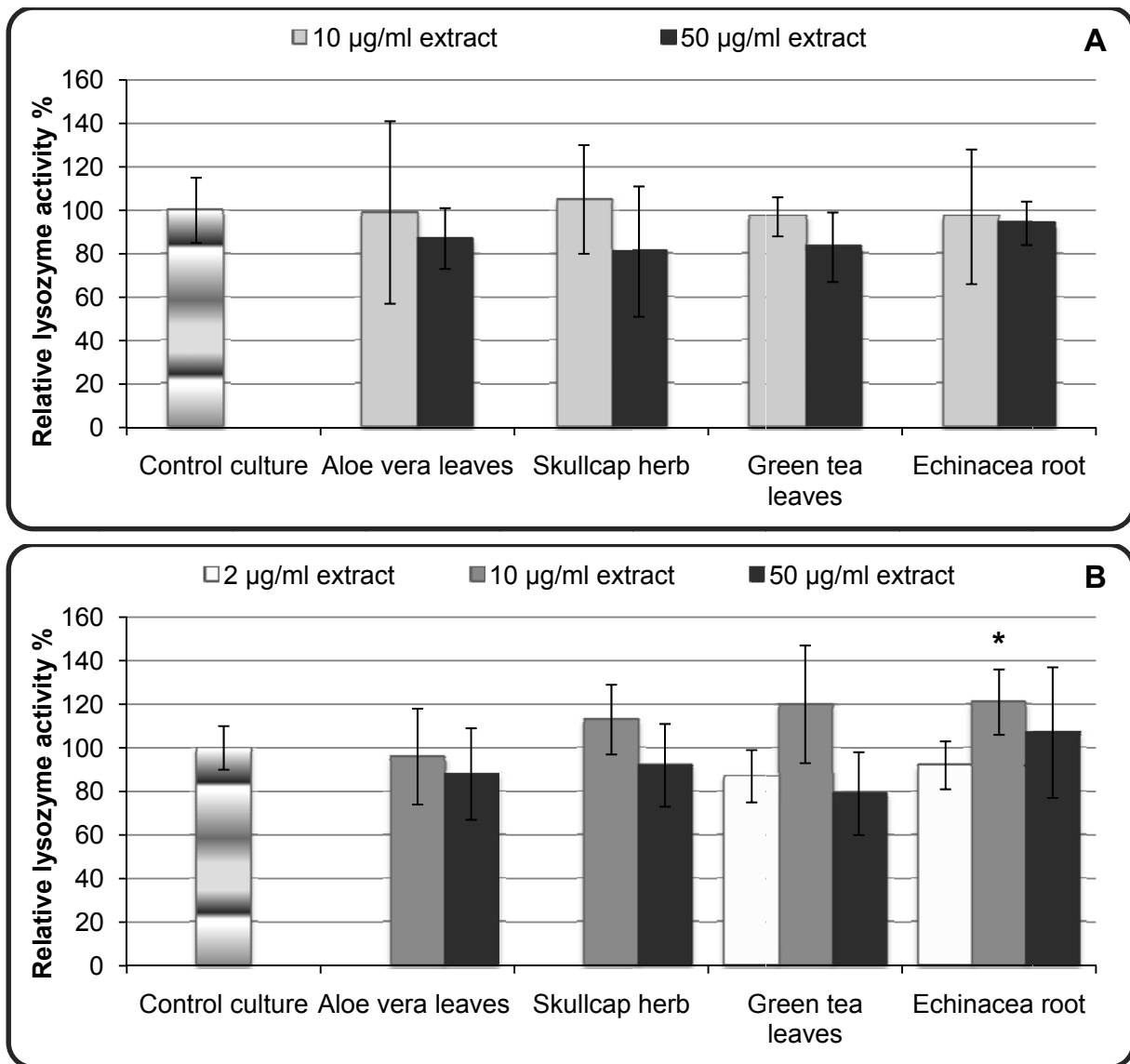
Contrary to *Rhodiola* extract, *Echinacea purpurea* root extract induced enhanced lysozyme release of epithelial cells after one hour treatment (Fig.42.B), while it had no significant stimulatory effects on THP-1 monocytes regardless of the concentration used (Fig.42.A). *Echinacea purpurea* root extract at 10µg/ml led to significant increase of 21% in the release of lysozyme activity when incubated with HT-29 epithelial cells for one hour (Fig.42.B).

An isolated alkamide of *Echinacea*, specifically Dodeca-2,4,8,10-tetraenoic acid isobutylamide, was also investigated concerning its influence on lysozyme activity of monocytes and epithelial cells in comparison to *Echinacea* extract.

*Echinacea* alkamide was demonstrated to induce enhanced lysozyme release of THP-1 monocytes after one hour exposure. Dose-dependent stimulatory effect of *Echinacea* alkamide was observed, since it caused significant increase of 92% in the secreted lysozyme at 50 $\mu$ g/ml, less significant increase of about 42% at both concentrations of 25 $\mu$ g/ml and 10 $\mu$ g/ml, and an increase of only 24% at 5 $\mu$ g/ml of *Echinacea* alkamide (Fig.43.A). On the contrary, *Echinacea* alkamide pronounced no effects on lysozyme secretion when incubated with HT-29 epithelial cells for one hour at concentrations up to 25 $\mu$ g/ml, whereas 50 $\mu$ g/ml led to slight but statistically significant decrease of 13% in lysozyme activity secretion (Fig.43.A).



**Fig.41.** Percent change in the secreted lysozyme activity of THP-1 monocytic cell cultures (A) and of HT-29 epithelial cell cultures (B) after 1 hour treatment with various herbal extracts at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without extract addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



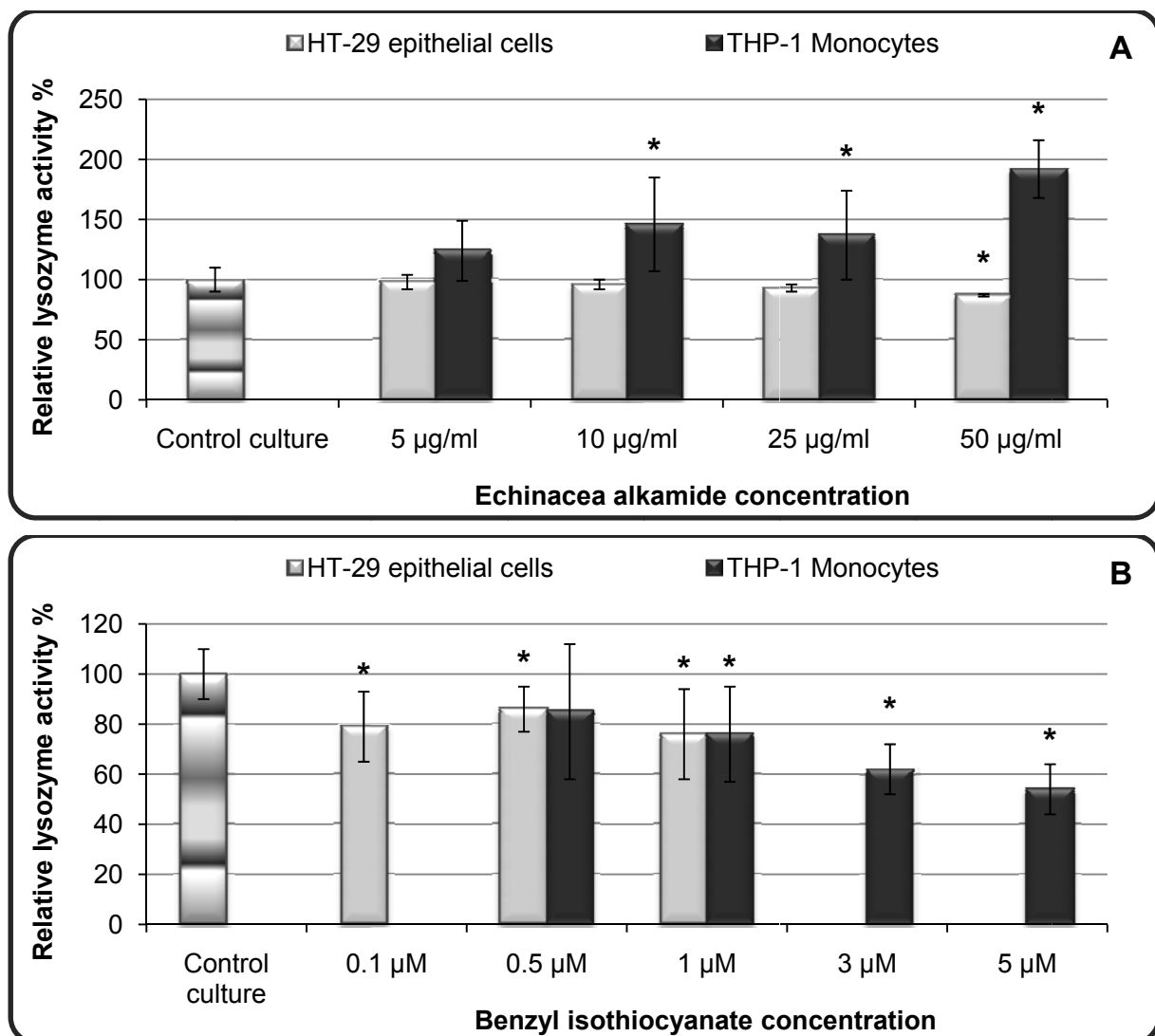
**Fig.42.** Percent change in the secreted lysozyme activity of THP-1 monocytic cell cultures (A) and of HT-29 epithelial cell cultures (B) after 1 hour treatment with various herbal extracts at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without extract addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Extracts of *Aloe vera* leaves, green tea leaves and *Scutellaria* (skullcap) herb pronounced no significant effects on the release of lysozyme activity when incubated for one hour either with monocytes or epithelial cells (Fig.42). Nevertheless, a tendency to increase in the released lysozyme activity of HT-29 epithelial cells was found after one hour incubation with 10µg/ml of green tea or skullcap extracts, but these stimulatory effects were not statistically significant (Fig.42.B).

On the other hand, CO<sub>2</sub>-extracts of hop flowers, rosemary leaves, Chinese licorice root and chamomile flowers inhibited significantly the release of lysozyme activity after one hour incubation with THP-1 monocytes. At non-toxic concentration of 50µg/ml they caused

significant strong decreases of 82%, 79%, 58% and 49%, respectively, in lysozyme activity release, whereas lower concentration of 10 $\mu$ g/ml had no significant effects (Fig.41.A).

Similar inhibitory effect of these extracts on lysozyme release was found when incubated with HT-29 epithelial cells for one hour, but with decreases less than that observed with monocytes. Significant decreases of 17% and 28% in the secreted lysozyme activity of epithelial cells were detected with hop flowers extract at both concentrations 10 $\mu$ g/ml and 50 $\mu$ g/ml, respectively. Rosemary leaves and licorice root extracts at 50 $\mu$ g/ml caused significant decreases of 10% and 29% respectively. Extract of chamomile flowers showed no significant effects on lysozyme secretion in HT-29 epithelial cells regardless of the concentration used (Fig.41.B).



**Fig.43.** Percent changes in the secreted lysozyme activity of THP-1 monocytes and of HT-29 epithelial cells after 1 hour treatment with isolated *Echinacea* alkamide (A) or with benzyl isothiocyanate (B) using sensitive fluorescence-based lysozyme assay. Control cultures without tested compounds addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Benzyl isothiocyanate exhibited significant inhibitory effects on lysozyme release of both THP-1 monocytes and HT-29 epithelial cells after one hour incubation. Significant decreases of the secreted lysozyme with direct dose-dependent relationship were detected upon exposure to increasing concentrations of benzyl isothiocyanate, particularly in THP-1 cell cultures. Increasing concentration range from 0.5 $\mu$ M to 5 $\mu$ M of benzyl isothiocyanate caused increasing decreases ranged from 15% to 46% in the release of lysozyme activity in THP-1 cells (Fig.43.B).

When HT-29 epithelial cells were exposed to benzyl isothiocyanate for one hour, a significant decrease of about 17% in lysozyme release was found at both concentrations of 0.1 $\mu$ M and 0.5 $\mu$ M, while 1 $\mu$ M of benzyl isothiocyanate caused further decrease of 24% (Fig.43.B).

In the matter of toxicity to cells, both *Echinacea* alkamide and benzyl isothiocyanate showed no toxic effects to the monocytes and epithelial cells after one hour exposure.

#### **3.3.7.1.1.2. Synthesis and secretion of lysozyme activity in cell cultures after long-term treatment**

Stimulatory effect on the secretion or expression of lysozyme activity upon long-term treatment was demonstrated with EGCG-rich green tea leaves extract. Statistically significant tendency to increase of about 17% in the expression of lysozyme activity was detected when THP-1 monocytes were treated with 50 $\mu$ g/ml green tea extract for 72 hours with no cytotoxicity (Table 11). Green tea extract had no remarkable influence on lysozyme activity of epithelial cells with long treatment periods up to 72 hours.

Extracts of *Scutellaria lateriflora* herb (skullcap), *Echinacea purpurea* root and *Aloe vera* leaves pronounced no significant effects on the expression or release of lysozyme activity of either THP-1 monocytes or HT-29 epithelial cells, irrespective of the extract concentration or the treatment period used (Table 12).

Benzyl isothiocyanate continued to exhibit significant dose-dependent inhibitory effects on the synthesis and release of lysozyme when incubated with THP-1 monocytes for incubation period of 24 hours, at the concentration range from 1 $\mu$ M to 5 $\mu$ M. This inhibitory influence of benzyl isothiocyanate on lysozyme activity of THP-1 disappeared unexpectedly after further 48 hours incubation, whereas significant dose-dependent stimulatory effects on lysozyme activity expression showed up after 72 hours incubation (Table 14). Benzyl isothiocyanate at concentrations up to 3 $\mu$ M showed no significant toxicity to the cells after 72 hours exposure, whereas 5 $\mu$ M started to pronounce toxic effects to THP-1 cells after 24 hours exposure. Upon incubation with HT-29 epithelial cells for periods up to 72 hours, benzyl isothiocyanate had no effects on the synthesis and release of lysozyme activity regardless of the concentration or the incubation period used (Table 14).

Extract of *Humulus lupulus* (hop) flowers pronounced strong and statistically significant inhibitory effects on the expression and release of lysozyme activity of both monocytes and epithelial cells at both concentrations 10µg/ml and 50µg/ml (Table 13). This inhibition showed direct time- and dose-dependent relationship. However, the high concentration of 50µg/ml showed high toxicity to THP-1 monocytes with treatment periods of 24 hours and longer, while its toxic effects to the HT-29 epithelial cells began to appear only after 72 hours incubation. The lower concentration of 10µg/ml of hop extract was non-toxic to both monocytes and epithelial cells with all tested treatment periods up to 72 hours.

Extracts of *Rhodiola rosea* root and Chinese licorice root were also demonstrated to inhibit the production and release of lysozyme activity in both cell lines, but with more significant inhibitory effects on THP-1 monocytes than epithelial cells (Table 13). Reverse dose-dependent inhibitory effect was observed with *Rhodiola* extract when incubated with THP-1 monocytes for 24 and 48 hours, since 50µg/ml of the extract caused more significant decreases in the total and secreted lysozyme activity than the higher concentration of 100µg/ml (Table 13). *Rhodiola* extract at both concentrations started to show toxicity to the THP-1 cells only after 72 hours incubation. Slight but sometimes statistically significant inhibitory effects were detected with *Rhodiola* extract when incubated with the epithelial cells for treatment periods up to 72 hours (Table 13). Regarding toxicity to HT-29 epithelial cells, *Rhodiola* extract at 50µg/ml was non-toxic with all treatment periods up to 72 hours, whereas the higher dose of 100µg/ml was toxic to the epithelial cells after 24 hours and longer treatment periods.

Extract of Chinese licorice root showed dose-dependent inhibition of lysozyme secretion and synthesis by HT-29 epithelial cells with long-term treatment periods up to 72 hours. Low extract concentration of 10µg/ml had no significant inhibitory effects, whereas strong and statistically significant inhibitory effects were found at 50µg/ml. Regarding cytotoxicity, licorice extract at the high dose of 50µg/ml started to pronounce toxic effects to the epithelial cells only after 72 hours incubation (Table 13). On the other hand, 10µg/ml of licorice root extract was enough to produce significant inhibitory effects on lysozyme secretion and synthesis of THP-1 monocytes with long treatment periods up to 72 hours with no cytotoxicity, whereas 50µg/ml of the extract was toxic to the monocytes after 24 hours and longer treatment periods (Table 13).

Furthermore, extracts of chamomile flowers and rosemary leaves showed inhibitory effects on the synthesis and secretion of lysozyme activity in THP-1 monocytes when incubated for long periods up to 72 hours. This inhibition was markedly significant at the high non-toxic extract concentration of 50µg/ml (Table 11). On the contrary, either extracts of chamomile and rosemary exhibited no significant influence on lysozyme activity of HT-29 epithelial cells regardless of the extract concentration or the incubation period used (Table 11).



Extract conc.	Cell Type	24 hours treatment		48 hours treatment		72 hours treatment	
		Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
<b>Chamomile flowers extract:</b>							
10 µg/ml	HT-29	- 1 % (±4)	- 6 % (±6)	- 3 % (±6)	- 3 % (±4)	0 % (±2)	- 4 % (±4)
	THP-1	+ 2 % (±2)	- 11 %* (±5)	- 4 % (±4)	- 9 % (±7)	- 8 % (±9)	- 1 % (±0)
50 µg/ml	HT-29	- 4 % (±4)	- 5 % (±3)	- 2 % (±9)	± 5 % (±2)	0 % ± 1)	- 3 % (±1)
	THP-1	- 25 %* (±2)	- 36 %* (±15)	- 26 %* (±1)	- 27 %* (±1)	- 27 %* (±5)	- 54 %* (±4)
<b>Rosemary leaves extract:</b>							
10 µg/ml	HT-29	+ 4 % (±6)	- 2 % (±5)	+ 6 % (±7)	+ 3 % (±4)	+ 3 % (±3)	+ 2 % (±4)
	THP-1	+ 1 % (±3)	- 15 %* (±12)	0 % (±9)	+ 1 % (±1)	+ 5 % (±5)	+ 4 % (±7)
50 µg/ml	HT-29	- 3 % (±4)	0 % (±3)	+ 4 % (±5)	+ 2 % (±4)	+ 1 % (±2)	+ 3 % (±7)
	THP-1	-63 %* (±0)	- 64 %* (±6)	- 45 %* (±2)	- 40 %* (±2)	- 33 %* (±2)	- 39 %* (±15)
<b>Green tea extract rich in EGCG:</b>							
2 µg/ml	HT-29	- 10 %* (±1)	- 8 %* (±4)	- 2 % (±8)	- 8 %* (±2)	- 2 % (±2)	- 1 % (±2)
10 µg/ml	HT-29	- 1 % (±9)	- 3 % (±5)	+ 2 % (±4)	- 1 % (±5)	- 2 % (±3)	0 % (±4)
	THP-1	- 7 % (±10)	+ 4 % (±3)	- 6 % (±1)	+ 5 % (±5)	+ 1 % (±2)	+ 2 % (±3)
50 µg/ml	HT-29	- 15 %* (±12)	- 16 %* (±7)	- 16 %* (±10)	- 6 % (±9)	- 18 %* (±4)	- 9 % (±6)
	THP-1	- 19 %* (±8)	- 7 % (±1)	- 2 % (±10)	+ 3 % (±7)	+ 2 % (±1)	+ 17 %* (±8)

**Table 11.** Percent change (to control) in the secreted and total lysozyme activity of THP-1 monocytes and HT-29 epithelial cells after treatment with various herbal extracts at different concentrations. Control cultures without extract addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of at least six separate experiments (±SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Extract conc.	Cell Type	24 hours treatment		48 hours treatment		72 hours treatment	
		Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
<b><i>Scutellaria lateriflora</i> (skullcap) herb extract:</b>							
10 µg/ml	HT-29	+ 2 % (±4)	- 1 % (±3)	+ 1 % (±4)	- 2 % (±3)	0 % (±3)	- 1 % (±2)
	THP-1	- 9 % (±3)	- 11 % (±4)	- 6 % (±3)	+ 7 % (±4)	+ 3 % (±2)	+ 2 % (±3)
50 µg/ml	HT-29	- 2 % (±8)	- 2 % (±6)	- 2 % (±7)	- 2 % (±8)	- 3 % (±7)	- 3 % (±8)
	THP-1	+ 11 % (±7)	+ 7 % (±1)	+ 12 % (±7)	+ 8 % (±4)	+ 8 % (±1)	+ 3 % (±4)
<b><i>Echinacea purpurea</i> root extract:</b>							
2 µg/ml	HT-29	- 1 % (±1)	- 4 % (±1)	- 7 % (±3)	- 7 % (±3)	- 4 % (±3)	- 8 % (±4)
10 µg/ml	HT-29	+ 2 % (±2)	+ 2 % (±3)	- 4 % (±4)	- 5 % (±4)	- 3 % (±3)	- 4 % (±7)
	THP-1	- 8 % (±4)	- 6 % (±5)	+ 4 % (±3)	+ 3 % (±2)	- 2 % (±8)	- 10 % (±0.5)
50 µg/ml	HT-29	- 6 % (±4)	+ 1 % (±8)	--	--	--	--
	THP-1	+ 13 % (±5)	- 2 % (±11)	- 5 % (±6)	+ 3 % (±11)	+ 2 % (±3)	+ 6 % (±10)
<b><i>Aloe vera</i> leaves extract rich in polysaccharides:</b>							
10 µg/ml	HT-29	- 1 % (±4)	- 3 % (±5)	+ 2 % (±4)	+ 2 % (±3)	+ 3 % (±4)	+ 1 % (±5)
	THP-1	- 5 % (±7)	- 5 % (±2)	- 7 % (±6)	- 3 % (±5)	+ 1 % (±2)	+ 2 % (±3)
50 µg/ml	HT-29	+ 2 % (±7)	+ 5 % (±6)	- 2 % (±6)	0 % (±9)	- 1 % (±7)	- 3 % (±3)
	THP-1	+ 2 % (±8)	+ 5 % (±2)	+ 7 % (±4)	- 2 % (±14)	+ 2 % (±6)	- 8 % (±21)

**Table 12.** Percent change (to control) in the secreted and total lysozyme activity of THP-1 monocytes and HT-29 epithelial cells after treatment with various herbal extracts at different concentrations. Control cultures without extract addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of at least six separate experiments (±SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Extract conc.	Cell Type	24 hours treatment		48 hours treatment		72 hours treatment	
		Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
<b><i>Humulus lupulus</i> (hop) flowers extract:</b>							
10 µg/ml	HT-29	- 59 %* (±14)	- 42 %* (±5)	- 67 %* (±4)	- 45 %* (±5)	- 64 %* (±2)	- 49 %* (±4)
	THP-1	- 51 %* (±1)	- 53 % (±4)	- 68 %* (±3)	- 69 %* (±2)	- 76 %* (±2)	- 71 %* (±1)
50 µg/ml	HT-29	- 81 %* (±11)	- 74 %* (±2)	- 94 %* (±5)	- 79 %* (±3)	--	--
	THP-1	--	--	--	--	--	--
<b><i>Rhodiola rosea</i> root extract:</b>							
50 µg/ml	HT-29	- 4 % (±3)	0 % (±6)	- 5 %* (±3)	- 12 %* (±7)	- 8 %* (±6)	- 4 % (±4)
	THP-1	- 26 %* (±6)	- 37 %* (±4)	- 37 %* (±2)	- 64 %* (±28)	- 21 %* (±4)	- 20 %* (±1)
100 µg/ml	HT-29	- 13 %* (±7)	- 5 % (±4)	- 9 %* (±5)	- 6 %* (±2)	- 3 % (±5)	- 2 % (±6)
	THP-1	- 16 % (±13)	- 21 %* (±12)	- 32 %* (±2)	- 44 %* (±1)	- 30 %* (±2)	- 32 %* (±2)
<b><i>Glycyrrhiza uralensis</i> (Chinese licorice) root extract:</b>							
10 µg/ml	HT-29	- 9 % (±11)	- 3 % (±5)	0 % (±7)	+ 2 % (±3)	- 6 % (±16)	- 2 % (±6)
	THP-1	- 32 %* (±5)	- 29 %* (±5)	- 43 %* (±5)	- 39 %* (±3)	- 48 %* (±2)	- 39 %* (±2)
50 µg/ml	HT-29	- 69 %* (±28)	- 56 %* (±17)	- 76 %* (±10)	- 54 %* (±28)	--	--
	THP-1	--	--	--	--	--	--

**Table 13.** Percent change (to control) in the secreted and total lysozyme activity of THP-1 monocytes and HT-29 epithelial cells after treatment with various herbal extracts at different concentrations. Control cultures without extract addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of at least six separate experiments (±SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Conc.	Cell Type	24 hours treatment		48 hours treatment		72 hours treatment	
		Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
<b>Benzyl isothiocyanate:</b>							
0.1 $\mu$ M	HT-29	0 % ( $\pm$ 4)	0 % ( $\pm$ 2)	+ 5 % ( $\pm$ 5)	+ 5 % ( $\pm$ 3)	+ 3 % ( $\pm$ 3)	+ 1 % ( $\pm$ 4)
0.5 $\mu$ M	HT-29	- 4 % ( $\pm$ 8)	- 4 % ( $\pm$ 4)	- 2 % ( $\pm$ 3)	- 1 % ( $\pm$ 2)	- 1 % ( $\pm$ 2)	- 2 % ( $\pm$ 2)
	THP-1	+ 10 % ( $\pm$ 8)	+ 10 % ( $\pm$ 15)	+ 0 % ( $\pm$ 7)	+ 6 % ( $\pm$ 9)	+ 6 % ( $\pm$ 9)	+ 10 % ( $\pm$ 9)
1 $\mu$ M	HT-29	+ 11 % ( $\pm$ 26)	- 7 % ( $\pm$ 9)	0 % ( $\pm$ 7)	- 1 % ( $\pm$ 3)	- 1 % ( $\pm$ 2)	0 % ( $\pm$ 3)
	THP-1	- 33 %* ( $\pm$ 43)	- 40 %* ( $\pm$ 43)	+ 1 % ( $\pm$ 7)	+ 9 % ( $\pm$ 8)	+ 2 % ( $\pm$ 9)	+ 27 %* ( $\pm$ 12)
3 $\mu$ M	THP-1	- 55 %* ( $\pm$ 2)	- 78 %* ( $\pm$ 14)	- 10 %* ( $\pm$ 3)	- 6 % ( $\pm$ 6)	+ 13 %* ( $\pm$ 6)	+ 32 %* ( $\pm$ 8)
5 $\mu$ M	THP-1	- 86 %* ( $\pm$ 1)	- 75 %* ( $\pm$ 0,5)	---	---	---	---

**Table 14.** Percent changes (to control) in the secreted and total lysozyme activity of THP-1 monocytes and HT-29 epithelial cells after treatment with benzyl isothiocyanate at different concentrations. Control cultures without tested compound addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of at least six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.1.2. Human gastric epithelial cells AGS

Extract of *Echinacea purpurea* root at concentration of 50 $\mu$ g/ml caused slight but statistically significant rise of 12% in the secreted lysozyme activity, whereas lower concentrations had no significant influence (Fig.45).

Quercetin, rutin, naringin and silymarin group flavonoids as well as polysaccharides extracted from *Aloe vera* had no significant effect on the secretion of lysozyme activity when incubated with AGS cells regardless of the concentration examined. Concentration ranges examined in our experiments are shown in Table 15.

On the other hand, extract of chamomile flowers at 50 $\mu$ g/ml caused statistically significant decrease of 26% in secreted lysozyme activity (Fig.45).

Curcumin showed dose-dependent inhibitory effect on lysozyme activity secreted in AGS cultures. Curcumin at 1 $\mu$ g/ml caused statistically significant decrease of 24% in the secreted lysozyme activity after one hour treatment, whereas more significant decrease of 80% was observed at final concentration of 10 $\mu$ g/ml (Fig.44.A).

Dose-dependent effect on lysozyme activity secreted in AGS cultures was also observed with green tea extract ( $\geq 90\%$  EGCG).  $10\mu\text{g/ml}$  final concentration had significant inhibitory effect of 16%, while  $50\mu\text{g/ml}$  of this flavonoid-rich green tea extract resulted in higher inhibition of 24% in the secretion of lysozyme activity after one hour treatment (Fig.44.B).

The significant decrease of secreted lysozyme activity induced in cell cultures supplemented with licorice root extract was likewise dose dependent (Fig.44.C). Licorice extract at final concentration of  $10\mu\text{g/ml}$  caused slight but significant inhibition of 8%, while  $50\mu\text{g/ml}$  resulted in significantly higher inhibition of 61% of lysozyme activity secretion after one hour treatment compared to control cultures.

Natural products and extracts	Examined concentration range
Quercetin	from 0.34 to 3.4 $\mu\text{g/ml}$
Rutin	from 10 to 50 $\mu\text{g/ml}$
Silymarin group	from 10 to 50 $\mu\text{g/ml}$
Naringin	from 10 to 50 $\mu\text{g/ml}$
<i>Echinacea purpurea</i> root extract	from 10 to 50 $\mu\text{g/ml}$
Chamomile flowers extract	from 10 to 50 $\mu\text{g/ml}$
<i>Aloe polysaccharides</i>	from 10 to 50 $\mu\text{g/ml}$

**Table 15.** Examined concentration ranges for the various plant extracts and natural products.

### 3.3.7.2. Homeopathic mother tinctures and its isolated compounds

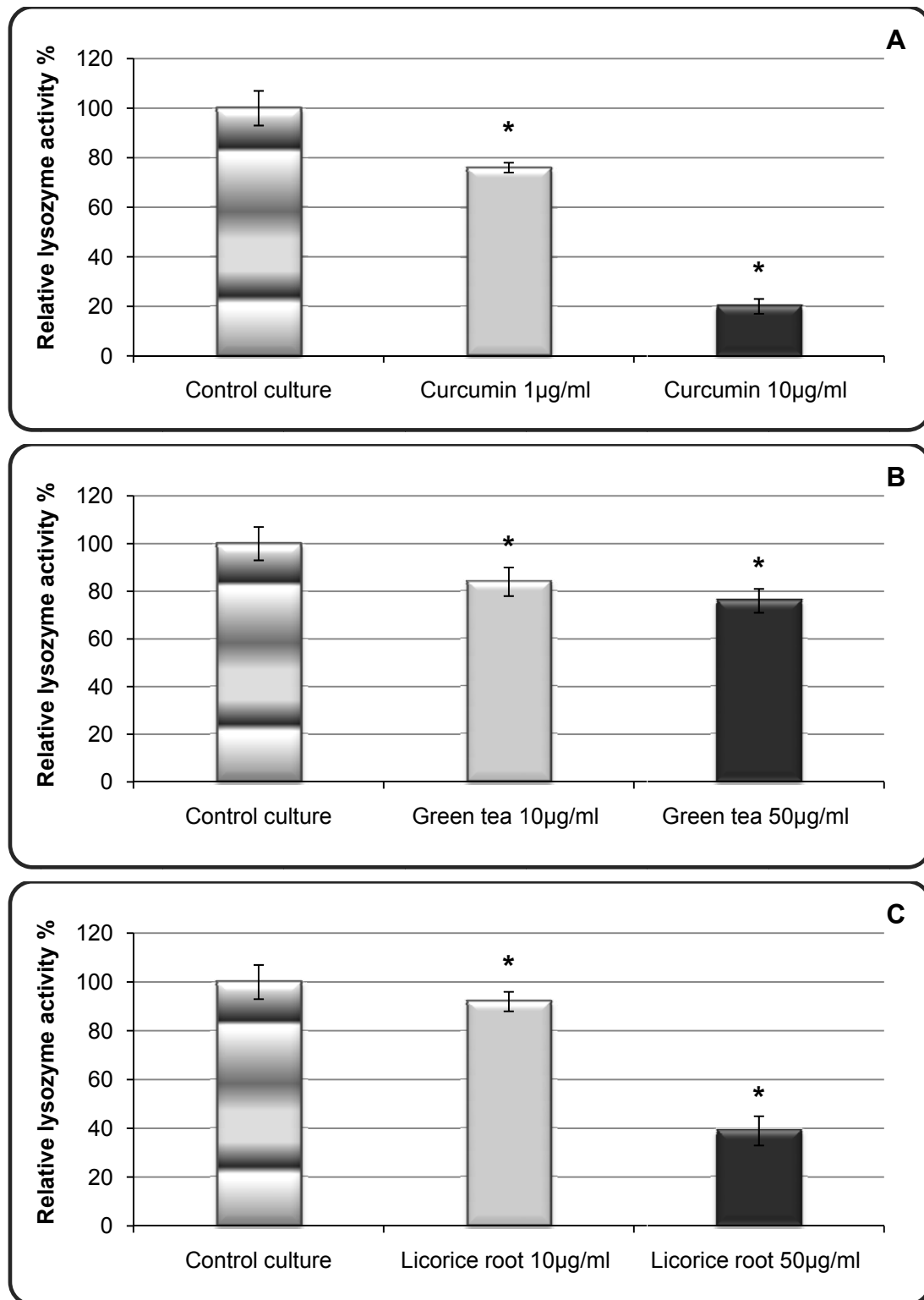
#### 3.3.7.2.1. Tomato extracts and its isolated compound tomatine

##### 3.3.7.2.1.1. Secretion of lysozyme activity in cell cultures after one hour treatment

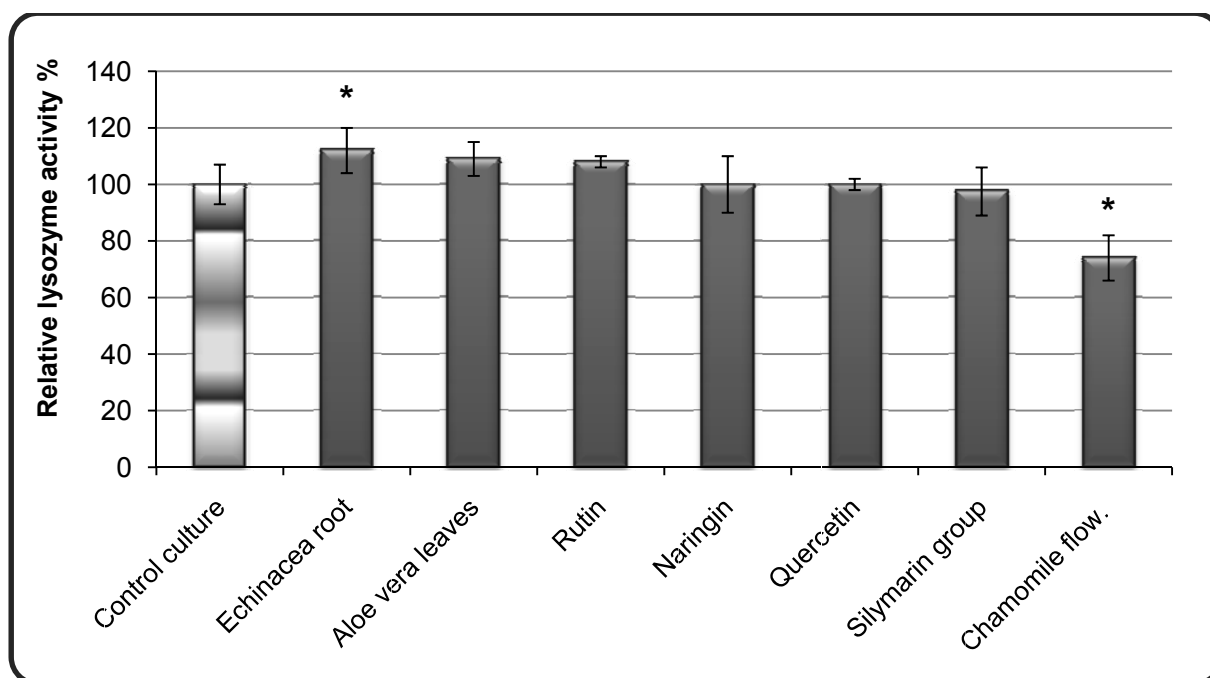
Tomatine was proved to stimulate lysozyme activity released after one hour treatment of undifferentiated monocytes. The stimulation of lysozyme activity secretion was significant with all examined concentrations of tomatine, but a distinct dose-dependence of this effect could not be found. The most significant increase of 77% in the secreted lysozyme activity of THP-1 cells was observed at tomatine concentration of 2nM (2.068ng/ml) (Fig.46.A).

On the contrary, no significant influence of tomatine could be demonstrated with the PMA-differentiated THP-1 monocytes as well as with HT-29 epithelial cells, regardless of tomatine concentration used (Fig.46.A).

To confirm the influence of tomatine on lysozyme activity of the cell cultures, tomatine-containing natural extracts of fresh and dried tomato were investigated. A similar effect of tomatine on undifferentiated THP-1 cells could be found to some extent with the extract of the dried tomato. The extract stimulated significantly lysozyme activity release after one hour treatment of THP-1 cells, but no dose-dependent effect could be found.



**Fig.44.** Percent decrease in the secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml AGS cell cultures after one hour treatment with curcumin (A), EGCG-rich green tea leaves extract (B) or Chinese licorice root extract (C) at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without any substance addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



**Fig.45.** Percent change in the secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml AGS cell cultures after one hour treatment with various natural products using sensitive fluorescence-based lysozyme assay. Concentration examined was  $50 \mu\text{g/ml}$ , except for quercetin was  $3.4 \mu\text{g/ml}$ . Control cultures without product addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

The most significant increase of 84% in the secreted lysozyme activity of THP-1 cells was detected at concentration of 0.3% dried-tomato extract (v/v), which equals to a final concentration of 105.3 ng/ml of tomatine (Fig.46.C). 0.3% of the dried-tomato extract was also able to enhance significantly the release of lysozyme activity when incubated with PMA-differentiated THP-1 cells for one hour. An increase of 44% in the secreted lysozyme levels was found when differentiated THP-1 cell cultures were incubated with 0.3% dried-tomato extract (v/v) (equals to a final concentration of 105.3 ng/ml of tomatine), whereas lower concentrations had no significant results (Fig.46.C). Like tomatine, the extract of the dried tomato had no significant effect on the lysozyme activity secreted by HT-29 epithelial cells after one hour incubation (Fig.46.C).

A significant stimulatory effect of fresh-tomato tincture on lysozyme activity secretion could be observed with the PMA-differentiated THP-1 cells. An increase of 30% in the secreted lysozyme activity was found when differentiated monocytes were incubated with 0.3% fresh-tomato tincture (v/v), which equals to a final concentration of 7.8 ng/ml of tomatine (Fig.46.B). Unlike tomatine, tomato tincture of the fresh fruits (tomatine content: 0.26%) had no significant effects on the release of lysozyme activity after one hour incubation with undifferentiated THP-1 cells at all investigated concentrations.

Tincture of the fresh tomato had no significant influence on HT-29 epithelial cells, which corresponded to the results of tomatine.

### 3.3.7.2.1.2. Synthesis and secretion of lysozyme activity in cell cultures after long-term treatment with tomatine

A significant effect of tomatine on lysozyme activity expression was observed only after 72 hours treatment of THP-1 cells. A significant increase of 20% in the total lysozyme activity expressed by THP-1 monocytes could be detected after 72 hours treatment with tomatine at 100nM (103.4ng/ml), whereas lower concentrations had no significant effects (Table 16).

At shorter incubation periods of 24 and 48 hours with THP-1 cells, tomatine had no significant outcome on lysozyme activity levels.

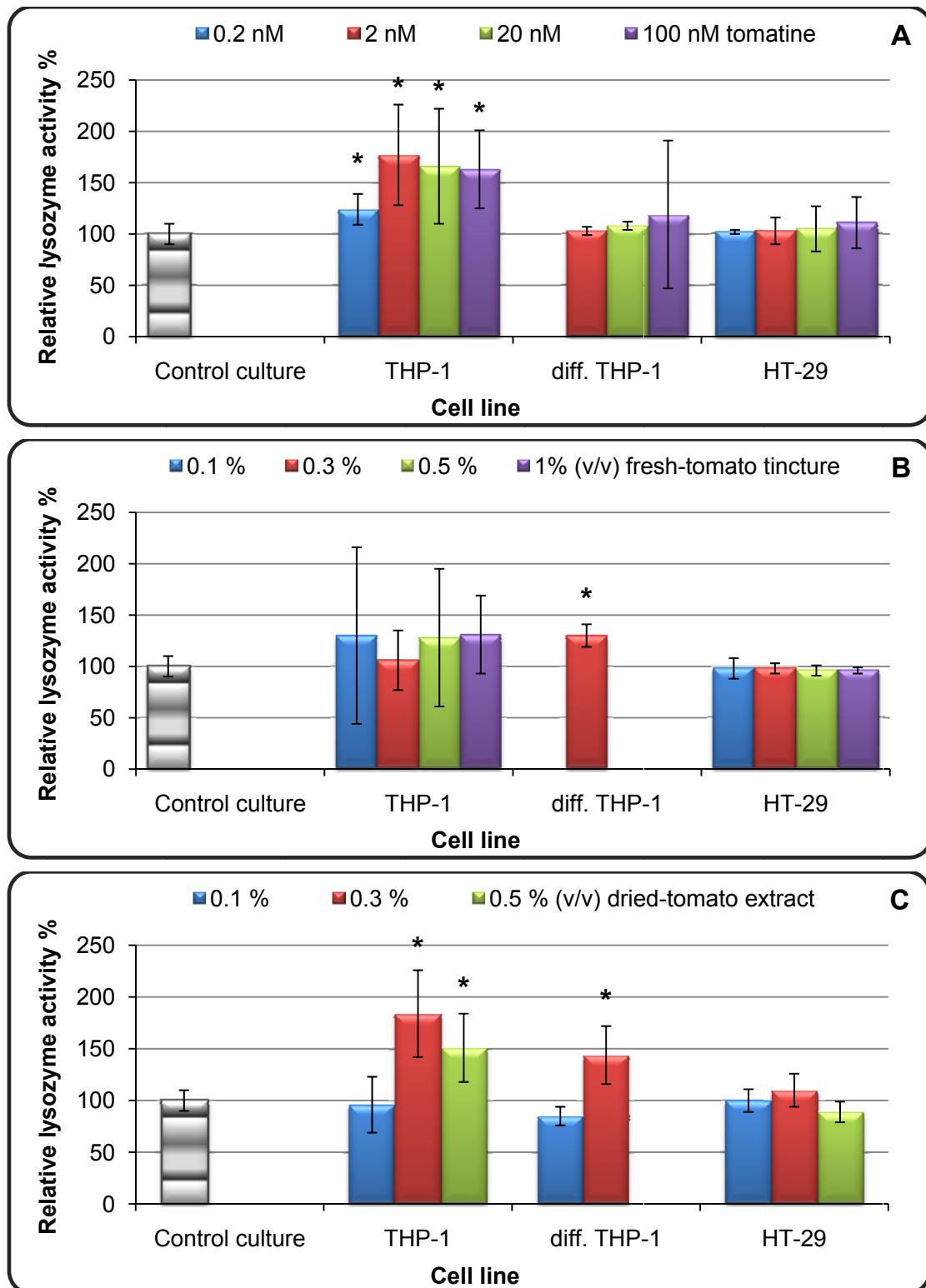
Corresponding to the results with the short-term incubation, lysozyme activity of HT-29 epithelial cells was at no time affected when treated with tomatine for periods up to 72 hours whatever concentration of tomatine was used (Table 16).

Tomatine-containing tomato extracts were not investigated for long-term incubation with cell cultures, since the results with tomatine were not sufficiently significant.

Tomatine conc.	Cell Type	24 hours treatment		48 hours treatment		72 hours treatment	
		Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
2 nM	HT-29	+ 1 % (±2)	+ 1 % (±6)	- 1 % (±1)	- 3 % (±6)	- 1 % (±1)	+ 4 % (±3)
	THP-1	+ 10 % (±8)	- 15 % (±22)	- 8 % (±12)	- 9 % (±9)	- 9 % (±8)	- 13 % (±12)
20 nM	HT-29	0 % (±2)	- 4 % (±7)	- 4 % (±3)	- 3 % (±4)	- 4 % (±3)	0 % (±4)
	THP-1	+ 10 % (±9)	+ 27 % (±24)	- 1 % (±19)	+ 4 % (±6)	+ 6 % (±12)	+ 7 % (±9)
100 nM	HT-29	- 3 % (±2)	- 3 % (±3)	- 5 % (±3)	- 4 % (±3)	- 5 % (±3)	- 1 % (±4)
	THP-1	+ 2 % (±7)	+ 11 % (±33)	+ 10 %* (±1)	- 6 % (±16)	+ 2 % (±3)	+ 20 %* (±10)

**Table 16.** Percent change (to control) in the secreted and total lysozyme activity of monocytic and epithelial cell cultures after treatment with tomatine at different non-toxic concentrations. Control cultures without tomatine addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments (±SD). Values with asterisk are significantly different (P<0.05, Mann-Whitney U test) from values of control cultures





**Fig.46.** Percent changes in the lysozyme activity secreted after 1 hour in the medium of various cell lines cultures treated with: (A) Tomatine

(B) Tomato tincture of the fresh fruits (tomatine content: 0.26%)

(C) Tomato extract of the dried fruits (tomatine content: 3.51%)

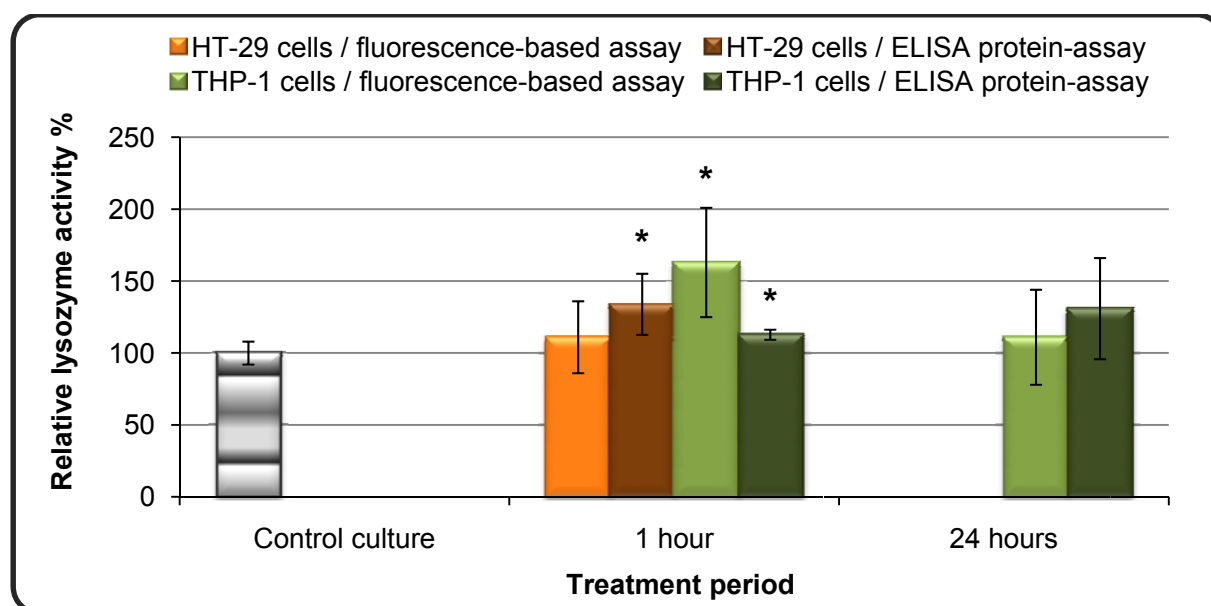
Control cultures without tested substances addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.2.1.3. Effect of tomatine on lysozyme of monocytes and epithelial cells measured using ELISA lysozyme protein assay compared to enzymatic activity assay

Tomatine at 100nM (103.4ng/ml) induced slight but statistically significant increase of 13% in the lysozyme secreted in the medium of THP-1 cell cultures after 1 hour treatment and detected using ELISA protein assay. This increase of the released lysozyme measured as a protein regardless of its enzymatic activity was significantly less than the 63% increase of the released lysozyme measured fluorometrically as an enzymatically active enzyme (Fig.47).

On the contrary, significant increase of 34% in the secreted lysozyme of HT-29 epithelial cells was found after one hour exposure to 100nM (103.4ng/ml) tomatine when was measured using ELISA protein assay, whereas no significant increase in the secreted active lysozyme measured fluorometrically was detected under the same conditions (Fig.47).

On the other hand, no significant differences were found between the effects of tomatine on the expression of lysozyme as a protein or as an active enzyme after 24 hours incubation with THP-1 cells (Fig.47).



**Fig.47.** Percent changes in the secreted lysozyme activity after 1 hour treatment, and in the total lysozyme activity after 24 hours treatment synthesized by THP-1 monocytes and HT-29 epithelial cells treated with 100nM (103.4ng/ml) tomatine using ELISA lysozyme protein assay compared to lysozyme activity assay. Control cultures without tomatine addition were considered to have 100% lysozyme activity. All points were measured in triplicate. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.2.2. Isolated aconitine

#### 3.3.7.2.2.1. Secretion of lysozyme activity in cell cultures after one hour treatment

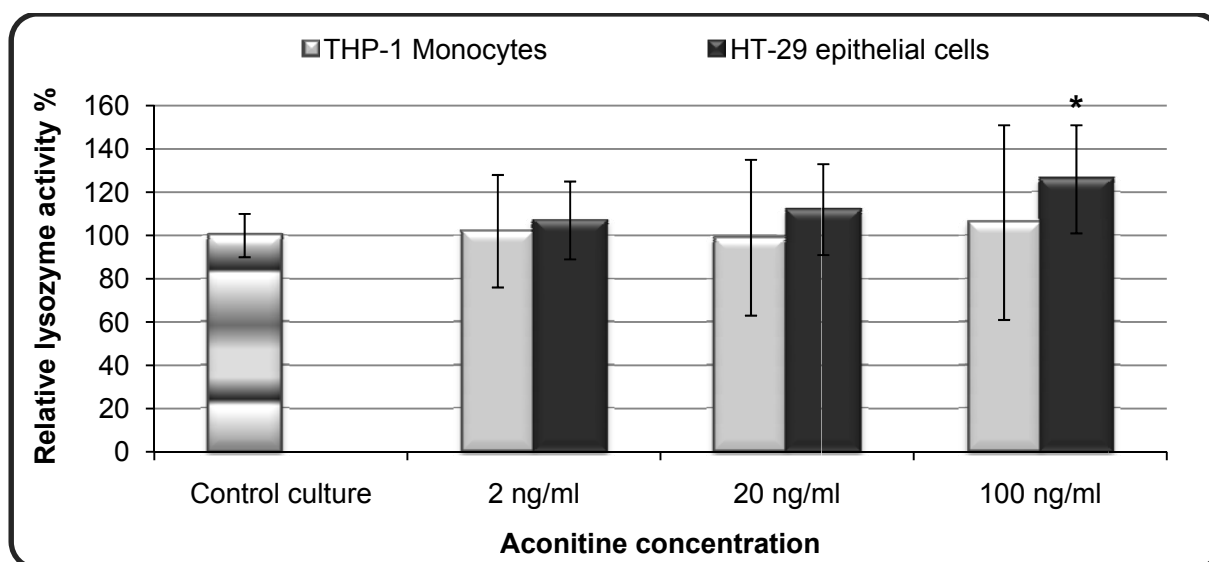
Aconitine was illustrated to stimulate the release of lysozyme activity of HT-29 epithelial cells after one hour treatment. This stimulatory effect was particularly significant at 100ng/ml (155nM) of aconitine (Fig.48). On the contrary, no significant influence of aconitine could be demonstrated on the secretion of lysozyme activity when incubated with THP-1 monocytes, regardless of aconitine concentration used (Fig.48).

#### 3.3.7.2.2.2. Synthesis and secretion of lysozyme activity in cell cultures after long-term treatment with aconitine

Aconitine at 100ng/ml (155nM) was found able to significantly up-regulate the expression and release of lysozyme activity in THP-1 monocytes upon treatment periods of 24 hours and 48 hours. This stimulatory effect of aconitine on THP-1 cells disappeared after 72 hours incubation (Table 17).

The stimulatory effect of aconitine on lysozyme activity release of HT-29 epithelial cells seen after one hour treatment was no more noticed upon longer treatment periods up to 72 hours regardless of the concentration used (Table 17).

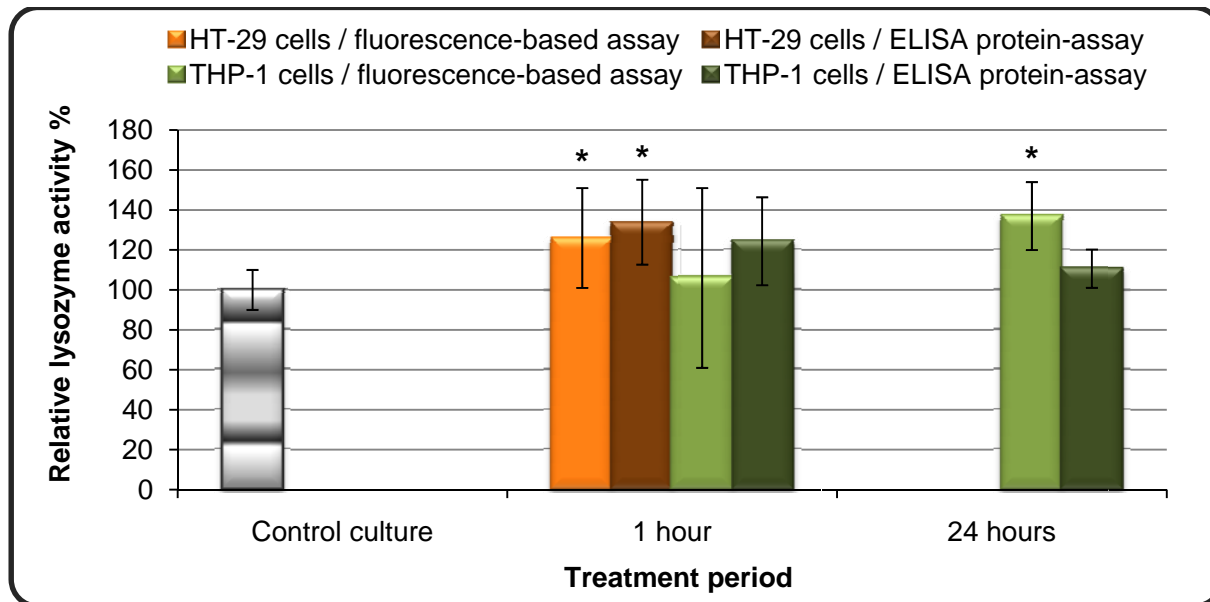
Regarding toxicity to cells, aconitine pronounced no toxic effects on monocytes or epithelial cells at all concentrations and incubation periods used.



**Fig.48.** Percent changes in the secreted lysozyme activity of THP-1 monocytic cell cultures and of HT-29 epithelial cell cultures after one hour treatment with aconitine at different non-toxic concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without aconitine addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Aconitine conc.	Cell Type	24 hours treatment		48 hours treatment		72 hours treatment	
		Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
2 ng/ml (3.097nM)	HT-29	- 5 % (±1)	- 7 % (±3)	- 4 % (±1)	- 6 % (±4)	- 4 % (±1)	- 1 % (±3)
20 ng/ml (30.97nM)	HT-29	- 6 % (±3)	- 5 % (±1)	- 2 % (±2)	- 4 % (±5)	- 2 % (±2)	- 4 % (±4)
	THP-1	+ 1 % (±6)	+ 10 % (±9)	- 9 % (±9)	- 6 % (±2)	+ 7 % (±20)	+ 1 % (±15)
100 ng/ml (155nM)	HT-29	- 4 % (±1)	- 4 % (±3)	- 9 % (±13)	- 4 % (±4)	- 9 % (±13)	0 % (±3)
	THP-1	+ 26 %* (±14)	+ 37 %* (±17)	+ 27 %* (±6)	- 11 % (±1)	+ 9 % (±10)	0 % (±15)

**Table 17.** Percent changes (to control) in the secreted and total lysozyme activity of monocytic and epithelial cell cultures after treatment with aconitine at different non-toxic concentrations. Control cultures without aconitine addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments (±SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



**Fig.49.** Percent changes in the secreted lysozyme activity after 1 hour treatment and in the total lysozyme activity after 24 hours treatment synthesized by THP-1 monocytes and HT-29 epithelial cells treated with 100ng/ml (155nM) aconitine using ELISA lysozyme protein assay compared to lysozyme activity assay. Control cultures without aconitine addition were considered to have 100% lysozyme activity. All points were measured in triplicate. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### **3.3.7.2.2.3. Effect of aconitine on lysozyme of monocytes & epithelial cells measured using ELISA lysozyme protein assay compared to enzymatic activity assay**

No significant differences were found between the effects of aconitine on the release of lysozyme as a protein and as an active enzyme after one hour treatment of either THP-1 monocytes and HT-29 epithelial cells. Aconitine induced statistically significant increase in the lysozyme secretion of epithelial cells upon treatment for one hour whether it was measured fluorometrically or using ELISA assay, while no statistically significant effect was detected when aconitine was incubated for one hour with monocytes (Fig.49).

On the other hand, the significant increase of 37% in the synthesis of active lysozyme of THP-1 cells found after 24 hours exposure was not detected when lysozyme was measured as a protein using ELISA lysozyme assay under the same conditions (Fig.49).

### **3.3.7.3. Isolated saponins**

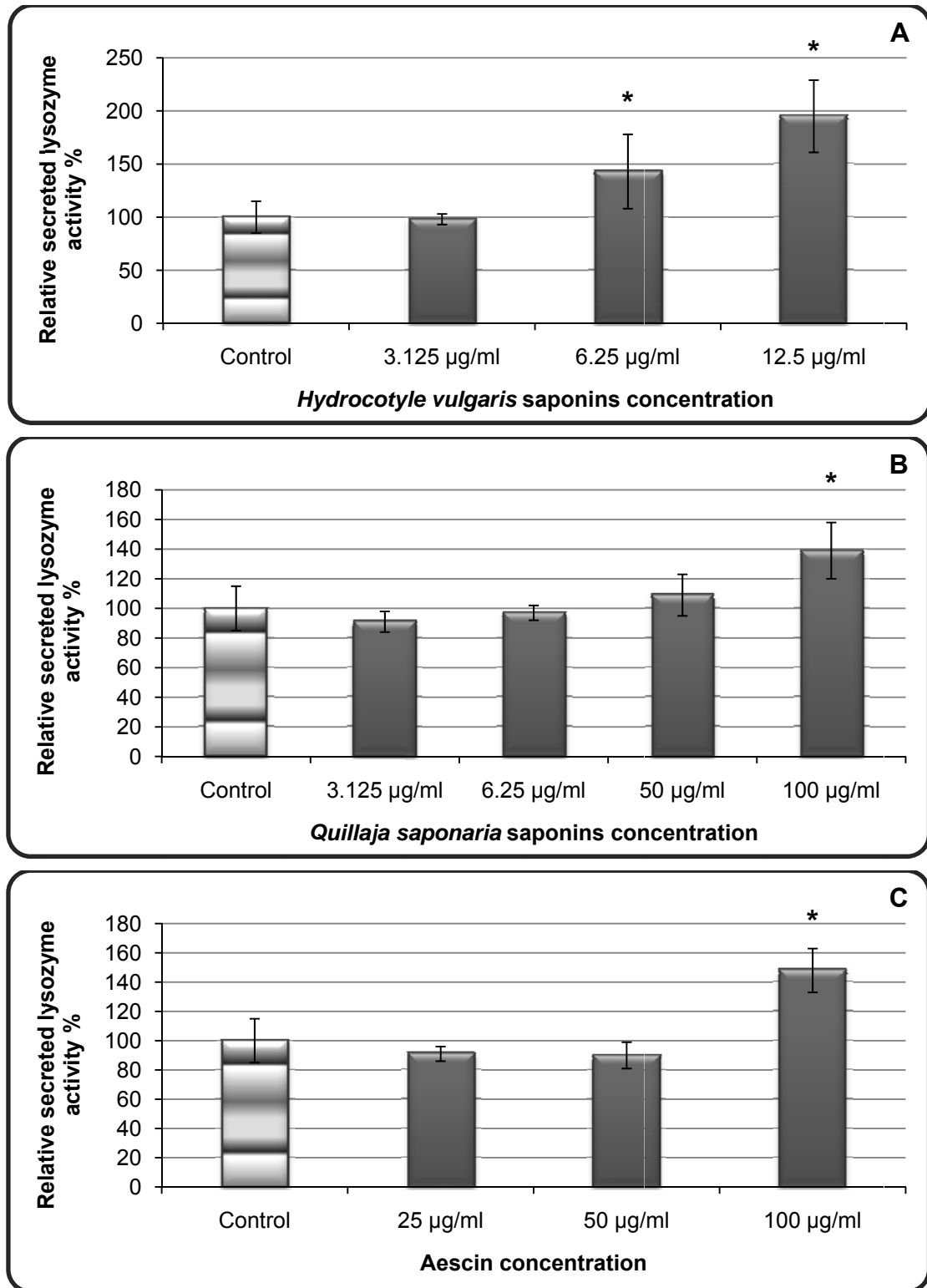
#### **3.3.7.3.1. Human monocytic cells THP-1**

##### **3.3.7.3.1.1. Secretion of lysozyme activity in cell cultures after one hour treatment**

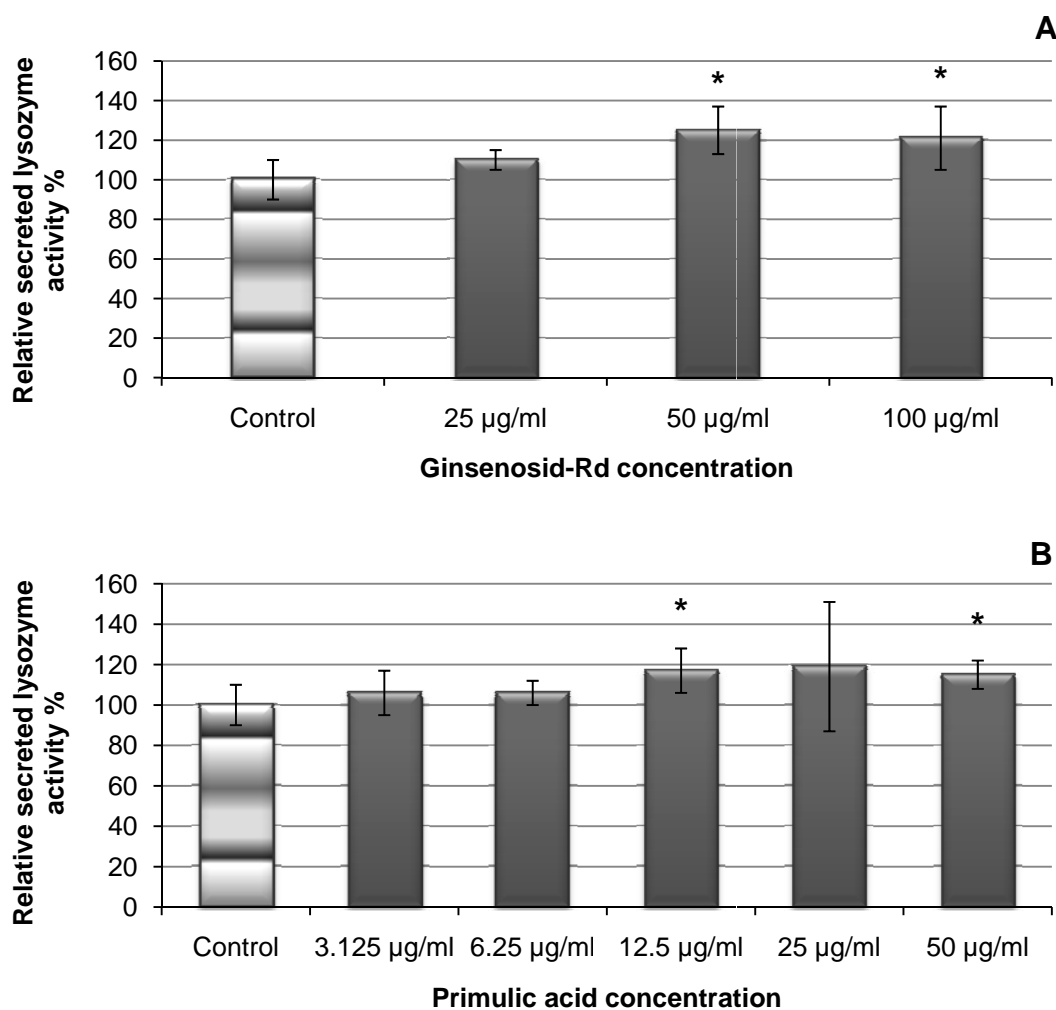
Dose-dependent effect on lysozyme activity secreted in THP-1 cultures was observed with *Hydrocotyle vulgaris* saponins. 6.25µg/ml final concentration of the saponin led to significant increase of 43% in the secreted lysozyme activity and 12.5µg/ml caused further significant increase of 95% after one hour incubation with the cells (Fig.50.A). However, 12.5µg/ml *Hydrocotyle* saponins started to show slight non-significant toxic effect on THP-1 cells after one hour treatment. Saponins from *Quillaja saponaria* bark showed also dose-dependent effect on lysozyme activity secretion in THP-1 cultures. *Quillaja* saponins at 100µg/ml caused statistically significant increase of 39% in the secretion of lysozyme activity after one hour treatment (Fig.50.B). In the same way, aescin at 100µg/ml caused statistically significant increase of 48% in the secreted lysozyme activity after one hour treatment (Fig.50.C).

Furthermore, ginsenosid-Rd and primulic acid showed statistically significant stimulation of lysozyme activity release in monocytes with no clear dose-dependent relationship (Fig.51).

On the other hand, another group of saponins illustrated in Fig.52 showed no influence on lysozyme activity secretion after one hour treatment, including hederacosid C, glycyrrhizinic acid, saponinum album and *Helianthus annuus* saponins. Furthermore, no significant differences were found when these saponins were used at different concentrations. Concentration ranges of saponins examined in our experiments are shown in Table 18.



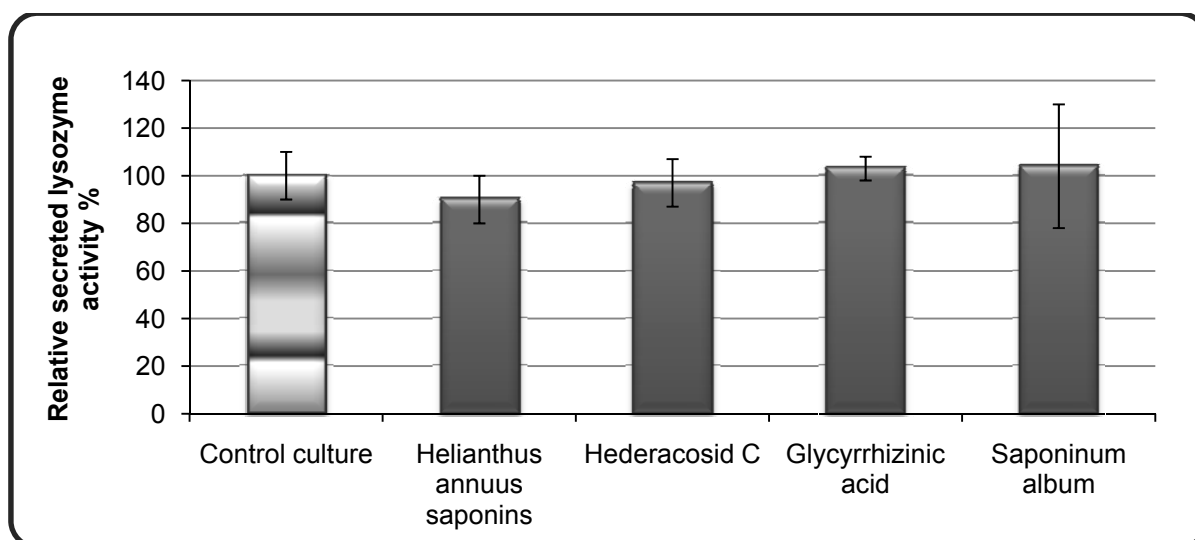
**Fig.50.** Percent changes in the secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after one hour treatment with *Hydrocotyle vulgaris* saponins (A), *Quillaja saponaria* saponins (B) or aescin (C) at different concentrations using fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



**Fig.51.** Percent changes in secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after one hour treatment with ginsenosid-Rd (A) or primulic acid (B) at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

saponin	Examined concentration range
<i>Helianthus annuus</i> saponins	from 12.5 to 100 µg/ml
Hederacosid C	from 12.5 to 100 µg/ml
Glycyrrhizinic acid	from 12.5 to 100 µg/ml
Saponinum album	from 12.5 to 50 µg/ml

**Table 18.** Examined concentration ranges for the various saponins illustrated in Fig.48.



**Fig.52.** Percent change in the secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after one hour treatment with  $12.5 \mu\text{g/ml}$  of various saponins using sensitive fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.3.1.2. Synthesis and secretion of lysozyme activity in cell cultures after long-term treatment

Lysozyme activity levels in THP-1 cultures supplemented with different saponins at non-toxic concentrations were measured at various incubation time points of up to 48 hours.

Contrary to the tendency towards increased lysozyme secretion observed with the short-term treatment with saponins, THP-1 cells incubated with saponins for longer incubation periods up to 48 hours showed tendency to decrease in the secretion and the expression of lysozyme activity in the examined cultures.

*Aescin* and *Quillaja saponaria* saponins had statistically significant inhibitory effects on the total lysozyme activity synthesized by THP-1 cells as well as on the release of lysozyme activity in the cell cultures incubated with each of these two saponins for 24 hours and for 48 hours, whereas *Hydrocotyle vulgaris* and saponinum album (*Gypsophila paniculata*) had significant inhibitory effects on both total and secreted lysozyme activity only after 48 hours incubation.

On the other hand, primulic acid and glycyrrhizinic acid caused decreases in the total lysozyme activity expressed by THP-1 cells after 24 hours incubation, while this inhibitory activity disappeared after 48 hours incubation (Table 19).



Examined saponin	Concentration	After 24h treatment		After 48h treatment	
		Secreted lysozyme activity %	Total lysozyme activity %	Secreted lysozyme activity %	Total lysozyme activity %
<i>Quillaja saponaria</i> saponins	6.25 µg/ml	- 20 ±7 %*	- 35 ±2 %*	- 21 ±11 %*	- 12 ±4 %*
Aescin	25 µg/ml	- 27 ±6 %*	- 38 ±4 %*	- 16 ±4 %*	- 22 ±5 %*
<i>Hydrocotyle vulgaris</i> saponins	3.125 µg/ml	- 7 ±16 %	- 13 ±27 %	- 23 ±8 %*	- 25 ±2 %*
Saponinum album	12.5 µg/ml	- 6 ±21 %	- 15 ±22 %	- 21 ±2 %*	- 26 ±3 %*
Primulic acid	6.25 µg/ml	+ 18 ±2 %	- 24 ±7 %*	+ 6 ±2 %	+ 3 ±2 %
Glycyrrhizinic acid	25 µg/ml	- 14 ±8 %*	- 12 ±6 %*	- 7 ±9 %	- 5 ±3 %
Hederacosid C	25 µg/ml	- 12 ±4 %*	- 9 ±6 %	- 5 ±6 %	- 3 ±4 %
<i>Helianthus annuus</i> saponins	25 µg/ml	- 9 ±9 %	- 7 ±11 %	- 4 ±1 %	+ 1 ±8 %
Ginsenosid-Rd	25 µg/ml	- 6 ±3 %	- 4 ±11 %	+ 8 ±7 %	- 8 ±7 %

**Table 19.** Percent change (to control) in the total and secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after treatment with various saponins using sensitive fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.3.2. Human colon epithelial cells HT-29

#### 3.3.7.3.2.1. Secretion of lysozyme activity in cell cultures after one hour treatment

Majority of the examined saponins stimulated lysozyme secretion after 1 hour treatment of the epithelial cells.

Primulic acid, *Quillaja saponaria* and saponinum album (from *Gypsophila paniculata*) stimulated the release of lysozyme activity with no clear dose-effect relationship (Fig.53).

Primulic acid caused a significant increase of 26% at a concentration of 25µg/ml, while lower concentration of 12.5µg/ml led to more significant increase of 37%. However, primulic acid at 25µg/ml exhibited some toxicity to the cells after one hour treatment (Fig.53.A).

*Quillaja saponaria* saponins at concentrations of 3.125µg/ml and 100µg/ml induced significant increases of 54% and 46% in the secreted lysozyme activity respectively, where 12.5µg/ml of this saponin caused less but significant increase of 12% (Fig.53.B).

Saponinum album caused slight but significant rise in the release of lysozyme activity, which remained the same when two concentrations of 12.5µg/ml and 50µg/ml of the saponin were used (Fig.53.C).

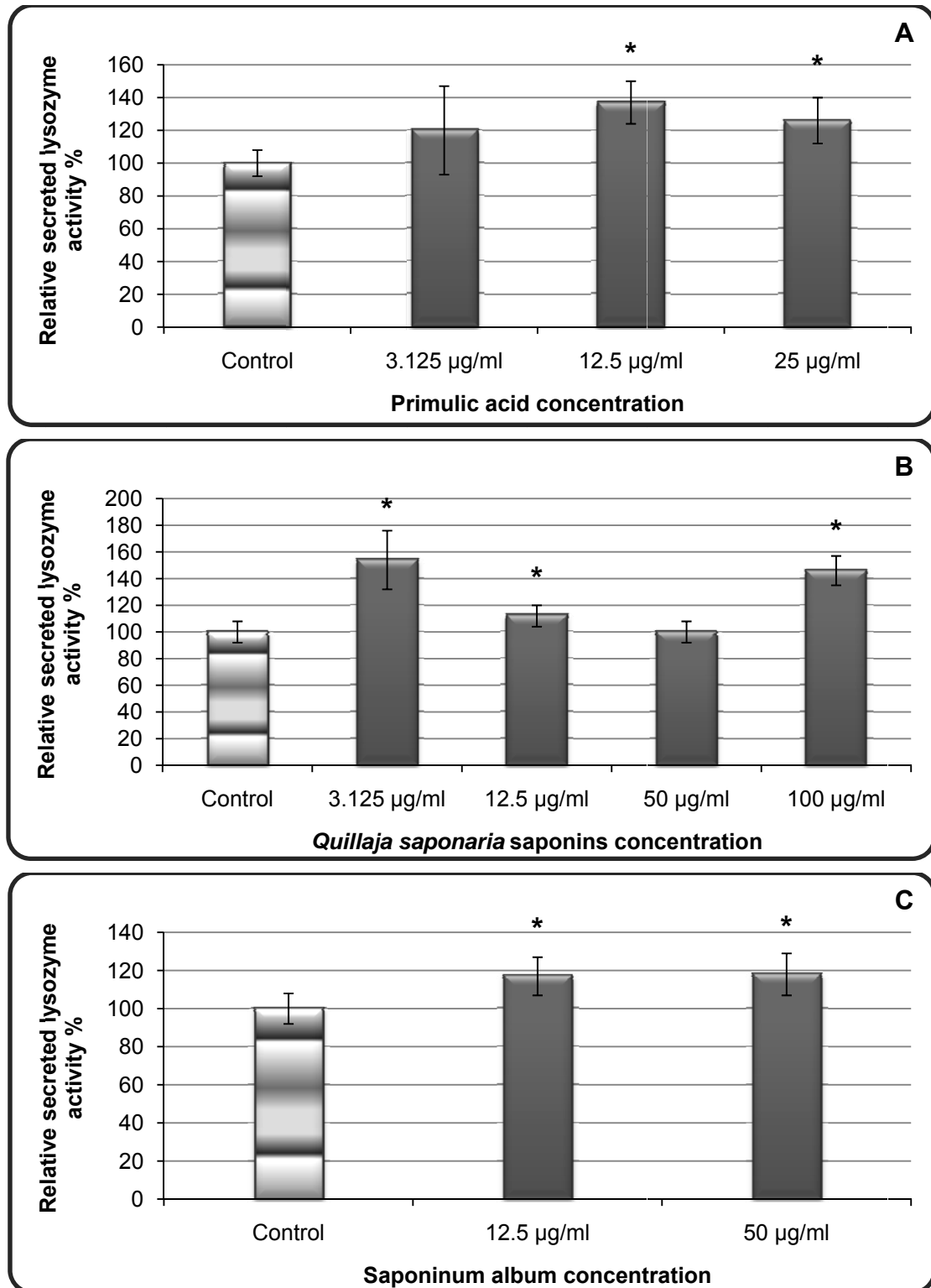
A direct dose-effect relationship could be found to some extent with *Hydrocotyle vulgaris* saponins, ginsenosid-Rd, glycyrrhizinic acid and *Helianthus annuus* saponins (Fig.54).

*Hydrocotyle vulgaris* saponins at 12.5µg/ml induced a significant increase of 37% in the secreted lysozyme, while lower doses of 3.125µg/ml and 6.25µg/ml of this saponin had no significant effects. A point to be considered that 12.5µg/ml of *Hydrocotyle* saponin showed some toxic effects to the epithelial cells (Fig.54.A).

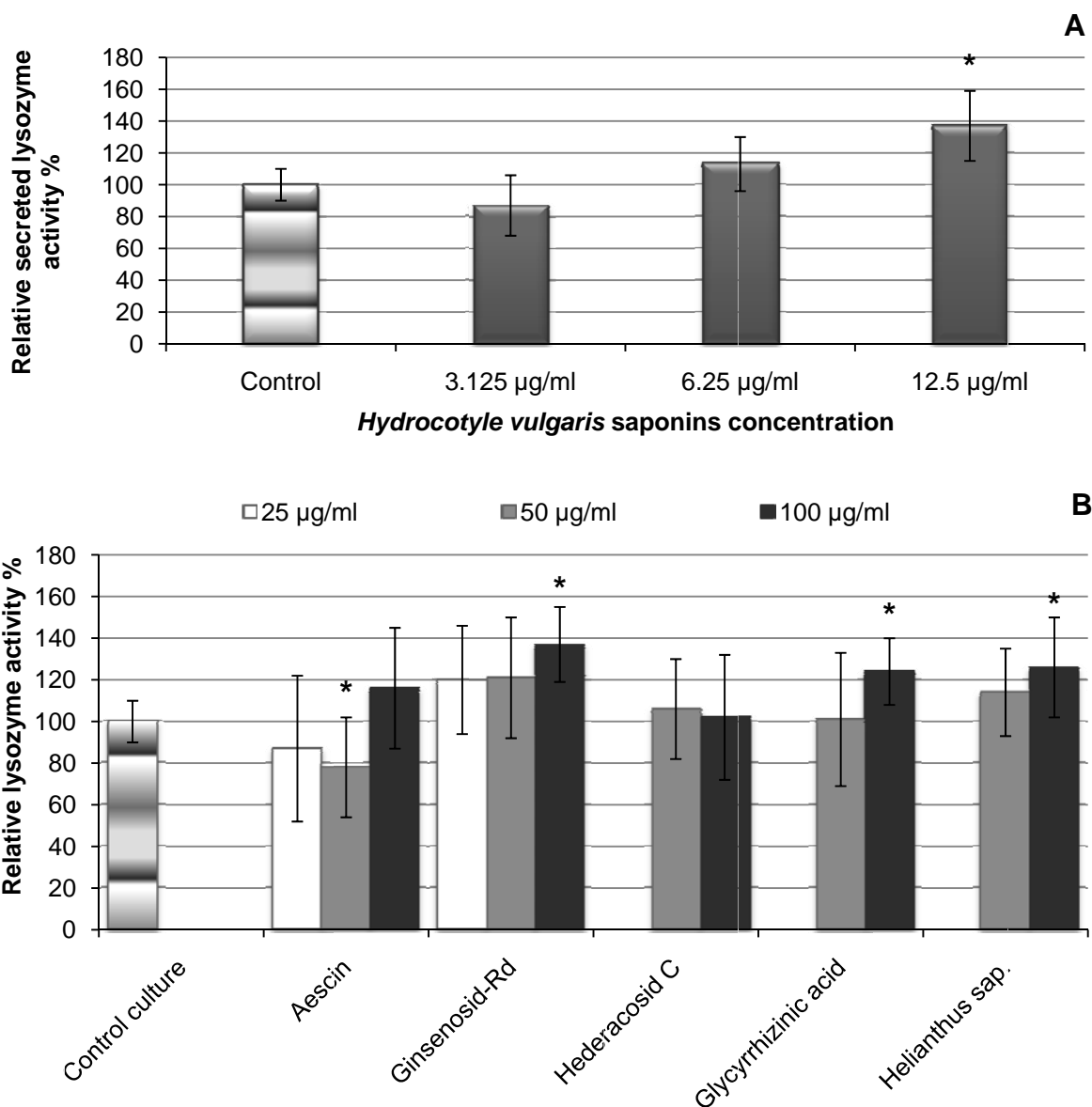
Ginsenosid-Rd, *Helianthus annuus* saponins and glycyrrhizinic acid at the non-toxic concentration of 100µg/ml resulted in significant increases of 37%, 26% and 24% respectively. Lower concentrations of 50µg/ml and 25µg/ml had no significant results on lysozyme secretion (Fig.54.B).

Hederacosid C was the only saponin with no significant effects on the release of lysozyme activity regardless of the saponin concentration (Fig.54.B).

The exceptional saponin tended to inhibit the release of lysozyme activity was aescin. However, this inhibitory effect was significant only at the non-toxic concentration of 50µg/ml (Fig.54.B).



**Fig.53.** Percent changes in the secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after one hour treatment with primulic acid (A), *Quillaja saponaria* saponins (B) or saponinum album (from *Gypsophila paniculata*) (C) at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



**Fig.54.** Percent changes in the secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after one hour treatment with *Hydrocotyle vulgaris* saponins (A) and with various saponins (B) at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.3.2.2. Synthesis and secretion of lysozyme activity in cell cultures after long-term treatment with saponins

Lysozyme activity levels in HT-29 cultures supplemented with different saponins were measured at various incubation time points of up to 72 hours (Table 20+21). Contrary to the tendency towards increased lysozyme secretion observed with the short-term treatment with saponins, epithelial cells incubated with saponins for 24 hours showed tendency to decrease

in the secretion and the expression of lysozyme activity in the examined cultures. However, this inhibitory effect was mostly associated with toxic effects of the saponins to the cells.

Primulic acid at concentrations higher than 12.5µg/ml as well as *Hydrocotyle vulgaris* saponins at concentrations higher than 6.25µg/ml were toxic to the epithelial cells with strong inhibition of lysozyme activity of the cells. These two saponins at the non-toxic concentration of 3.125µg/ml caused slight decreases of lysozyme activity after 24 hours treatment.

Furthermore, aescin and saponinum album at 50µg/ml exhibited toxic effects to the cells after 24 hours treatment, which led to significant decreases of total and released lysozyme activity. These two saponins at lower non-toxic concentrations resulted in slight decreases of lysozyme activity. *Quillaja saponaria* saponins at non-toxic concentration of 50µg/ml caused a significant decrease of 17% and 27% of both total and secreted lysozyme activity respectively after 24 hours treatment of the epithelial cells. Lower concentrations of this saponin had no effects on lysozyme activity.

Another group of the tested saponins showed no significant influences on the synthesis and release of lysozyme activity of the epithelial cells after 24 hours exposure regardless of the used non-toxic doses, including hederacosid C, Helianthus annuus saponins, ginsenosid-Rd and glycyrrhizinic acid (Table 21). Influence of longer incubation periods up to 72 hours on the expression of lysozyme of epithelial cells was investigated using glycyrrhizinic acid. As a result, no significant effect of the saponin was found after long exposure to the cells up to 72 hours regardless of the used non-toxic doses of the saponin (Table 20).

Glycyrrhizinic acid conc.	24 hour treatment		48 hours treatment		72 hours treatment	
	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
10 µg/ml	+ 0 % (±4)	- 2 % (±3)	+ 3 % (±3)	+ 2 % (±3)	+ 1 % (±3)	+ 1 % (±4)
50 µg/ml	- 5 % (±18)	- 1 % (±4)	+ 10 % (±16)	+ 1 % (±10)	+ 2 % (±11)	+ 1 % (±9)

**Table 20.** Percent change (to control) in the total and secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after incubation in culture medium supplemented with glycyrrhizinic acid using sensitive fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

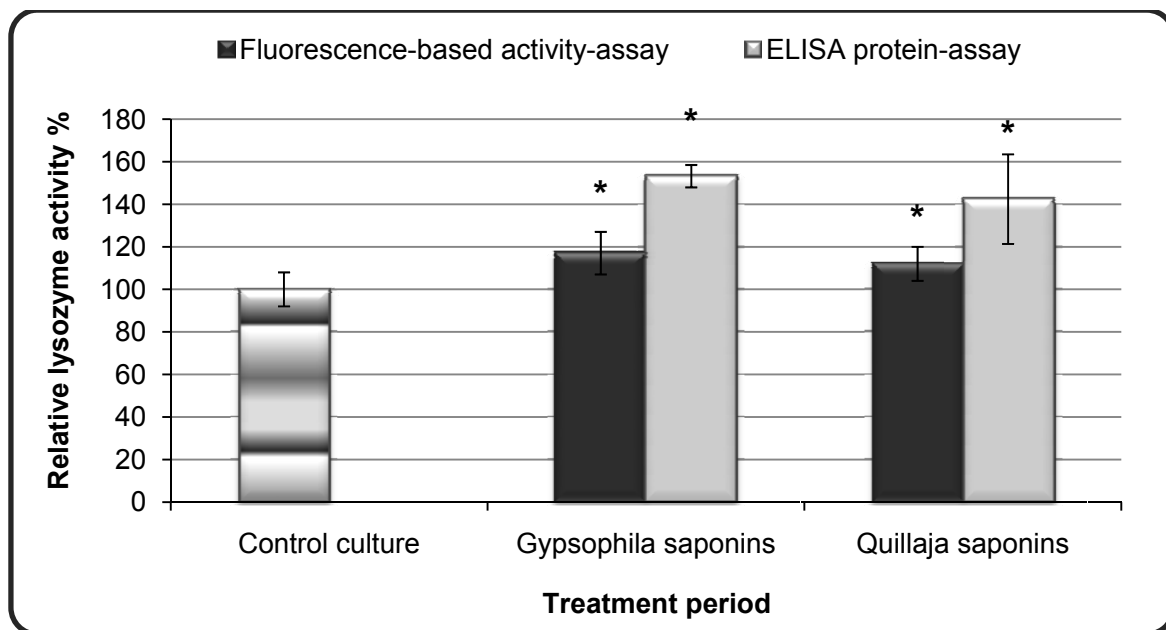
Examined saponin	Concentration	After 24h treatment	
		Secreted lysozyme activity %	Total lysozyme activity %
Primulic acid	3.125 µg/ml	- 7 ±5 %	+ 2 ±3 %
	12.5 µg/ml	- 16 ±3 %*	0 ±2 %
	25 µg/ml	- 71 ±2 %*	- 67 ±1 %*
<i>Hydrocotyle vulgaris</i> saponins	3.125 µg/ml	- 11 ±3 %*	- 5 ±4 %
	6.25 µg/ml	- 33 ±2 %*	- 25 ±3 %*
	12.5 µg/ml	- 80 ±3 %*	- 79 ±2 %*
Aescin	25 µg/ml	- 10 ±6 %*	- 4 ±5 %
	50 µg/ml	- 30 ±1%*	- 21 ±2 %*
Saponinum album	12.5 µg/ml	- 7 ±18 %	- 8 ±8 %
	50 µg/ml	- 59 ±1 %*	- 53 ±2 %*
<i>Quillaja saponaria</i> saponins	3.125 µg/ml	- 6 ±1 %	+ 1 ±3 %
	50 µg/ml	- 27 ±0.5 %*	- 17 ±2 %*
Hederacosid C	50 µg/ml	- 0 ±5 %	+ 6 ±4 %
	100 µg/ml	- 1 ±3 %	+ 5 ±3 %
<i>Helianthus annuus</i> saponins	50 µg/ml	- 4 ±5 %	+ 5 ±9 %
	100 µg/ml	- 2 ±7 %	+ 8 ±7 %
Ginsenosid-Rd	25 µg/ml	- 3 ±4 %	0 ±4 %

**Table 21.** Percent change (to control) in the total and secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after treatment with various saponins using sensitive fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of at least six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.3.2.3. Effects of selected saponins on the release of lysozyme measured using ELISA lysozyme protein assay compared to enzymatic activity assay

The stimulatory effects of saponins which enhanced the release of enzymatically active lysozyme levels in epithelial cell cultures were found to be associated with significantly stronger stimulation that enhanced the release of elevated lysozyme levels measured as a protein. HT-29 epithelial cells showed significant increase of the released lysozyme activity of about 17% upon exposure to 12.5µg/ml of saponinum album (from *Gypsophila paniculata*) for one hour, whereas an increase of lysozyme levels as protein of 53% was detected using ELISA lysozyme assay (Fig.55). Moreover, 12.5µg/ml of *Quillaja saponaria* saponins stimulated similarly significant increase of 12% in the release of lysozyme activity measured fluorometrically, while an increase of lysozyme levels as protein of 43% was detected using ELISA lysozyme assay (Fig.55).

Based on these results we have concluded that each of these saponins induced significant increase of about 50% in the levels of released lysozyme of epithelial cells after one hour treatment, of which only about 30% was enzymatically active lysozyme.

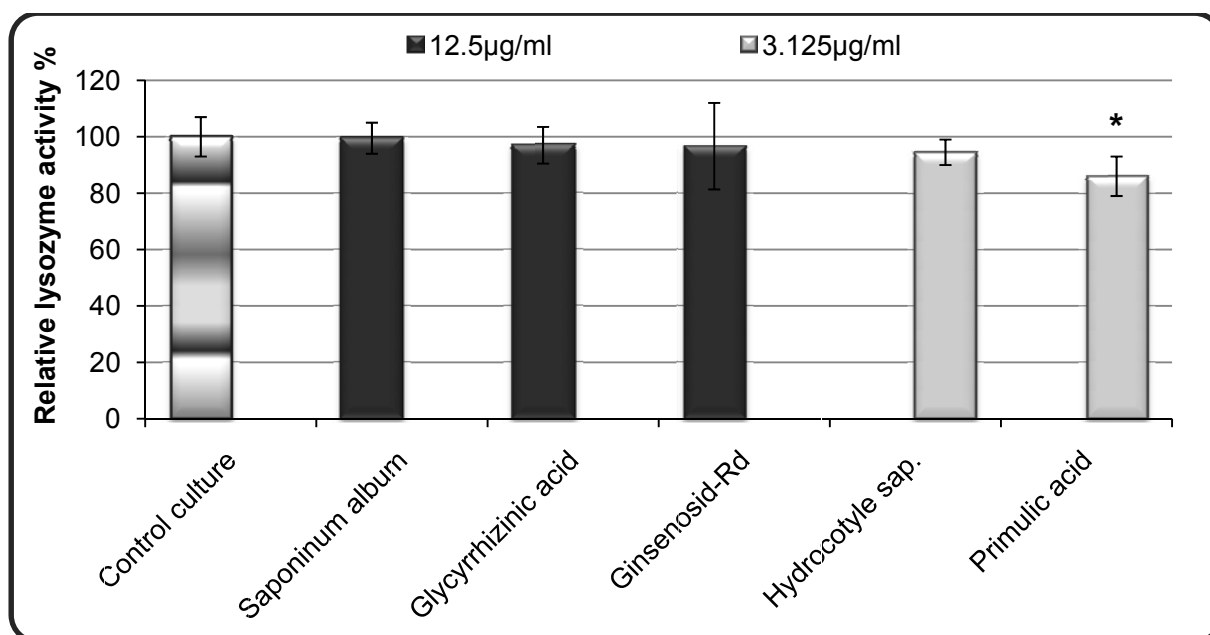


**Fig.55.** Percent changes in the secreted lysozyme activity of HT-29 epithelial cell cultures after one hour treatment with 12.5µg/ml of saponinum album (from *Gypsophila paniculata*) or *Quillaja saponaria* saponins using ELISA lysozyme protein assay compared to lysozyme activity assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.3.3. Human gastric epithelial cells AGS

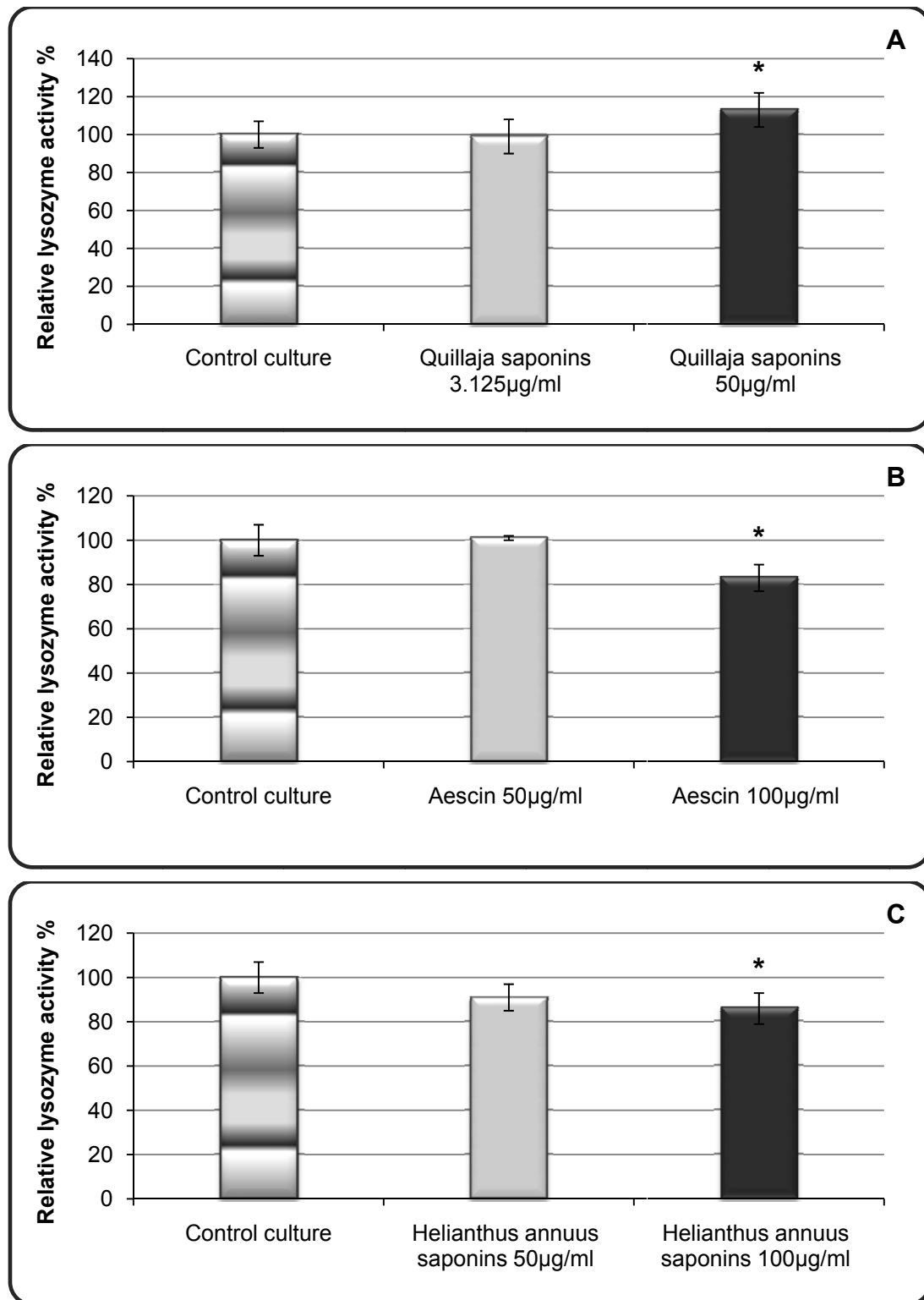
*Quillaja saponaria* saponins showed dose-dependent stimulatory effect on lysozyme activity secreted in AGS cultures. *Quillaja* saponins at 50µg/ml caused statistically significant increase of 13% in secreted lysozyme activity after one hour treatment, whereas no significant effect was observed with final concentration of 3.1µg/ml (Fig.57.A). Dose-dependent effect on lysozyme activity secreted in AGS cultures was also observed with aescin and *Helianthus annuus* saponins. 50 µg/ml final concentration of these two saponins had no significant effect, while 100 µg/ml led to significant decrease of about 15% in the secreted lysozyme activity after one hour treatment (Fig.57.B,C). Primulic acid at 3.125µg/ml exerted statistically significant inhibition of 14% on the secretion of lysozyme activity of the gastric cells after one hour incubation (Fig.56). It showed no significant differences in its inhibitory influence on lysozyme activity secreted in cell cultures when used at higher non-toxic concentrations up to 12.5µg/ml.

Glycyrrhizinic acid (12.5µg/ml), Ginsenosid-Rd (12.5µg/ml), saponinum album (12.5µg/ml) and *Hydrocotyle vulgaris* saponins (3.125µg/ml) showed no significant influences on lysozyme activity secreted in cell cultures after one hour treatment, with no significant differences when these oils were tested at higher end-concentrations (Fig.56).



**Fig.56.** Percent change in secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml AGS cell cultures after one hour treatment with various saponins using sensitive fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.





**Fig.57.** Percent change in secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml AGS cell cultures after one hour treatment with *Quillaja saponaria* saponins (A), aescin saponin (B) or *Helianthus annuus* saponins (C) at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without saponin addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.4. Essential oils

#### 3.3.7.4.1. Human monocytic cells THP-1

##### 3.3.7.4.1.1. Secretion of lysozyme activity in cell cultures after one hour incubation

Orange blossom oil caused significant increase of 35% in the secreted lysozyme activity at 0.5 $\mu$ L/ml (Fig.59.C). Furthermore, THP-1 cells incubated with thyme oil at 1 $\mu$ L/ml exhibited lysozyme activity release significantly higher than control culture of 85%, while concentrations between 0.025 and 0.5 $\mu$ L/ml led to significant decrease in the released lysozyme activity of 20% after the same incubation time (Fig.59.B).

Dose-dependent effect on the release of lysozyme activity was illustrated with *Salvia officinalis* oil. 0.5 $\mu$ L/ml of *Salvia* oil showed significant decrease of 22% in the released lysozyme activity, whereas 2 $\mu$ L/ml caused increase of 13% after 1 hour treatment. However, 2 $\mu$ L/ml of *Salvia* oil pronounced toxic effect on THP-1 cells at this treatment time (Fig.60.A).

*Nigella sativa* seed oil caused little but significant increase of 18% in the secreted lysozyme activity at a concentration of 1 $\mu$ L/ml. On the contrary, non-toxic concentration of 2 $\mu$ L/ml had a highly significant inhibitory effect of 40% on the release of lysozyme activity after one hour incubation with THP-1 cells (Fig.60.B).

Cinnamon oil, anise oil and lavender oil exhibited dose-dependent reverse inhibitory effects on the release of lysozyme activity after one hour treatment. Each of these oils caused significant inhibitory effect on the secreted lysozyme activity of THP-1 cells at a concentration of 1 $\mu$ L/ml, while this inhibitory effect was higher at 0.5 $\mu$ L/ml. The most significant inhibitory effect was observed with cinnamon oil, with a decrease of 50% in the secreted lysozyme activity at 0.5 $\mu$ L/ml, and of 35% at 1 $\mu$ L/ml (Fig.58).

A slight but significant inhibitory effect was also detected with clove oil at concentration equal to or less than 0.2 $\mu$ L/ml, whereas this effect was non-significant at 0.5 $\mu$ L/ml (Fig.59.A).

*Melissa officinalis* oil caused significant decrease of about 15% in the secreted lysozyme activity at the concentrations of 0.1 and 0.2 $\mu$ L/ml. Lower concentrations of this oil exerted no significant influence on lysozyme activity, while higher concentrations were toxic to the cells (Fig.60.C).

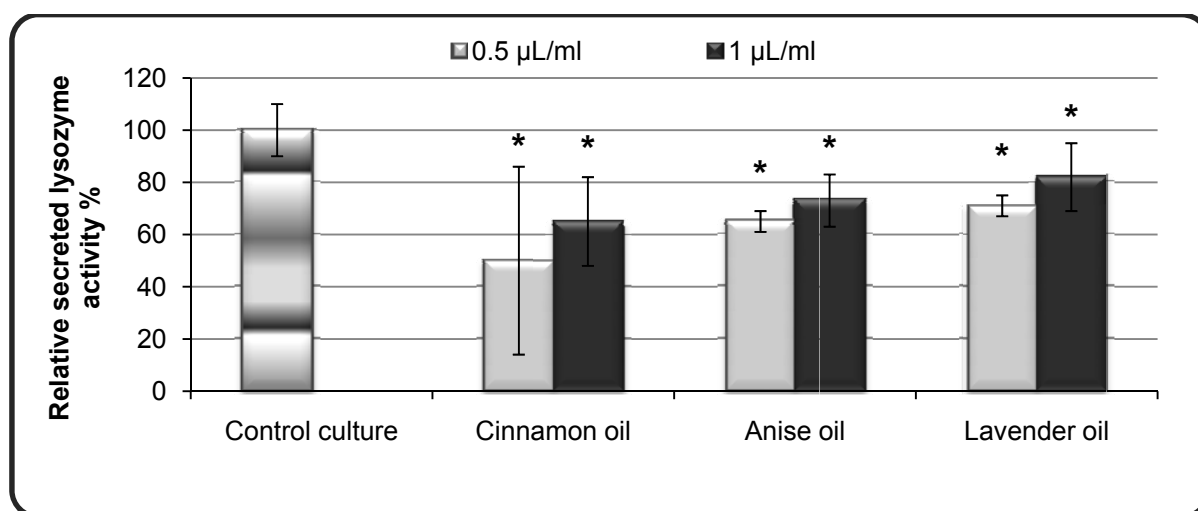
A significant decrease of the released lysozyme activity was observed with pine needle and chamomile oil at concentrations equal to or higher than 0.5 $\mu$ L/ml (Fig.61.A). These two oils were toxic to monocytes at the concentration of 1 $\mu$ L/ml after one hour treatment, however. Majoran, tea tree, eucalyptus and rosemary oil at 0.5 $\mu$ L/ml showed no influences on lysozyme activity secreted in cell cultures after one hour treatment, with no significant differences when these oils were tested at higher or lower end-concentrations (Fig.61.B).

### 3.3.7.4.1.2. Synthesis and secretion of lysozyme activity in cell cultures after long-term treatment with saponins

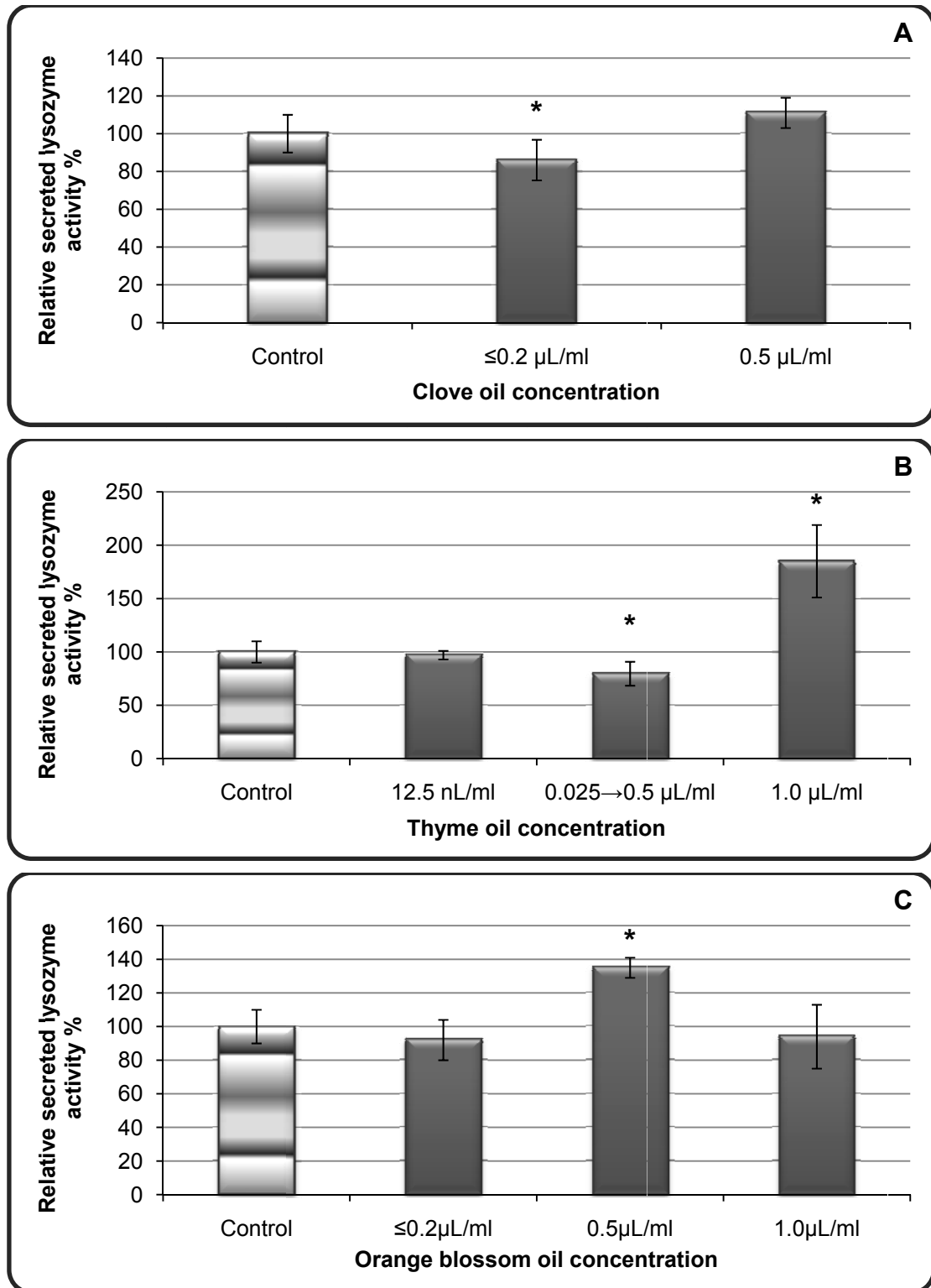
Lysozyme activity levels in THP-1 cultures supplemented with selected essential oils at different concentrations were measured after longer incubation period of 24 hours (Table 22). THP-1 cells incubated with essential oils for 24 hours exhibited no tendency to increase in the secretion and the expression of lysozyme activity in the examined cultures.

An exception was thyme oil at a concentration as low as 12.5nL/ml, which caused a significant increase of 18% in the total lysozyme activity synthesized by THP-1 cells after 24 hours incubation. Higher concentrations of 25 and 50nL/ml of thyme oil had no significant influence on lysozyme activity expression and release. A significant dose-dependent inhibitory effect started to occur at concentrations higher or equal to 0.1 $\mu$ L/ml of thyme oil.

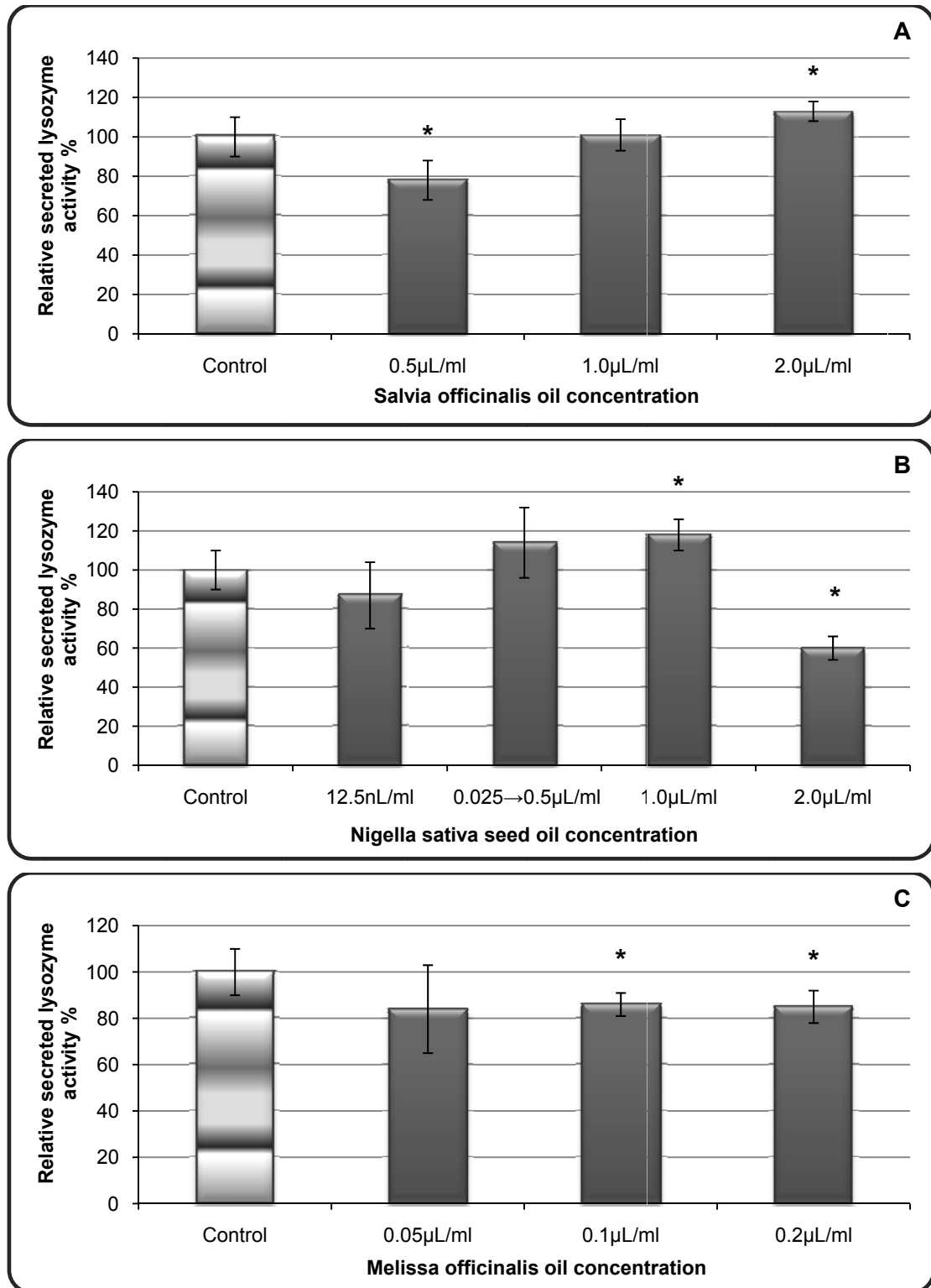
A point to be considered is that thyme oil at 1 $\mu$ L/ml showed toxicity to THP-1 cells after 24 hours treatment. Clove oil at 0.2 $\mu$ L/ml exerted also statistically significant inhibitory effect of 27% on the lysozyme secreted in the culture, whereas a concentration of 0.5 $\mu$ L/ml had significant inhibitory effects on both total and secreted lysozyme activity. Clove oil at concentration equal to or higher than 0.5 $\mu$ L/ml exhibited toxic effect on the cells after 24 hours incubation. A dose-dependent reverse effect, to some extent, was observed with *Nigella sativa* seed oil. Low concentrations of this oil ( $\leq$  50nL/ml) pronounced inhibitory effects on both total and released lysozyme activity, whereas a concentration as high as 1 $\mu$ L/ml had no significant effect. *Melissa officinalis* oil at 0.2 $\mu$ L/ml caused significant decrease of 15% in the lysozyme activity secreted in the culture. Lower concentrations of this oil were ineffective, while higher concentrations pronounced toxic effects on THP-1 cells.



**Fig.58.** Percent changes in the secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after one hour treatment with cinnamon oil, anise oil or lavender oil at two concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



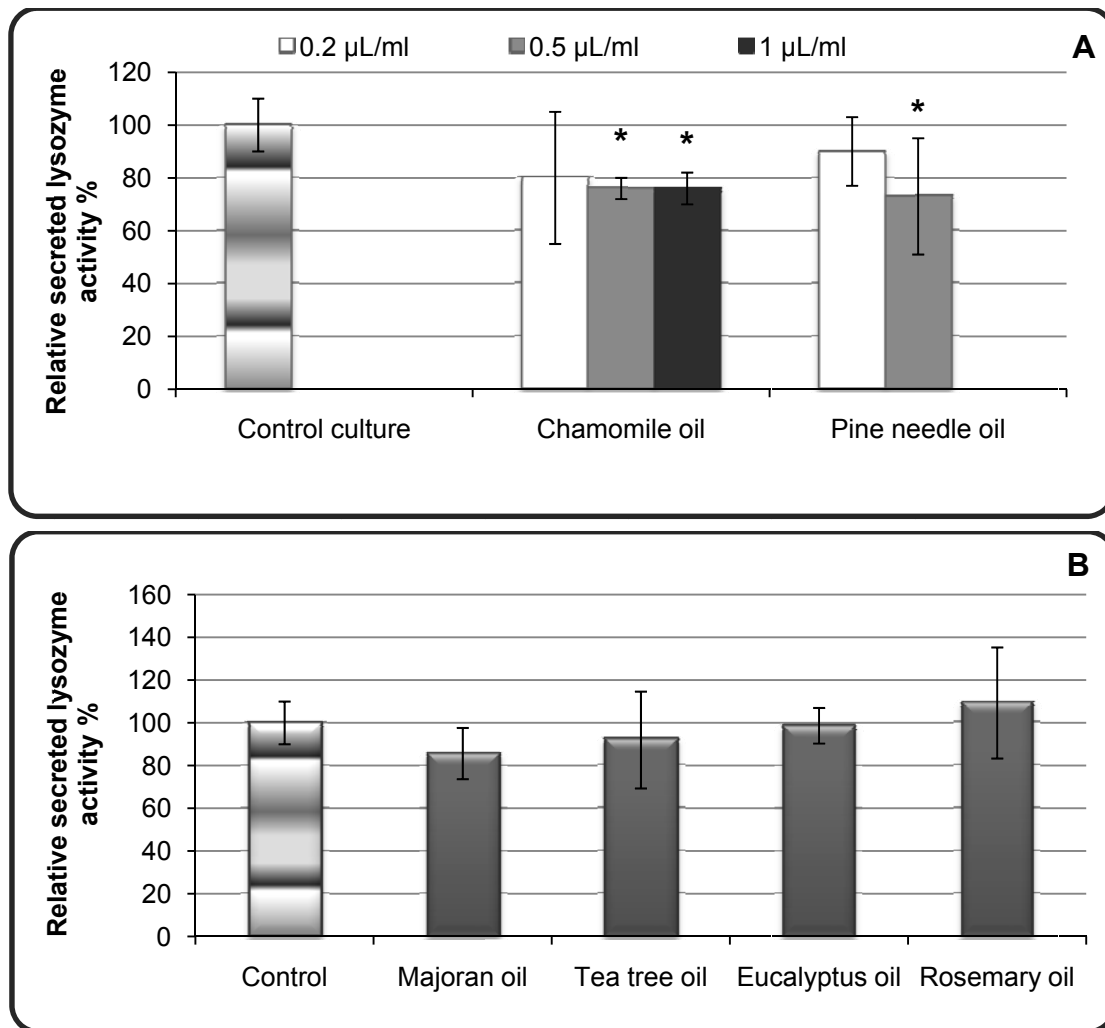
**Fig.59.** Percent changes in the secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after one hour treatment with clove oil (A), thyme oil (B) or orange blossom oil (C) at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



**Fig.60.** Percent changes in the secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after one hour treatment with *Salvia officinalis* oil (A), *Nigella sativa* seed oil (B) or *Melissa officinalis* oil (C) at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Examined essential oil	Concentration	After 24h treatment with the examined essential oil	
		Secreted lysozyme activity %	Total lysozyme activity %
Clove oil	0.5 $\mu\text{L/ml}$	- 51 $\pm$ 2 %*	- 33 $\pm$ 10 %*
	0.2 $\mu\text{L/ml}$	- 27 $\pm$ 18 %*	- 15 $\pm$ 15 %
	0.1 $\mu\text{L/ml}$	- 11 $\pm$ 11 %	- 5 $\pm$ 5 %
	50 nL/ml	0 $\pm$ 10 %	0 $\pm$ 10 %
	25 nL/ml	- 1 $\pm$ 6 %	- 9 $\pm$ 12 %
	12.5 nL/ml	+ 10 $\pm$ 10 %	- 1 $\pm$ 6 %
	Thyme oil	1 $\mu\text{L/ml}$	- 23 $\pm$ 1 %*
0.2 $\mu\text{L/ml}$		- 39 $\pm$ 3 %*	- 48 $\pm$ 6 %*
0.1 $\mu\text{L/ml}$		- 13 $\pm$ 5 %*	- 8 $\pm$ 8 %
50 nL/ml		0 $\pm$ 13 %	+ 6 $\pm$ 15 %
25 nL/ml		- 9 $\pm$ 5 %	+ 5 $\pm$ 5 %
12.5 nL/ml		- 2 $\pm$ 8 %	+19 $\pm$ 5 %*
<i>Nigella sativa</i> seed oil		1 $\mu\text{L/ml}$	+ 4 $\pm$ 1 %
	50 nL/ml	- 22 $\pm$ 6 %*	- 14 $\pm$ 13 %
	25 nL/ml	- 13 $\pm$ 6 %*	- 11 $\pm$ 4 %*
	12.5 nL/ml	- 21 $\pm$ 8 %*	+ 1 $\pm$ 2 %
<i>Melissa officinalis</i> oil	0.2 $\mu\text{L/ml}$	- 15 $\pm$ 2 %*	+ 2 $\pm$ 5 %
	0.1 $\mu\text{L/ml}$	- 4 $\pm$ 4 %	0 $\pm$ 4 %
	50 nL/ml	- 6 $\pm$ 7 %	- 4 $\pm$ 3 %

**Table 22.** Percent changes (to control) in the total and secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after treatment with various essential oils using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



**Fig.61.** Percent changes in the secreted lysozyme activity of  $1 \times 10^6$  cells/ml THP-1 cell cultures after one hour treatment with various essential oils using sensitive fluorescence-based lysozyme assay. Examined concentration for oils in (B) was  $0.5 \mu\text{L/ml}$ . Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.4.2. Human colon epithelial cells HT-29

#### 3.3.7.4.2.1. Secretion of lysozyme activity in cell cultures after one hour treatment

Stimulation of lysozyme activity release of epithelial cells was demonstrated with tea tree, majoran and anise oils. A significant increase of 24% of secreted lysozyme activity was observed with majoran oil at a concentration of  $1 \mu\text{L/ml}$ , with no significant effect at  $0.5 \mu\text{L/ml}$  (Fig.62.A). Compared to majoran oil, tea tree oil exerted opposite stimulatory effects regarding the dose, since it caused significant increase of 26 % at  $0.5 \mu\text{L/ml}$ , with no significant effect at  $1 \mu\text{L/ml}$  (Fig.62.A). Anise oil exhibited to some extent reverse dose-dependent induction of lysozyme activity secretion of epithelial cells. A slight but significant

increase of 14% was detected at 1 $\mu$ L/ml of anise oil, and of further 19% at 0.5 $\mu$ L/ml of the oil (Fig.62.B).

On the other hand, pine needle oil and cinnamon oil exhibited dose-dependent reverse effect on the release of lysozyme activity after one hour treatment. Each of these two oils caused significant inhibitory effects on the secreted lysozyme activity of epithelial cells at a concentration of 0.5 $\mu$ L/ml, while this inhibitory effect was non-significant at higher concentration of 1 $\mu$ L/ml. An inhibition of 21% was detected with 0.5 $\mu$ L/ml pine needle oil, and of 26% with 0.5 $\mu$ L/ml cinnamon oil (Fig.62.A). *Melissa officinalis* oil showed to the contrary direct dose-dependent inhibitory effect on the secreted lysozyme activity of HT-29 cells. We found a significant drop of 21% in the released lysozyme at 1 $\mu$ L/ml of *Melissa* oil, whereas lower concentration of 0.5 $\mu$ L/ml had no significant effect (Fig.62.A).

Another group of selected essential oils showed no significant influences on the release of lysozyme activity of the epithelial cells after one hour exposure regardless of the used doses, including orange blossom, lavender, eucalyptus, rosemary, *Nigella sativa* seed, clove, *Salvia officinalis*, thyme and chamomile oils (Fig.62.B+C).

A point for consideration that all tested concentrations of above mentioned essential oils were non-toxic after one hour treatment of the HT-29 epithelial cells.

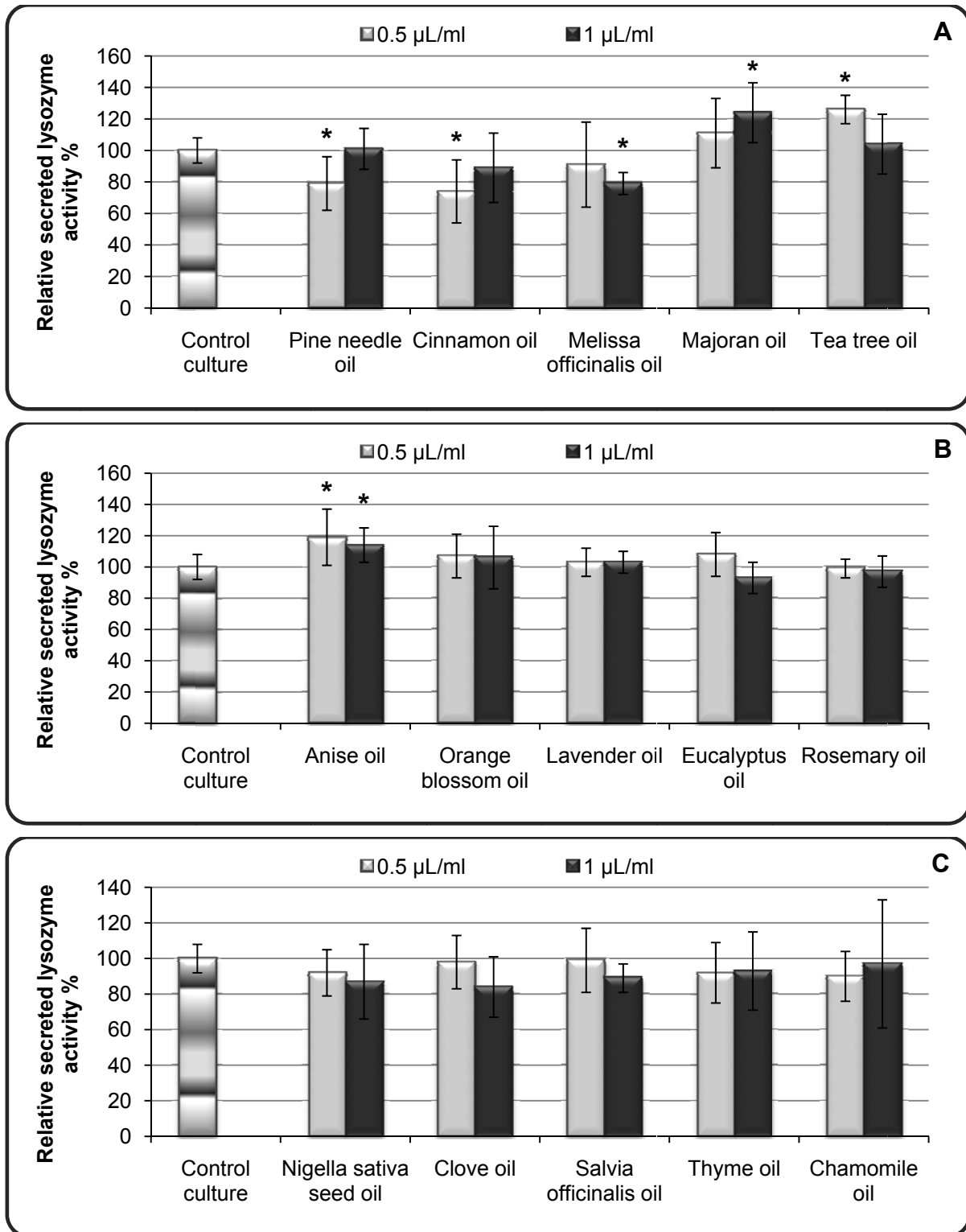
#### **3.3.7.4.2.2. Synthesis and secretion of lysozyme activity in cell cultures after long-term treatment with saponins**

Lysozyme activity levels in HT-29 cultures supplemented with selected essential oils at different concentrations were measured after longer incubation period of 24 hours (Table 23+24). Chamomile, clove, cinnamon and *Melissa officinalis* oils caused strong inhibition of the secreted and total lysozyme, with significant toxic effects to the cells at both concentrations of 0.5 $\mu$ L/ml and 1 $\mu$ L/ml (Table 23).

Thyme, pine needle and majoran oils were toxic to the epithelial cells at 1 $\mu$ L/ml with strong inhibition of lysozyme activity of the cells. At the non-toxic concentration of 0.5 $\mu$ L/ml pine needle and majoran oils had no significant effects on both secreted and total lysozyme, whereas thyme oil caused slight but significant decrease of 10% of the total lysozyme activity expressed by the cells (Table 23). Tea tree and anise oils inhibited slightly but significantly the cell expression of lysozyme activity at a non-toxic concentration of 1 $\mu$ L/ml, while 0.5 $\mu$ L/ml had no significant effect (Table 24).

Another group of selected essential oils showed no significant influences on the synthesis and release of lysozyme activity of the epithelial cells after 24 hours exposure regardless of the used non-toxic doses, including rosemary, lavender, orange blossom, eucalyptus, *Salvia officinalis* and *Nigella sativa* seed oils (Table 24).





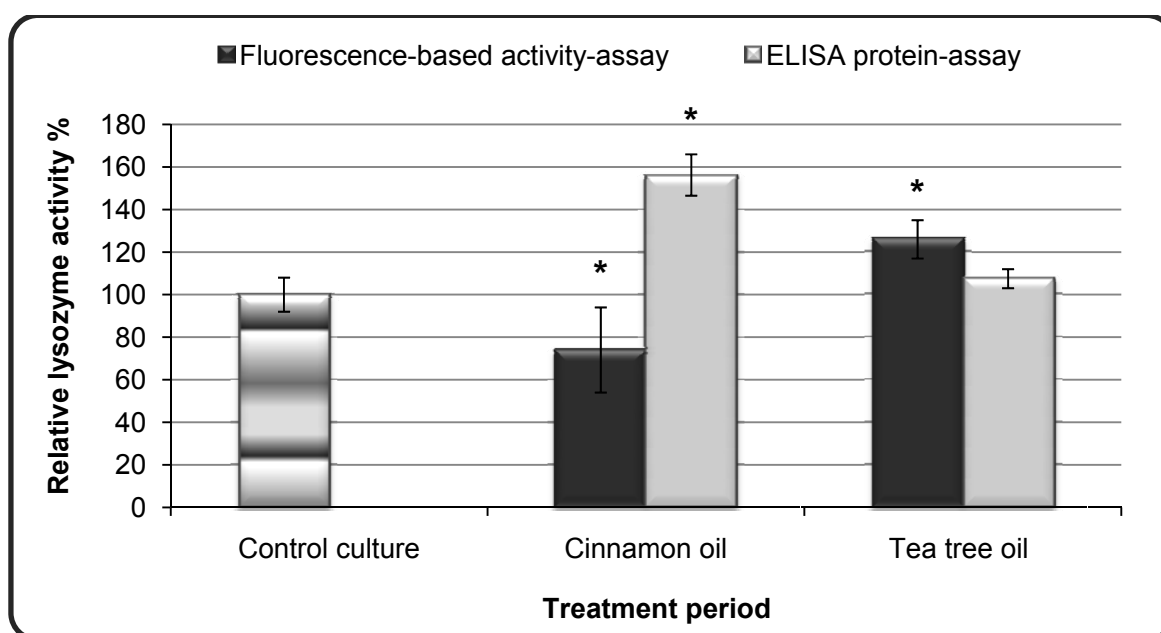
**Fig.62.** Percent changes in the secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after one hour treatment with various essential oils at two concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.3.7.4.2.3. Effects of selected essential oils on lysozyme release of epithelial cells measured using ELISA lysozyme protein assay compared to enzymatic activity assay

The inhibitory effects of cinnamon oil which caused significant drop of 26% in the released enzymatically active lysozyme levels of epithelial cells were found to be associated with unexpected significant stimulation that induced an increase of 56% in the levels of secreted lysozyme in the cultures measured as a protein using ELISA (Fig.63).

Based on these findings we have deduced that cinnamon oil induced significant increase in the levels of released lysozyme after one hour treatment of the epithelial cells, while it was able simultaneously to cause significant inhibition of the enzymatic activity of lysozyme secreted in the cell cultures.

On the other hand, no statistically significant differences were found between the stimulatory effects of tea tree oil on the levels of secreted lysozyme measured as a protein and as an active enzyme after one hour incubation with HT-29 epithelial cells (Fig.63).



**Fig.63.** Percent changes in the secreted lysozyme activity of HT-29 epithelial cell cultures after one hour treatment with 0.5 $\mu$ L/ml of cinnamon oil or tea tree oil using ELISA lysozyme protein assay compared to lysozyme activity assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Examined essential oil	Concentration	After 24h treatment with the examined essential oil	
		Secreted lysozyme activity %	Total lysozyme activity %
Chamomile oil	1 $\mu$ L/ml	- 76 $\pm$ 6 %*	- 68 $\pm$ 12 %*
	0.5 $\mu$ L/ml	- 67 $\pm$ 22 %*	- 53 $\pm$ 29 %*
Clove oil	1 $\mu$ L/ml	- 82 $\pm$ 2 %*	- 72 $\pm$ 8 %*
	0.5 $\mu$ L/ml	- 59 $\pm$ 26 %*	- 33 $\pm$ 18 %*
<i>Melissa officinalis</i> oil	1 $\mu$ L/ml	- 92 $\pm$ 3 %*	- 82 $\pm$ 4 %*
	0.5 $\mu$ L/ml	- 84 $\pm$ 6 %*	- 75 $\pm$ 7 %*
Cinnamon oil	1 $\mu$ L/ml	- 92 $\pm$ 1 %*	- 80 $\pm$ 3 %*
	0.5 $\mu$ L/ml	- 98 $\pm$ 2 %*	- 86 $\pm$ 3 %*
Thyme oil	1 $\mu$ L/ml	- 52 $\pm$ 41 %*	- 44 $\pm$ 37 %*
	0.5 $\mu$ L/ml	- 31 $\pm$ 28 %	- 10 $\pm$ 6 %*
Pine needle oil	1 $\mu$ L/ml	- 83 $\pm$ 7 %*	- 77 $\pm$ 6 %*
	0.5 $\mu$ L/ml	- 3 $\pm$ 6 %	- 29 $\pm$ 40 %
Majoran oil	1 $\mu$ L/ml	- 40 $\pm$ 33 %*	- 39 $\pm$ 23 %*
	0.5 $\mu$ L/ml	- 9 $\pm$ 7 %	- 7 $\pm$ 6 %

**Table 23.** Percent changes (to control) in the total and secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after treatment with various essential oils using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of at least six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Examined essential oil	Concentration	After 24h treatment with the examined essential oil	
		Secreted lysozyme activity %	Total lysozyme activity %
Tea tree oil	1 $\mu$ L/ml	- 11 $\pm$ 6 %	- 11 $\pm$ 3 %*
	0.5 $\mu$ L/ml	- 10 $\pm$ 6 %	- 2 $\pm$ 6 %
Anise oil	1 $\mu$ L/ml	- 5 $\pm$ 6 %	- 9 $\pm$ 4 %*
	0.5 $\mu$ L/ml	- 3 $\pm$ 4 %	- 1 $\pm$ 4 %
Rosemary oil	1 $\mu$ L/ml	- 5 $\pm$ 1 %	- 1 $\pm$ 8 %
	0.5 $\mu$ L/ml	+ 1 $\pm$ 3 %	- 10 $\pm$ 3 %
Lavender oil	1 $\mu$ L/ml	- 9 $\pm$ 1 %	- 10 $\pm$ 5 %
	0.5 $\mu$ L/ml	+ 3 $\pm$ 6 %	- 9 $\pm$ 3 %
Orange blossom oil	1 $\mu$ L/ml	- 9 $\pm$ 2 %	- 7 $\pm$ 2 %
	0.5 $\mu$ L/ml	0 $\pm$ 2 %	- 7 $\pm$ 1 %
Eucalyptus oil	1 $\mu$ L/ml	- 8 $\pm$ 4 %	- 9 $\pm$ 5 %
	0.5 $\mu$ L/ml	- 10 $\pm$ 2 %	- 4 $\pm$ 6 %
<i>Salvia officinalis</i> oil	1 $\mu$ L/ml	- 4 $\pm$ 3 %	- 3 $\pm$ 5 %
	0.5 $\mu$ L/ml	+ 2 $\pm$ 1 %	- 10 $\pm$ 3 %
<i>Nigella sativa</i> seed oil	1 $\mu$ L/ml	+ 1 $\pm$ 3 %	- 5 $\pm$ 3 %
	0.5 $\mu$ L/ml	- 7 $\pm$ 15 %	+ 1 $\pm$ 10 %

**Table 24.** Percent changes (to control) in the total and secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after treatment with various essential oils using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of at least six separate experiments ( $\pm$ SD). Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

#### 3.3.7.4.3. Human gastric epithelial cells AGS

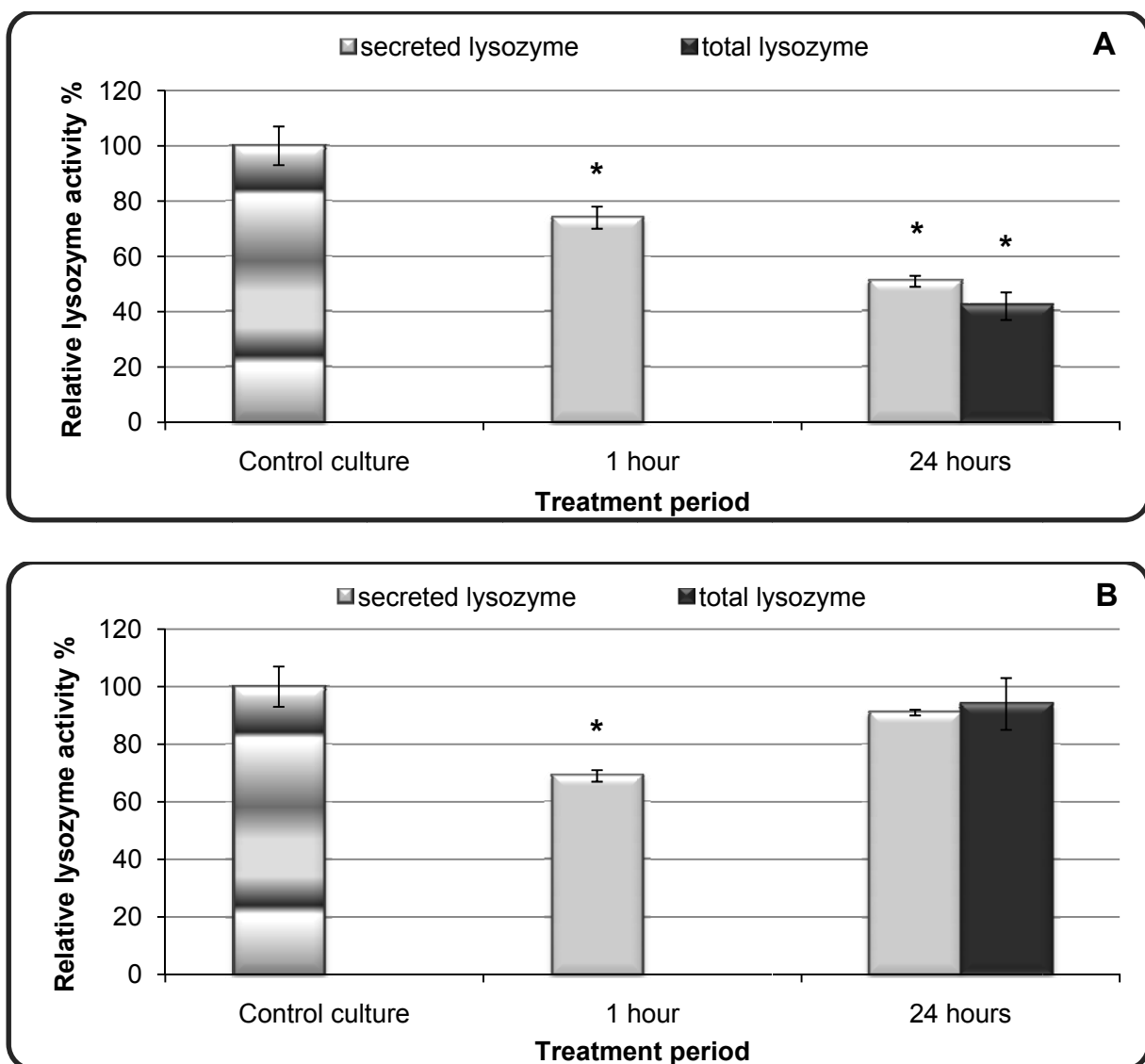
*Melissa officinalis* oil at final concentration of 0.1  $\mu$ L/ml showed statistically significant inhibitory effect on lysozyme activity in AGS cell cultures. This inhibitory effect was found proportional to the treatment period. One hour treatment with *Melissa* oil caused 26% inhibition in lysozyme activity secretion in cell cultures, whereas 24 hours treatment caused 49% inhibition of lysozyme activity secretion and 58% suppression of lysozyme activity production (Fig.64.A).

Thyme oil at final concentration of 0.1  $\mu$ L/ml exhibited inhibitory effect on lysozyme activity in AGS cell cultures as well. Inhibition of lysozyme activity secretion by 31% was statistically

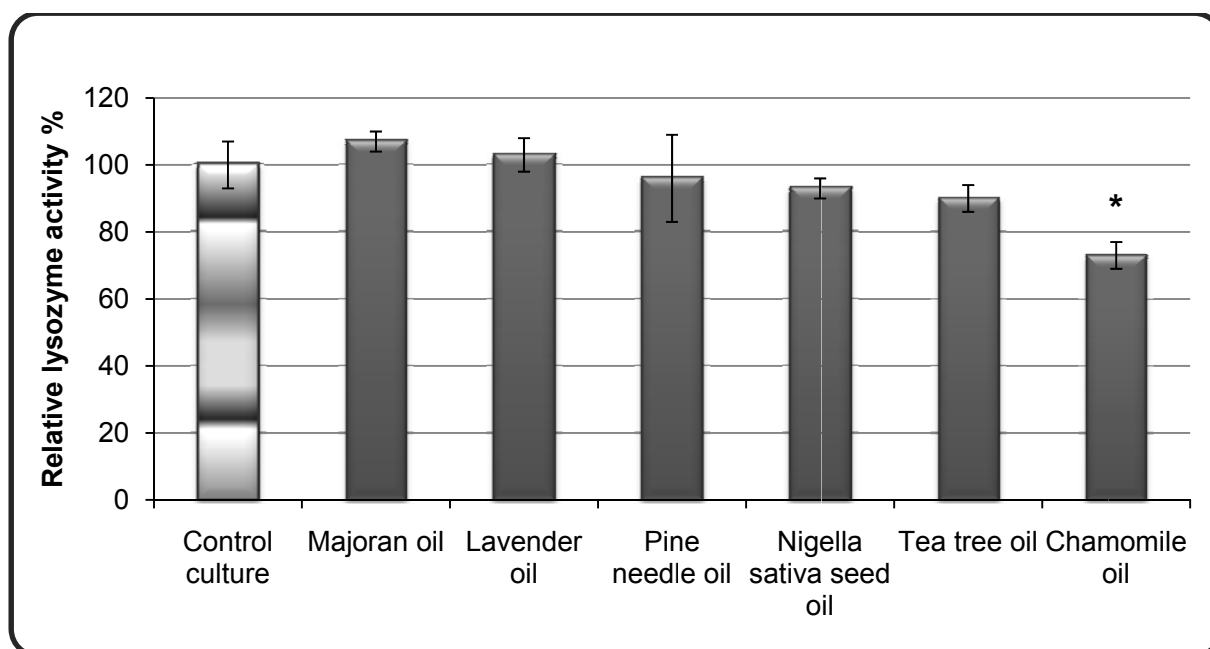
significant after one hour treatment with this oil, while 24 hours treatment caused slight non-significant suppression of both secreted and total lysozyme (Fig.64.B).

Furthermore, a statistically significant influence was observed with chamomile oil at a final concentration of 0.5 $\mu$ l/ml, which suppressed lysozyme activity secretion of 27% after one hour treatment of AGS cells (Fig.65).

Another group of essential oils showed no significant influences on the release of lysozyme activity of AGS cells, including majoran, lavender, pine needle, tea tree and Nigella sativa seed oil (Fig.65).



**Fig.64.** Percent decrease in total and secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml AGS cell cultures after 1 hour and after 24 hours treatment with 0.1 $\mu$ l/ml *Melissa officinalis* oil (A) or thyme oil (B) using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.



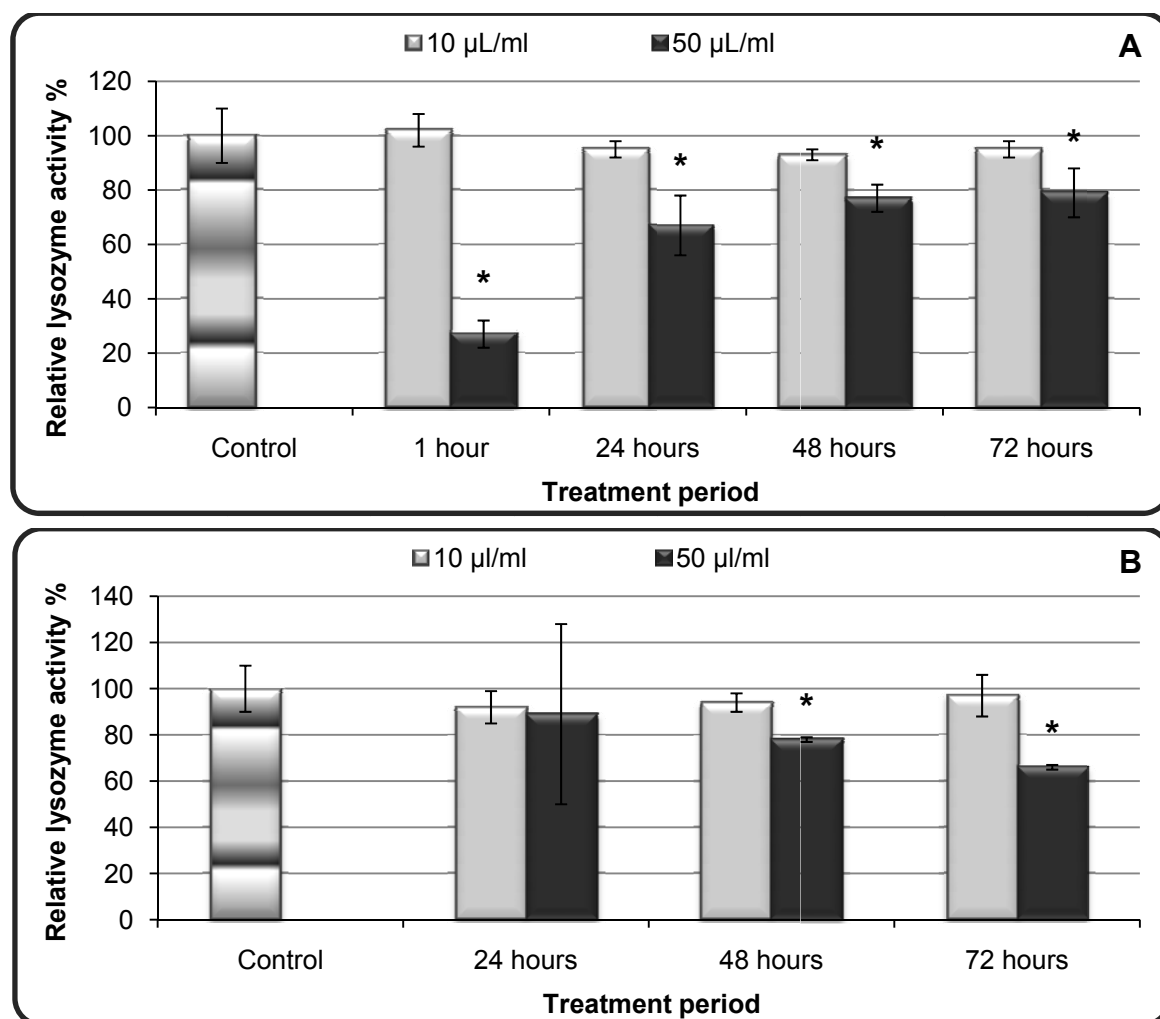
**Fig.65.** Percent change in secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml AGS cell cultures after one hour treatment with  $0.5 \mu\text{l/ml}$  end-concentration of various essential oils using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

#### 3.3.7.4.4. Effects of the essential oil of *Lippia dulcis* on lysozyme activity of monocytes and epithelial cells

THP-1 cell cultures supplemented with the essential oil at a concentration of  $50 \mu\text{L/ml}$  showed time-dependent reverse inhibitory effect on the release of lysozyme activity in the culture medium. This inhibitory effect was statistically significant at the various treatment periods of up to 72 hours. The most significant inhibition of 73% on the secreted lysozyme activity was observed after one hour treatment with the essential oil (Fig.66.A). The essential oil of *Lippia dulcis* at concentration of  $50 \mu\text{L/ml}$  had time-dependent inhibitory effects on the total lysozyme expressed by THP-1 cells as well. It caused a significant inhibition of 22% on lysozyme activity synthesized by cells within 48 hours, and of 34% within 72 hours (Fig.66.B). Lower concentration of the essential oil of  $10 \mu\text{L/ml}$  pronounced no significant effects either on lysozyme activity release or on lysozyme expression of THP-1 cells.

On the other hand, HT-29 cell cultures incubated with the essential oil at different concentrations for periods up to 72 hours demonstrated no changes in the synthesis and secretion of lysozyme activity in comparison to the control cultures (Table 25).

The essential oil of *Lippia dulcis* showed no toxic effects on monocytes or epithelial cells at the tested concentrations even after 72 hours incubation.



**Fig.66.** Percent changes in the secreted lysozyme activity (A) and in the total lysozyme activity (B) synthesized by  $1 \times 10^6$  cells/ml THP-1 cell cultures treated with *Lippia dulcis* essential oil at two concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without oil addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

Essential oil conc.	1 hour treatment	24 hours treatment		48 hours treatment		72 hours treatment	
	Secreted lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme	Secreted lysozyme	Total lysozyme
10 µg/ml	0 ± 12 %	- 3 ± 5 %	- 6 ± 7 %	- 4 ± 6 %	- 3 ± 2 %	- 3 ± 6 %	+ 1 ± 7 %
50 µg/ml	- 1 ± 10 %	- 3 ± 3 %	- 4 ± 6 %	- 3 ± 3 %	- 3 ± 2 %	- 2 ± 3 %	+ 2 ± 7 %

**Table 25.** Percent changes (to control) in the total and secreted lysozyme activity of  $0.5 \times 10^6$  cells/ml HT-29 cell cultures after treatment with *Lippia dulcis* essential oil. Control cultures without oil addition were considered to have 100% lysozyme activity. Points were measured in triplicate. Values are representative of average value of six separate experiments (±SD).

### **3.3.8. Effects of propolis**

Effects of propolis on the release of lysozyme activity of monocytes and epithelial cells were investigated. THP-1 monocytes showed enhanced lysozyme activity release upon exposure to propolis for one hour. This stimulatory effect of propolis had to some extent reverse dose-dependent relationship, since the most significant increase of 64% in the secreted lysozyme activity of monocytes was detected at the low concentration of 1µg/ml, while less but still significant increases were observed at higher concentrations of German propolis up to 100µg/ml (Fig.67.A).

Compared to German propolis, a similar significant stimulatory effect was found with propolis from Syrian origin, which caused a significant increase of 51% in the secreted lysozyme activity of monocytes at the low concentration of 1µg/ml (Fig.67.B).

On the other hand, propolis from both origins exerted no significant effects when incubated with HT-29 epithelial cells for one hour, regardless of the concentration used (Fig.67.A+B).

Regarding toxicity to cells, propolis pronounced no toxic effects on either of examined two cell lines at all used concentrations.

## **3.4. Establishing lysozyme activity assay for human whole saliva and tears using the microplate turbidimetric assay**

### **3.4.1. Optimization of salivary samples dilution for lysozyme assay**

Series of saliva dilutions were done to determine the optimal dilution.

Saliva sample was diluted 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280 and 1:2560 with the phosphate buffer solution.

Lysozyme activity in the saliva samples with each of the previous dilutions was assayed turbidimetrically.

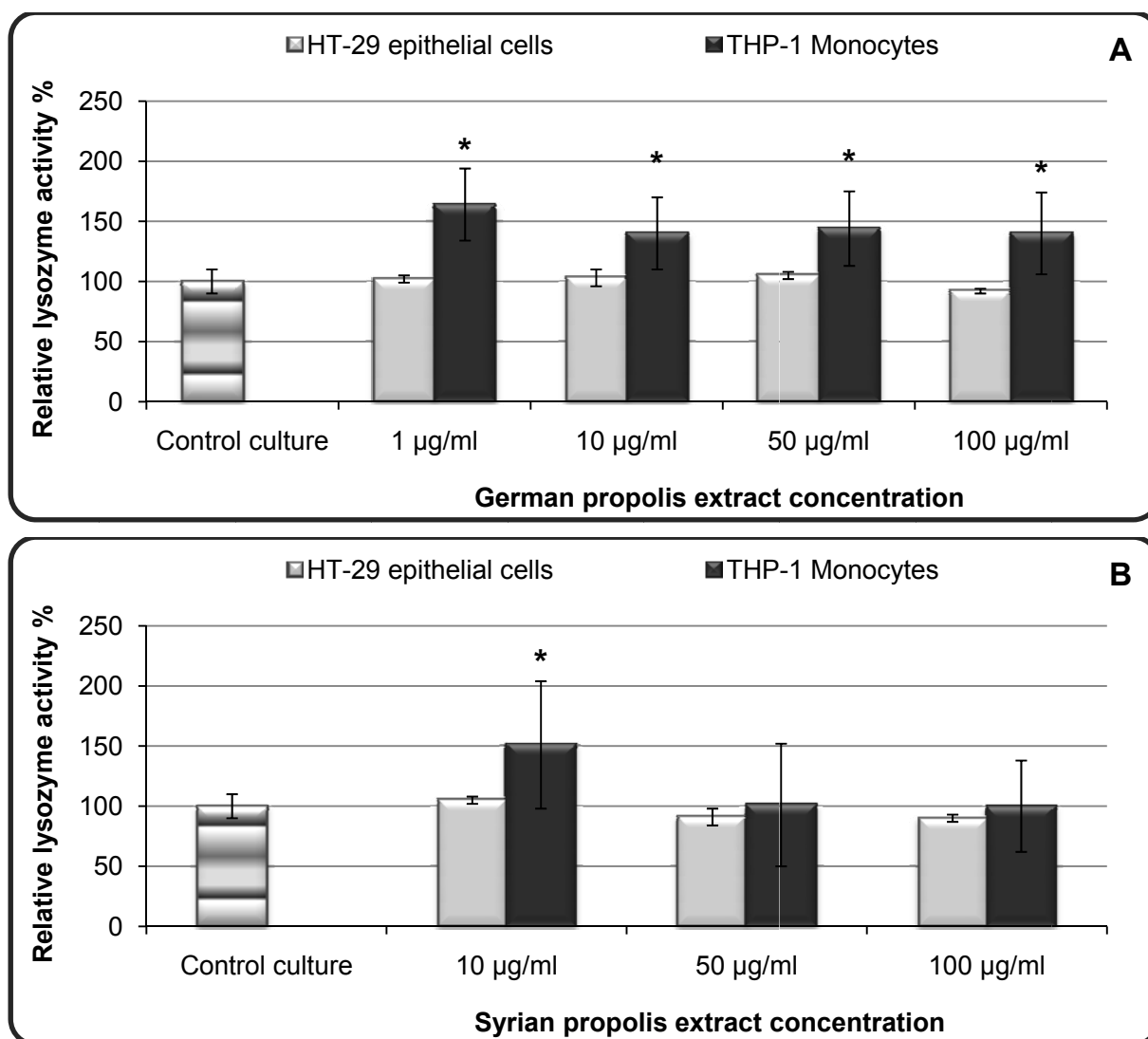
The slope values of the curves started to be proportional to lysozyme concentration at dilutions higher than 1:320. With the dilutions of 1:80 and 1:160, lysozyme activity was too high which exceeded the linear range of the turbidity assay. With the dilutions of 1:1280 and lower, lysozyme activity was too low and started to be out of the linear range of the assay (Table 26).

Comparing correlation coefficient of the curves of previous dilutions, the best correlation coefficient value was achieved with the sample diluted 1:640.

Thus, we have chosen a dilution of 1:500 as optimal final dilution for the saliva samples.

In few samples, where low lysozyme levels were detected, additional dilution of 1:400 was necessary.





**Fig.67.** Percent changes in the secreted lysozyme activity of THP-1 monocytic cell cultures and of HT-29 epithelial cell cultures after one hour treatment with propolis extract from German origin (A) or with propolis extract from Syrian origin (B) at different concentrations using sensitive fluorescence-based lysozyme assay. Control cultures without propolis addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the columns are representative of average value of at least six separate experiments. Values with asterisk are significantly different ( $P < 0.05$ , Mann-Whitney U test) from values of control cultures.

### 3.4.2. Optimization of tear samples dilution for lysozyme assay

Series of tear sample dilutions were done to determine the optimal dilution.

Tear sample was first diluted 1:10, 1:20, 1:40, 1:80 and 1:160 with the phosphate buffer solution. Lysozyme activity assayed in the tear sample with each of the previous dilutions was still too high to be measured turbidimetrically.

Hence, further dilutions of 1:320, 1:640, 1:1280, 1:2560, 1:5120, 1:10240, and 1:20480 were made to improve the linearity of the assay.

The slope value of the assay curve started to be proportional to lysozyme concentration at dilutions higher than 1:5120.

Regarding correlation coefficient of the curves of previous dilutions, the best correlation coefficient value of  $R^2 = 0.9987$  was achieved with the sample diluted 1:10240.

Thus, we chose the dilution of 1:10240 as an optimal final dilution for tear samples.

Salivary Sample	Dilution	Regression equation	correlation coefficient
<b>Sample 1</b>	1:10	$y = 0,0025x + 0,5001$	$R^2 = 0,7757$
	1:20	$y = 0,0034x + 0,4834$	$R^2 = 0,7665$
	1:40	$y = 0,0119x + 0,4194$	$R^2 = 0,87$
	1:80	$y = 0,018x + 0,3263$	$R^2 = 0,9501$
	1:160	$y = 0,0214x + 0,2456$	$R^2 = 0,9676$
	1:320	$y = 0,0186x + 0,0863$	$R^2 = 0,9787$
	1:640	$y = 0,0082x + 0,0217$	$R^2 = 0,997$
	1:1280	$y = 0,002x + 0,0055$	$R^2 = 0,9696$
<b>Sample 2</b>	1:2560	$y = 0,0001x + 0,0075$	$R^2 = 0,1006$
	1:80	$y = 0,0265x + 0,2529$	$R^2 = 0,9484$
	1:160	$y = 0,0264x + 0,1422$	$R^2 = 0,9693$
	1:320	$y = 0,0209x + 0,0627$	$R^2 = 0,9697$
<b>Sample 3</b>	1:640	$y = 0,0129x + 0,0107$	$R^2 = 0,9982$
	1:80	$y = 0,0212x + 0,1994$	$R^2 = 0,975$
	1:160	$y = 0,0194x + 0,1116$	$R^2 = 0,9775$
	1:320	$y = 0,017x + 0,028$	$R^2 = 0,9912$
	1:640	$y = 0,0085x + 0,005$	$R^2 = 0,9994$

**Table 26.** Regression equations and correlation coefficients of the turbidimetric assay curves of lysozyme activity in different human salivary samples using serial dilutions. All points for each regression equation were measured in triplicate. Salivary samples are of three healthy non-medicated non-smoking women aged 27-30 years.

### **3.4.3. Effects of storage time & temperature after salivary collection on lysozyme activity**

The influence of storage of the collected salivary samples on lysozyme activity in regard to time and temperature was investigated. Our data demonstrated decrease in lysozyme activity of human saliva with the time course regardless of storage temperature of the collected salivary, as shown in Fig.68 and Fig.69. Therefore, it was preferable to measure lysozyme activity of saliva immediately after collection.

However, if the direct measurement of lysozyme activity is not possible, changes in lysozyme activity showed in the Fig.68 and Fig.69 should be considered, depending on storage time and temperature of the collected salivary samples before lysozyme activity assay.

If the storage time does not exceed 24 hours, it is preferable to store the collected salivary samples in the refrigerator at 2-4°C. Nevertheless, lysozyme activity in salivary samples decreased about 7% after 2 hours, 30% after 4 hours, 35% after 5 hours and about 50% after 24 hours storage at 2-4°C, as illustrated in Fig.69. The highest rate of lysozyme activity decrease was observed when the collected salivary samples were stored at room temperature in comparison to refrigerator and room temperature storage. Effect of long term storage (more than 24 hours) of salivary samples on lysozyme activity was not studied.

In our work, lysozyme activity levels of saliva samples were measured directly after collection.

### **3.4.4. Sex-related changes in lysozyme activity levels of human saliva**

There was no clear correlation found between saliva lysozyme activity levels and sex in the 20-30 years age group (Fig.70).

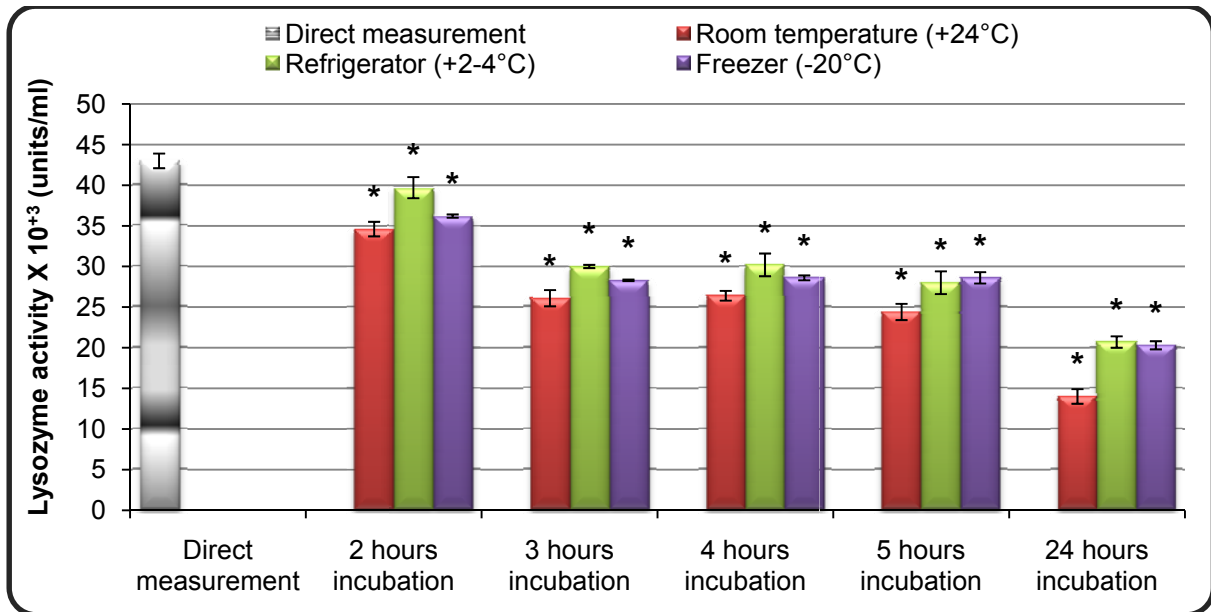
On the other hand, females between 31 and 50 years old showed higher saliva lysozyme activity up to 36417 units/ml (950 µg/ml) in comparison to that of men of the same age group, which had lysozyme activity less than 5067 units/ml (132 µg/ml).

Moreover, males over 50 years old had relatively low saliva lysozyme activity down to 7000 units/ml (183 µg/ml) (Table 27).

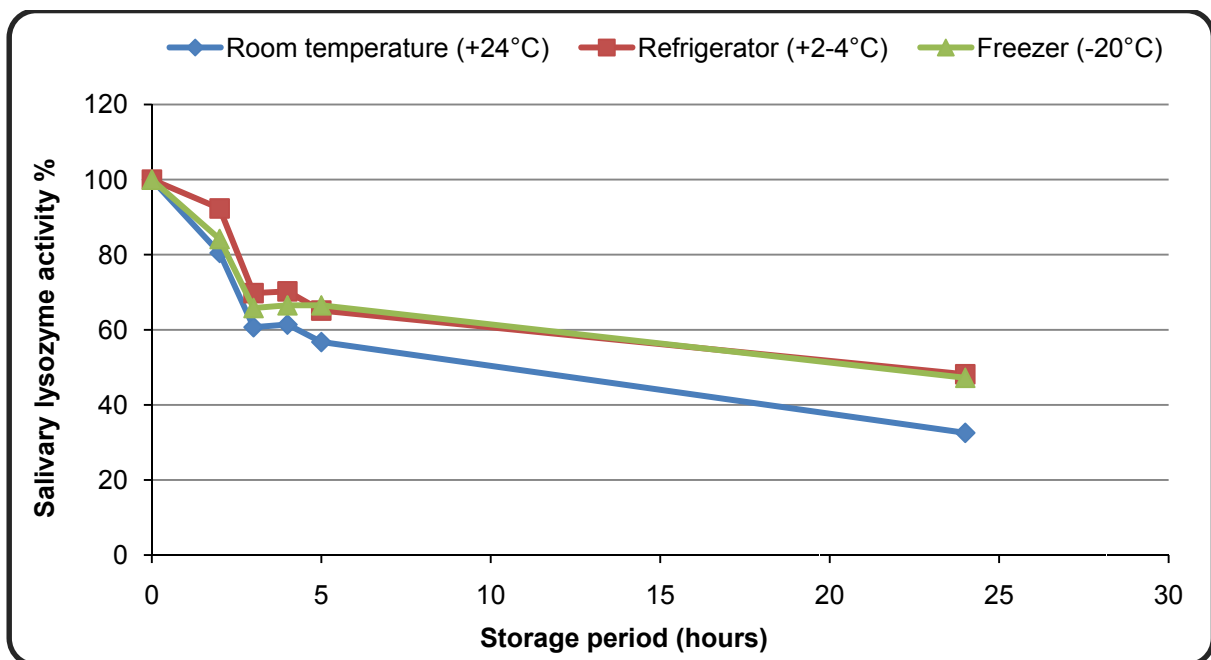
### **3.4.5. Age-related changes in lysozyme activity levels of human saliva**

Negative correlation between saliva lysozyme activity levels and age with some exceptions could be suggested (Fig.70). Out of 24 healthy volunteers (males and females) in the 20-30 years age group, 17 showed saliva lysozyme activity in the range of 20333 – 43000 units/ml (530 – 1122 µg/ml); whereas only 7 volunteers showed activity lower than 16833 units/ml (439 µg/ml). Saliva lysozyme activity in the volunteers between 31 and 50 years old was in the range of 3667 – 36417 units/ml (96 – 950 µg/ml).

Healthy volunteers older than 50 years didn't show saliva lysozyme activity more than 7000 units/ml (183 µg/ml) (Table 27).



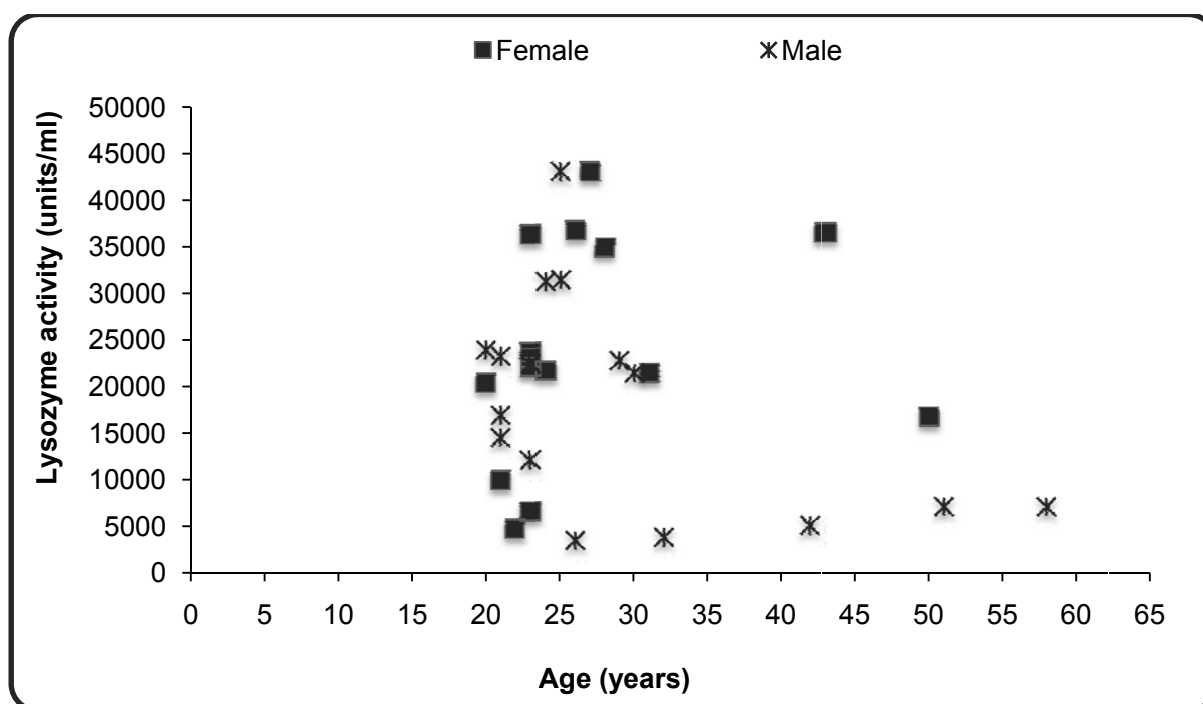
**Fig.68.** Lysozyme activity in units per ml of salivary sample measured turbidimetrically. Assay was done immediately after collection and at different time points during storage at different temperatures. All points were measured in triplicate. Lysozyme values with asterisk are significantly different (P<0.05, Mann-Whitney U test) from lysozyme value of the direct measurement.



**Fig.69.** Percent decrease in lysozyme activity of salivary sample during storage at different temperatures. Salivary sample assayed immediately after collection was considered to have 100% lysozyme activity. All points were measured in triplicate.

Sex	Age group ( years )	No. per group	Lysozyme ( units/ml )
Males	20-30	8	21333 - 43000
		3	12000 - 16833
	1	3333	
	31-50	2	3667 - 5067
	51-58	2	7000
Females	20-30	9	20333 - 43000
	31-50	3	4667 - 9867
		3	16667 - 36417

**Table 27.** Saliva lysozyme activity levels in 31 healthy non-medicated volunteers. Lysozyme activity levels of the salivary samples were measured turbidimetrically and expressed in units per ml. Assay was done immediately after saliva collection. All points were measured in triplicates.



**Fig.70.** Comparison of saliva lysozyme activity levels in males and females. Lysozyme activity of the salivary samples was measured turbidimetrically and expressed in units per ml. Assay was done immediately after saliva collection. All points were measured in triplicate. The figure is representative of 31 healthy non-medicated volunteers.

### 3.4.6. Lysozyme activity levels of human tears

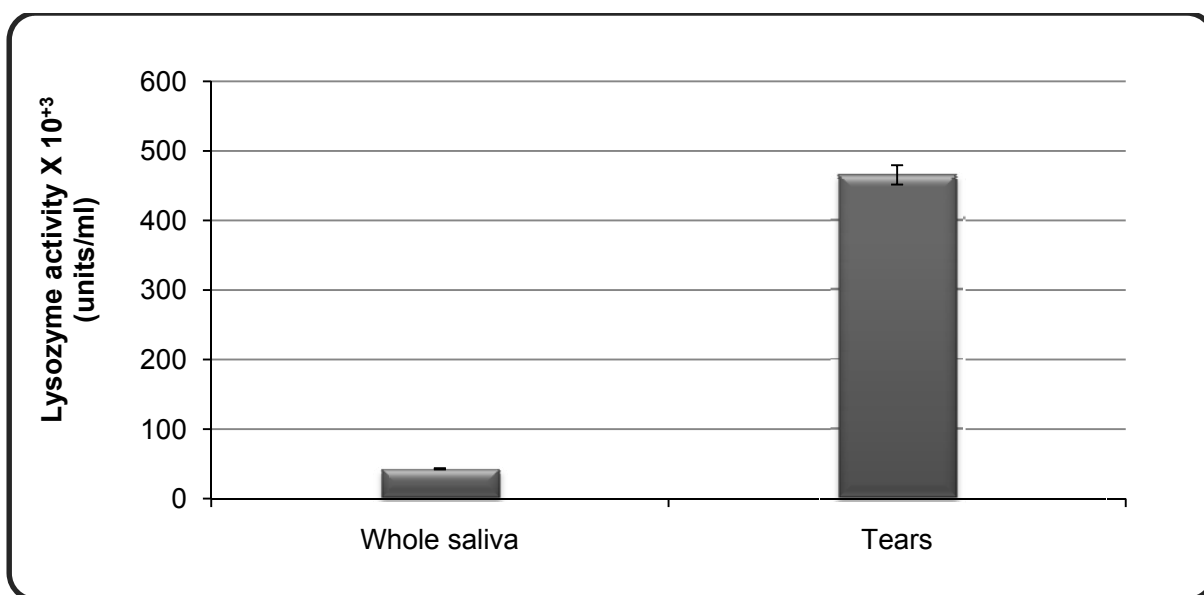
Using a final dilution of 1:10240 of the tear sample, lysozyme activity levels were determined with the turbidimetric assay.

The slope values of the kinetic curves were used to determine the lysozyme concentration in comparison with the standard using the regression equation of the standard.

As a result, lysozyme activity in human tears was determined to be  $465556 \pm 13878$  units/ml (or  $12145 \pm 362$   $\mu\text{g/ml}$ ) (Fig.71).

Lysozyme activity in human whole saliva of the same volunteer was  $43000 \pm 882$  units/ml (or  $1113 \pm 25$   $\mu\text{g/ml}$ ). As illustrated in Fig.71, lysozyme activity of human tears was about 11 times higher than that of whole saliva of the same volunteer using the same assay method.

The high levels of lysozyme activity in human tears indicate the importance of this enzyme as a natural defense molecule against ocular infections due to the antimicrobial and antiviral properties of this enzyme.



**Fig.71.** Comparison of lysozyme activity levels in human tears and saliva of a healthy non-medicated 27 years old female. Lysozyme activity was measured turbidimetrically and expressed in units per ml. Values were measured in triplicate.

## 4. DISCUSSION

### 4.1. Lysozyme determination methods

In the present work, we described two simple, rapid and versatile assay methods of lysozyme activity: the classical turbidimetric microplate assay and the more recent EnzChek<sup>®</sup> fluorescence-based assay. The calibration of the lysozyme standards into units of activity under our used conditions was essential for an accurate comparison between these two assay methods.

The EnzChek<sup>®</sup> fluorescence-based lysozyme activity assay was proved to be good in regard to sensitivity and linearity, basing on the catalytic effect of lysozyme to labelled *Micrococcus lysodeikticus* cells and the increase in fluorescence that is proportional to lysozyme activity. This method had detection limit of lysozyme concentration down to 4units/ml (Fig.11). However, this detection limit of lysozyme activity is equivalent to 0.47 calibrated units/ml, considering that calibrated unit of lysozyme activity is defined as decreasing a 0.001 absorption value at 450nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* suspension as substrate, under the conditions of pH 6.24 and 37°C, in a 250µl reaction mixture using 96-well microtiter plate (Fig.14). On the other hand, the turbidimetric microplate lysozyme assay was proved to have good linearity but less sensitivity in comparison with the EnzChek<sup>®</sup> fluorescence-based assay. It had detection limit down to 12.5units/ml (Fig.5). This detection limit of lysozyme activity is equivalent to 0.8 calibrated units/ml, taking into consideration the definition of calibrated unit of lysozyme activity mentioned above (Fig.10). Nevertheless, this detection limit of the turbidimetric microplate lysozyme assay has been enough to provide a simple, accurate and inexpensive method to determine lysozyme levels in tissue or body fluids such as saliva and tears, which contain high lysozyme levels, whereas the EnzChek<sup>®</sup> fluorescence-based assay can provide a high-sensitivity method for detecting lysozyme activity at low levels e.g. in cell culture systems.

In order to measure lysozyme as a protein, we investigated the enzyme-linked immunosorbent assay (ELISA) of human lysozyme. Expressing lysozyme as nanograms of enzyme per millilitre, lysozyme standard curve using ELISA was linear in the range of 0.156-10ng/ml (Fig.15). This immunologically based assay is not dependent on the expression of biological activity to determine the concentration of lysozyme. Because the level of active lysozyme may be more relevant for elucidating a defensive role for this protein, an assay method that can determine the active lysozyme in body fluids and cell culture supernatants, such as the turbidimetric and fluorometric assay used mainly in our investigations, would provide more meaningful results. However, human lysozyme ELISA was beneficial in our study to investigate the possible change in the influence of some substances examined in this work on lysozyme levels of the human cell lines upon using various determination

methods which measure lysozyme as an active enzyme (fluorometric assay) or as a protein (ELISA).

#### **4.2. Lysozyme production in monocytic cell lines**

As a part of their surveillance functions in the immune system, the major cell types responsible for the production of lysozyme seem to be the monocytes/macrophages and neutrophilic granulocytes. Lysozyme has been shown to be present in macrophages in high concentrations, in monocytes and in the lysosomes of granulocytes (Bandlow & Kiihne, 1980). We investigated three monocytic cell lines, THP-1, HL-60 and U-937 regarding their ability to produce lysozyme activity in cell cultures.

THP-1 is a human cell line, established from the peripheral blood of a 1-year-old boy with acute monocytic leukaemia. This cell line has distinct monocytic markers. During culture, THP-1 cells maintain these monocytic characteristics for over 14 months (Tsuchiya *et al.*, 1980). The cells can be used for induction of differentiation studies. PMA, for example, has been reported to induce the differentiation of THP-1 cells into macrophage-like cells which mimic native monocyte derived macrophages in several respects (Auwerx, 1991; Ries *et al.*, 1998; Tsuchiya *et al.*, 1980). Furthermore, THP-1 cells have been described to produce lysozyme and to be phagocytic (Tsuchiya *et al.*, 1980). Compared to other human myeloid cell lines, such as HL-60, U-937, KG-1, or HEL cell lines, THP-1 cells are more mature, and the differentiated cells behave more like native monocyte-derived macrophages. Because of these characteristics, THP-1 cell line provides a valuable model for studying the mechanisms involved in macrophage differentiation and lysozyme secretion (Auwerx, 1991; Morgan & Singer, 1998).

HL-60 is a human cell line established from the peripheral blood of a 35-year-old woman with acute myeloid leukemia, and consists predominantly of promyelocytes. However, these promyelocytic cells can be induced to terminally differentiate to morphologically mature granulocytes by incubation with a wide variety of compounds, such as angelmicin B (Yokoyama *et al.*, 1996), DMSO (dimethyl sulfoxide) (Breitman *et al.*, 1980) and phorbol esters (PMA) (Brock *et al.*, 2003; Huberman *et al.*, 1981; Solanki *et al.*, 1981). Moreover, these induced HL-60 cells have many of the functional characteristics of normal peripheral blood granulocytes, including phagocytosis, complement receptors, chemotaxis, and the ability to reduce nitroblue tetrazolium (NBT) (Breitman *et al.*, 1980). HL-60 cells have been reported to produce lysozyme, and this production and release could be induced by several differentiation compounds like angelmicin B (Yokoyama *et al.*, 1996) and PMA (Huberman *et al.*, 1981; Solanki *et al.*, 1981).

Both human acute monocytic leukemia cell lines THP-1 and HL-60 were proved in our experiments to produce and release lysozyme activity in detectable amounts (Fig.17; Fig.18).



Moreover, the synthesis and the release of lysozyme activity into the culture medium increased with the time course.

U-937 is a human monocyte-like cell line established from human histiocytic lymphoma. It has monoblastic and immature monocytic characteristics and can be induced to differentiate into monocyte/macrophage-like cells with various agents such as interferon- $\gamma$  (IFN- $\gamma$ ), and phorbol myristate acetate (PMA) (Tsukamoto *et al.*, 1992). Differentiated U-937 cells adhere to a plastic or glass substrate coated or not with matrix components, i.e. vitronectin, fibrinogen, fibronectin and collagen. The mature differentiated U-937 cells acquire all the peculiar features of macrophage-like cells: irregular shape, a large number of pseudopodia on their surface and an intense phagocytic activity (Pagliata *et al.*, 2005). It has been reported that human promonocytic U-937 cells can produce lysozyme. It has been also found that the production and release of lysozyme in U-937 can be activated by several agents such as PMA and interferon- $\gamma$  (IFN- $\gamma$ ) (Balboa *et al.*, 2003; Harris *et al.*, 1985; Lemansky & Hasilik, 2000; Radons *et al.*, 1994; Takashi *et al.*, 2006).

Contrary to the previous reports, we couldn't find any evidence that human leukemic monocyte lymphoma cell line U-937 was able to produce and secrete detectable levels of active lysozyme, neither with nor without addition of inducing factors such as PMA, taking into account that the used U-937 was fresh and low-passage cell line derived from the cell bank. In addition, this cell line was previously tested in our laboratory to have the typical characteristics of monocytes, such as differentiation and phagocytic activity (Weng *et al.*, 2008; Weng, 2009). Another important point for consideration is that ELISA was the most used method to determine lysozyme in the previous reports of U-937, which measures the protein but not the actual enzymatic activity of lysozyme. Another method used in these previous studies was the turbidimetric assay, which has been recently proved to lack sensitivity in comparison with the highly sensitive fluorescence-based lysozyme assay utilized in this paper (Helal & Melzig, 2008).

### **4.3. Lysozyme production in epithelial cell lines and primary tumor cells**

#### **4.3.1. Human epithelial cell lines of the digestive system**

At mucosal surfaces, lysozyme expression is confined to specialized epithelial cells, including the serous gland cell of the respiratory epithelium and the Paneth's cell of the gastrointestinal epithelium. Mechanisms responsible for cell-specific expression are poorly understood (Kai *et al.*, 1999). We investigated two epithelial cell lines derived from the gastrointestinal tract, HT-29 and AGS regarding their ability to produce lysozyme activity in the cell cultures.

HT-29 is a human colon adenocarcinoma cell line established from the primary tumor of a 44-year-old Caucasian woman with colon adenocarcinoma. Ultrastructural features reported for HT-29 cells include microvilli, microfilaments, large vacuolated mitochondria with dark

granules, smooth and rough endoplasmic reticulum with free ribosomes, lipid droplets, few primary and many secondary lysosomes. The cells express urokinase receptors, but do not have detectable plasminogen activator activity. These intestinal cells have been reported to synthesize and secrete lysozyme *in vitro* into the culture medium (Clarke *et al.*, 1996; Alderman *et al.*, 1985; Fett *et al.*, 1986).

AGS is a human gastric adenocarcinoma cell line derived from fragments of a tumor resected from a 54-year-old Caucasian woman who had received no prior therapy (Barranco *et al.*, 1983a,b). To our knowledge, no reports have been published which study the ability of AGS cells to produce lysozyme in the cell cultures.

Our results illustrated that both human colon adenocarcinoma cell line HT-29 and gastric adenocarcinoma cell line AGS were able to produce and secrete lysozyme activity in cell cultures at levels markedly higher than those of the cultivated monocytic cells (Fig.19; Fig.20). Detectable amounts of lysozyme activity could be determined intracellularly as well as extracellularly in the cell cultures of HT-29 and AGS cells. We found also increases in the levels of lysozyme activity with the time course.

Based on the present results, these two gastrointestinal cell lines provide convenient, reliable and physiologically relevant culture systems for studies regarding expression and release of lysozyme activity in the human epithelial cells.

#### **4.3.2. Human lung epithelial cell lines**

Among the antimicrobial peptides, lysozyme is considered as one of the most important host defense molecules in epithelial cells. Lysozyme is highly enriched in mucosal surfaces, airway epithelia, lungs and airway surface fluid (Kai *et al.*, 1999; Suico *et al.*, 2009). Smith *et al.* (1996) showed that inhibition of cationic antimicrobial proteins such as lysozyme predisposed the airway epithelium to infection. On the basis of these reports, we investigated whether human lung epithelial cell lines, specifically A549 and BEAS-2B, were capable of secreting active lysozyme in the culture medium of these cells.

A-549 is a human lung carcinoma cell line established from an explanted lung tumor which was removed from a 58-year-old Caucasian man in 1972; cells were described to induce tumors in athymic mice and to synthesize lecithin (Giard *et al.*, 1973; Lieber *et al.*, 1976).

The human lung carcinoma cell line A549 was mentioned in few reports to secrete lysozyme activity *in vitro* into the culture medium (Suico *et al.*, 2009). On the contrary, conflicting studies reported that A549 cell line continued synthesizing a number of molecules such as MUC2, but not lysozyme (Finkbeiner *et al.*, 1994). Some studies indicated that myeloid elf-1-like factor (MEF), which was suggested to play a role in regulating innate immunity (Hedvat *et al.*, 2004), is required for the activation of lysozyme transcription in the human non-small cell lung carcinoma cell line A549. They analyzed the expression of MEF mRNA in human

cancer cell lines and non-cancer cell lines. MEF expression was found to be undetectable or low in A549 cancer cell line compared with non-cancer cell line. A549 tumors were proved to be poorly differentiated, whereas high-level expression of lysozyme was seen exclusively after A549 epithelial cells differentiation through differentiation-inducing factors that induce the expression of myeloid elf-1-like factor (MEF) (Kai *et al.*, 1999; Seki *et al.*, 2002).

Detectable amounts of lysozyme activity in A549 cell cultures, which is a marker of gland serous cell differentiation, were reportedly found upon exposure to differentiation-inducing factors that can activate MEF expression in these cells (Suico *et al.*, 2009).

Based on our results combined with these reports we concluded that lysozyme expression and secretion in A549 cell line is undetectable, which may be due to the poor differentiation of these cells (Fig.21).

BEAS-2B is an adenovirus 12-SV40 hybrid virus-transformed human bronchial epithelial cell line. Epithelial cells were isolated from normal human bronchial epithelium obtained from autopsy of non-cancerous individuals. Cells were infected with an adenovirus 12-SV40 virus hybrid (Ad12SV40) and cloned. Cells retain the ability to undergo squamous differentiation in response to serum, and can be used to screen chemical and biological agents for ability to induce or affect differentiation and/or carcinogenesis (Reddel *et al.*, 1989).

Zhang *et al.* (2007) reported that low levels of lysozyme mRNA were detected in BEAS-2B bronchial epithelial cells. On the other hand, no reports, to our knowledge, studied yet the levels of active lysozyme secreted in the medium of BEAS-2B epithelial cell cultures. Our experimental results suggested that BEAS-2B virus-transformed bronchial epithelial cells were not able to secrete detectable amounts of lysozyme activity in the medium of the cell cultures of this cell line.

To conclude, the present investigation illustrated that no detectable amounts of lysozyme activity could be measured in the supernatants of A549 and BEAS-2B lung epithelial cell cultures even after 4 days incubation of cells in culture medium, taking into consideration the highly sensitive fluorescence-based lysozyme assay utilized to measure lysozyme activity (Helal & Melzig, 2008).

#### **4.3.3. Human oral squamous cell carcinoma cell line BHY & oral tumor cells**

Lysozyme has been shown to exert antimicrobial activity on a wide range of oral microorganisms (Anil & Samaranayake, 2002). It has been frequently reported that lysozyme is produced by epithelial cells in general. In order to investigate the capability of epithelial cells in the oral cavity to secrete lysozyme activity, lysozyme activity levels secreted in the culture medium of BHY cells were measured.

BHY is a human oral squamous cell carcinoma cell line established from the tumor of a 52-year-old Japanese man with highly differentiated squamous cell carcinoma of the lower alveolus which was highly invasive to the mandibular bone and the muscle layer of the oral

floor; described as anchorage-independent and to be tumorigenic in nude mice (Kawamata *et al.*, 1997). To the best of our knowledge, there have been no reports concerning the ability of cultured oral squamous cell carcinoma cells to release lysozyme activity in the culture medium.

Human oral squamous cell carcinoma cells (BHY) were grown in culture medium for 24 hours and for 48 hours, then lysozyme activity levels secreted in the culture medium at these two time points were measured using the EnzChek<sup>®</sup> fluorescence-based assay. No detectable levels of lysozyme activity could be measured in the culture medium of BHY cells even after 48 hours incubation, taking into consideration the use of a highly sensitive fluorescence-based assay for measuring lysozyme activity.

To compare lysozyme secretion in the BHY oral cell line with primary oral tumor cells, lysozyme activity levels in the cultures of human primary tumor cells derived from the tongue of a patient with oral tumor were investigated. Primary cells were maintained in culture medium for 6 days, and then lysozyme activity levels secreted in the medium were measured using the EnzChek<sup>®</sup> fluorescence-based assay. On the contrary to BHY oral cell line, primary oral tumor cells were able to secrete active lysozyme in detectable concentration of about 5 units/ml after 6 days incubation in culture medium (Fig.22).

Concluding, our measurement results suggested that primary oral tumor cells secrete lysozyme activity in the culture medium, whereas the oral tumor cell line was not able to secrete detectable levels of this active enzyme.

#### **4.4. Effects of differentiation-inducing agents**

##### **4.4.1. PMA**

Phorbol esters are tetracyclic diterpenoids that mimic the action of diacyl glycerol (DAG), activator of protein kinase C, which regulates different signal transduction pathways and other cellular metabolic activities. They occur naturally in many plants of the family Euphorbiaceae and Thymelaeaceae (Goel *et al.*, 2007). PMA has been reported to affect many enzymatic activities through its interaction with protein kinase C (PKC). PMA has been shown to provoke the concentration- and time-dependent decrease of mRNA coding for the enzymes such as phosphoenol pyruvate carboxykinase (Chu & Granner, 1986). Conflicting reports about the effects of phorbol esters on lysozyme release and expression have been published. Phorbol esters were stated in most reports to increase the synthesis and discharge of lysozyme in THP-1 cells (Ries *et al.*, 1998). Only few reports mentioned that lysozyme activity in supernatants of THP-1 cell cultures was not affected by phorbol esters treatment (Tsuchiya *et al.*, 1982). The ability of PMA to induce monocytic differentiation and affect many enzymatic activities, as well as the conflicting published reports about PMA influence on lysozyme release and expression suggested necessity to investigate its effects on lysozyme levels of various cell lines.

Our data illustrated that PMA could induce only slight increases in the release of lysozyme activity of THP-1 monocytes, which was frequently not stable in the repeated experiments (Fig.32.A). On the other hand, when incubated with HL-60 monocytes PMA had no inducing effect on the release of lysozyme activity after one hour exposure, whereas inhibiting effects were observed upon longer exposure periods (Fig.35). Regarding lysozyme synthesis, PMA had no significant effects on the production of lysozyme activity expressed by THP-1 and HL-60 monocytes for long exposure periods up to 72 hours.

No significant differences were found between the effects of PMA on the lysozyme of monocytes when lysozyme levels were measured as a protein using ELISA compared to the active enzyme measured fluorometrically (Fig.33).

In order to compare the influence of differentiation-inducing agents of monocytes on lysozyme activity of monocytes with other lysozyme-producing cell types, PMA was incubated with epithelial cells to investigate its possible effects for periods up to 72 hours.

PMA had no significant effects either on the expression or on the release of lysozyme activity of epithelial cells regardless of the concentration or the exposure period used.

#### 4.4.2. hIFN- $\gamma$

Interferon-gamma (IFN- $\gamma$ ) is a dimerized soluble cytokine that is the only member of the type II class of interferons (Gray & Goeddel, 1982). The initial discovery of human interferons was based on their ability to inhibit viral growth in target cells (Friedland, 1996). Their biological effects *in vivo* include antiviral activity, cell growth inhibitor and immunomodulatory activity (Pitha, 2007; Dheda *et al.*, 2005; Perez *et al.*, 1990; Chen *et al.*, 1992). IFN- $\gamma$  is more potent than the other two IFNs ( $\alpha$ , $\beta$ ) in its immunomodulatory activities. This interferon was originally called macrophage-activating factor, a term now used to describe a larger family of proteins to which IFN- $\gamma$  belongs. IFN- $\gamma$ , or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. The importance of IFN- $\gamma$  in the immune system stems in part from its ability to inhibit viral replication directly, and most importantly from its immunostimulatory and immunomodulatory effects. IFN- $\gamma$  is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops (Entrez Gene, 2010; Schoenborn & Wilson, 2007). In 1986, hIFN- $\gamma$  cDNA was cloned and expressed in *Escherichia coli* (Pitha, 2007). rhIFN- $\gamma$  is of particular interest in view of its well-defined immunomodulatory effects, including stimulation of natural killer (NK) and lymphokine-activated killer (LAK) cell-mediated cytotoxicity, activation of macrophages, stimulation of Fc receptor expression on mononuclear cells and antibody-dependent cellular cytotoxicity, and enhancement of HLA class II antigen expression that can increase cytotoxic lymphocyte function (Jaffe & Herberman, 1988).

Above mentioned immunoregulatory, antiviral, anti-tumor and macrophage-activating properties of hIFN- $\gamma$  suggested the value of investigating the ability of hIFN- $\gamma$  to regulate lysozyme activity in monocytes and other lysozyme-producing cell lines.

Our experiments showed that the influence of hIFN- $\gamma$  on the release and production of lysozyme activity of monocytes was not similar when incubated with different monocytic cell lines. The results demonstrated that hIFN- $\gamma$  at 500 and 1000U/ml could induce only slight increases in the release of lysozyme activity of THP-1 monocytes, while higher concentration of 2000 U/ml caused to the contrary a significant drop in the release of lysozyme activity after one hour exposure (Fig.32.B). Compared to THP-1 monocytes, hIFN- $\gamma$  had no influence on the release of lysozyme activity of HL-60 monocytes after one hour treatment.

With longer exposure period of 24 hours, hIFN- $\gamma$  resulted in no significant effects on the production and release of lysozyme activity of THP-1 cells without regard to the concentration used, while when incubated with HL-60 monocytes for 24 hours hIFN- $\gamma$  caused significant tendency to decrease in the production and release of lysozyme activity even though it showed no cytotoxicity at the tested concentrations (Fig.35).

#### 4.4.3. Retinoic acid

The vital roles that vitamin A and its metabolite retinoic acid (RA) play in the homeostatic control of the immune system have been known for decades, given the observation that vitamin A-deficient individuals are incapable of controlling bacterial, viral, and protozoan diseases. The absence of RA over the course of normal human development leads to defects in the immune system, embryonic development, vision, brain function, and other systems (Pino-Lagos *et al.*, 2008). Recent reports of the essential role of RA in maintaining gut lymphocyte homing and T regulatory cell differentiation have brought this small metabolite to the forefront as a critical regulator of immunity (Pino-Lagos *et al.*, 2008).

RA exerts profound influence on leukocyte proliferation and differentiation. It has been evaluated as to its effects on the differentiation, activation and function of monocytes/macrophages and the ability of macrophages to initiate immune response against pathogens. In general, the effect of RA on monocytes, macrophages, and macrophage cell lines suggests that RA inhibits the production of cytokines (Semba, 1994; Mehta & Lopez-Berestein, 1986). RA can also suppress carcinoma cell growth and is currently used in treatment of some cancers (Noy, 2010). Due to the suggested role of RA in immunity to infectious diseases, monocytes differentiation and cancer treatment, we studied its ability to affect lysozyme activity of monocytes and epithelial cells. Takenaga (1981) reported retinoic acid to induce lysozyme activity in mouse myeloid leukemia M1 cells.

When we treated THP-1 monocytes with retinoic acid, it inhibited significantly the expression and release of lysozyme activity, which was observed with the short-term as well as long-term exposure periods (Fig.34). This inhibitory effect of RA on lysozyme production is similar

to its reported inhibitory effect on the production of cytokines, which may be due to the ability of RA to suppress the growth of carcinoma cells.

Compared to monocytes, retinoic acid incubated with HT-29 epithelial cells had no significant effects on the expression and release of lysozyme activity of epithelial cells regardless of the concentration or the incubation period used.

The results may indicate that monocytes/ macrophages are important target of RA in comparison to epithelial cells.

#### **4.4.4. Forskolin**

Forskolin, a direct adenylyl cyclase-stimulating agent, has been reported to increase cAMP levels in U937 cells and to induce monocytes differentiation (Rubin *et al.*, 1986; Shayo *et al.*, 1997; Brodsky *et al.*, 1998; López-Lluch *et al.*, 1998). Regenhard *et al.* (2001) proved that forskolin did not stimulate lysozyme RNA expression in HD11 myelomonocytic cells even after 12 hours exposure. The results of Regenhard *et al.* seem to agree with our results that forskolin had no significant effects on the expression and release of lysozyme activity of THP-1 monocytes, which was observed with the short-term as well as long-term exposure periods up to 72 hours.

Since lysozyme is also important antimicrobial molecule of airway surface liquid, effect of forskolin on lysozyme release by cultures of human airway epithelium was frequently reported with conflicting results. Ramesh Babu *et al.* (2004) found that forskolin decreased lysozyme activity secretion of Calu-3 epithelail cells using the turbidimetric assay, whereas other publications reported that forskolin increased Calu-3 lysozyme secretion using ELISA assay (Joo *et al.*, 2004; Duszyk, 2001; Dubin *et al.*, 2004).

Due to the conflicting reports about forskolin effects on lysozyme levels of epithelial cells, we investigated its influence on the release and production of lysozyme activity in HT-29 epithelial cells. Forskolin had no significant influence on the production and release of lysozyme activity measured fluorometrically in HT-29 epithelial cells, which was observed with the short-term as well as long-term incubation periods up to 72 hours.

#### **4.4.5. Conclusion**

Differentiation-inducing agents, especially PMA, have been frequently reported to induce enhanced expression and release of lysozyme in monocytes even after one hour treatment.

Our results suggested that PMA and other differentiation-inducing agents were not able to stimulate lysozyme activity of monocytes constantly in a regular manner. Therefore PMA was not suitable to be used as positive control for our experiments.

Differentiated monocytes have been proved to express higher levels of lysozyme activity compared to undifferentiated monocytes. Therefore, the rise in lysozyme activity levels of monocytes treated with differentiation-inducing agents previously reported may be explained

due to the differentiation process which makes the monocytes more mature, and not related to the differentiation-inducing agents themselves.

#### **4.5. Effects of the cholinergic agonist carbachol**

Carbachol is a well-known cholinergic agonist, which has been frequently reported in the literature to induce increases in the expression and release of various enzymes and chemokines *in vitro* as well as *vivo* (Zheng *et al.*, 2004; Wang *et al.*, 2007; Schmidt *et al.*, 1993; Dubick *et al.*, 1988). The cholinergic agonist carbachol is a potent secretagogue for mucus secretion from airway submucosal glands. However, Joo *et al.* (2004) found that lysozyme secretion in Calu-3 epithelial cells was only modestly stimulated by carbachol using ELISA assay (Joo *et al.*, 2004). We investigated the influence of carbachol at non-toxic concentrations on the expression and release of lysozyme in monocytes and epithelial cells. Our results showed that treatment with carbachol for short exposure periods up to 24 hours had no significant influence on lysozyme release and production in both THP-1 monocytes and HT-29 epithelial cells. Slight but statistically significant inhibitory effects of carbachol on lysozyme activity levels started to appear with treatment periods longer than 24 hours, while no clear dose-dependent relationship of this inhibitory effect was found (Table 5).

#### **4.6. Effects of the immunosuppressant corticosteroids**

Glucocorticoids (GCs) are hormone products of the adrenal gland, which have long been recognized to have a profound impact on immunological processes (Turnbull & Rivier, 1999). Macrophages derive from monocytes and play a central role in innate immunity and at the initiation of adaptive immunity. Their activation by microbial pattern recognition receptors (e.g. toll-like receptors, TLRs) and their large repertoire to release inflammatory regulators make these cells a prominent relevant target for anti-inflammatory therapy by steroids (Baschant & Tuckermann 2010). GCs have been shown to negatively regulate numerous macrophage functions (Guyre & Munck, 1989; Baybutt & Holsboer, 1990). Activation of hypothalamic pituitary adrenal (HPA) axis as well as addition of corticosterone to the cultures of macrophages resulted in a suppression of the production of tumor necrosis factor alpha (TNF- $\alpha$ ) and reactive nitrogen intermediates by macrophages (Brown & Zwillig, 1994; Bishayi & Ghosh, 2007). Furthermore, a number of cytokines were efficiently suppressed by GCs (Reichardt *et al.*, 2001; Tuckermann *et al.*, 2007). We investigated the effect of the corticosteroid prednisolone on the production and release of the innate immune molecule lysozyme in THP-1 monocytes. Prednisolone is the active metabolite of prednisone (Davis *et al.*, 1978), which is a corticosteroid drug with predominantly glucocorticoid and low mineralocorticoid activity (Czock *et al.*, 2005).

Our results demonstrated that prednisolone inhibited significantly the production and release of lysozyme activity measured fluorometrically in monocytes, which was observed with the



short-term as well as long-term exposure periods up to 72 hours (Fig.36). Surprisingly, the significant decrease in the release and synthesis of active lysozyme of THP-1 monocytes was not detected when lysozyme was measured as a protein using ELISA under the same conditions (Fig.37). Based on these results we concluded that prednisolone did not affect the production and release of lysozyme as protein by monocytes, but it was able to inhibit the enzymatic activity of lysozyme produced by these cells. The proven inhibitory effect of prednisolone on lysozyme activity produced and released by THP-1 monocytes has suggested a new mechanism of action which can partly explain the ability of prednisolone to suppress the immune system and negatively regulate macrophage functions

On the contrary, reported investigations regarding the airway epithelium have supported the concept that GCs enhance innate immunity by enhancing the survival and/or function of neutrophils and alveolar macrophages, while they suppress adaptive immunity by inducing the apoptosis of airway dendritic cells. While suppressing systemic adaptive immune responses, GCs were reported to exert little or no inhibitory effect on the ability of the epithelium to express antimicrobial substances such as complement, collectins, lactoferrin, defensins and lysozyme and, in some cases, may even elevate their production (Schleimer, 2004). Relatively little has been published in the recent literature on the effects of GCs on the expression of lysozyme in epithelial cells. Ali and coworkers (1996) found that dexamethasone had no effect on spontaneous release of lysozyme and lactoferrin by human nasal explants *in vitro*.

In agreement to some extent with the results of Ali and coworkers, we found that prednisolone incubated with HT-29 epithelial cells for treatment periods up to 48 hours had no significant influence on the release and expression of lysozyme activity synthesized by the cells. A slight but statistically significant inhibitory effect of prednisolone at high concentration (36µg/ml) on both secreted and total lysozyme activity of epithelial cells was detected only after 72 hours exposure (Table 6).

To conclude, we suggest that the significant inhibitory effects of prednisolone on the lysozyme activity of monocytes participate in the mechanism of action for the immunosuppressive effect of prednisolone, taking into consideration the surveillance functions of monocytes/macrophages in the immune system as well as the important role of lysozyme as a part of the innate immune system.

#### **4.7. Effects of inflammatory response inducers**

##### **4.7.1. Sodium Thioglycolate**

Thioglycolate is often used as an eliciting agent in the studies with peritoneal exudate macrophages. This stimulus has the advantage of recruiting a large number of cells to the site of inflammation but does not increase the microbicidal activity of macrophages and therefore does not activate macrophages. After an intraperitoneal injection of thioglycolate,

the resistance of mice to infecting microorganisms is decreased, probably due to insufficient bactericidal activity of the elicited macrophages (Leijh *et al.*, 1984). The question of which mechanism underlies this insufficient defense mechanism has not yet been answered.

Due to the important role of lysozyme in innate immunity and inflammatory response, we investigated whether sodium thioglycolate can influence lysozyme activity synthesis and release of human monocytes as well as epithelial cells. A slight tendency to increase in the secretion of lysozyme activity was detected when THP-1 monocytes were treated with thioglycolate. The stimulatory effect on the production of lysozyme activity in THP-1 monocytes started to be statistically significant after 72 hours treatment (Fig.38; Table 7). Contrary to previous reports, the obtained results suggested that thioglycolate was able to activate slightly but significantly the microbicidal activity of monocytes through its stimulatory effect on lysozyme activity levels produced by monocytes especially after long-term treatment.

On the other hand, thioglycolate exhibited slight inhibitory effect on the release and production of lysozyme activity in HT-29 epithelial cells with exposure periods up to 72 hours (Fig.38; Table 7). This inhibitory effect may explain to some extent the reported decrease in the resistance of mice to infecting microorganisms after an intraperitoneal injection of thioglycolate mentioned above.

#### 4.7.2. Bacterial Components

Bacterial endotoxins activate immunologic and inflammatory responses in the acute phase of bacterial infection, particularly in cells of immunologic systems including B and T lymphocytes and macrophages (Goethe & Phi-van, 1998). Macrophages can be activated and induced to release nitric oxide (NO) upon stimulation with bacterial products (Ribeiro-Dias *et al.*, 1998). Rabbit alveolar macrophage stimulated by the injection of dead BCG (*Bacillus Calmette-Guérin tuberculosis vaccine*) exhibited reportedly increased levels of various hydrolytic enzymes, including lysozyme activity, as compared to alveolar macrophages from unstimulated rabbits (Cohn & Wiener, 1963). Lipopolysaccharides from *Salmonella enteritidis* (LPS), through its lipid A component, can interact with various host cell types including mononuclear cells, endothelial and smooth muscle cells, polymorphonuclear granulocytes, and thrombocytes, among which macrophages/monocytes are of particular importance. Thus, LPS-induced activation of macrophages resulted in the production of bioactive lipids, reactive oxygen species, and in particular peptide inflammatory mediators such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukins (Rietschel *et al.*, 1994) (Fang *et al.*, 2004). Moreover, LPS was reported to stimulate intestinal epithelial cells to secrete IL-8, a chemotactic peptide that also activates neutrophils (Lee *et al.*, 2005). In animal studies, LPS was reported to activate lysozyme gene in the chicken myelomonocytic cell line HD11. LPS caused a 15-fold increase in the transcription rate of the lysozyme gene. Furthermore, LPS-

induced increase in nuclear lysozyme transcripts was found to greatly exceed the increase in transcription rate, and the nuclear lysozyme transcripts in untreated cells were more unstable than those accumulated in LPS-activated cells (Goethe & Phi-van, 1998).

Based on these reports, we investigated whether various bacterial components can affect positively lysozyme activity synthesis and release of human monocytes as well as epithelial cells. In agreement to previous reports which concerned lysozyme gene activation, LPS caused significant increase in the lysozyme activity secreted in the medium of THP-1 monocytic cell cultures after 1 hour exposure (Fig.39.A). This increase in the release of lysozyme activity measured fluorometrically was accompanied with significantly higher increase in the released lysozyme measured as a protein using ELISA after one hour treatment with LPS (Fig.40).

Moreover, dose-dependent stimulatory effect of LPS on secreted lysozyme activity was observed with PMA-differentiated THP-1 monocytes (Fig.39.A).

Upon long-term treatment, significant inducing effect of LPS on the production of lysozyme activity in THP-1 monocytes was observed after 72 hours treatment (Fig.39.B; Table 8). Furthermore, LPS stimulated PMA-differentiated monocytes to express and secrete significantly higher lysozyme activity levels after 72 hours exposure (Fig.39.B; Table 8). No significant differences were found between the stimulatory effects of LPS on the production of lysozyme in monocytes as a protein or as an active enzyme after long-term treatment.

Effects of Leiber-Beta S and Auxoferm YGT Plus were not similar to those produced by LPS on the release of lysozyme activity in the cell cultures after one hour exposure. Neither of them had stimulatory effect on the secretion of lysozyme activity in THP-1 monocytes or HT-29 epithelial cells.

Concluding, the obtained results have suggested LPS as immunostimulant agent through the stimulation of production and release of lysozyme activity in monocytes and differentiated macrophages, the important defense molecule of the innate immune system which is able to control the growth of susceptible bacteria and to modulate host immunity against infections and depressions of immune responses.

#### **4.8. Effects of antibacterial Chito-Oligosaccharide**

Chito-oligosaccharide (COS) is generally known to possess many specific biological functions depending on its size, among which the antibacterial activity is of particular importance. Chitosan is a natural, non-toxic, co-polymer of GlcN (glucosamine) and GlcNAc (*N*-acetylglucosamine) obtained after partial de-*N*-acetylation of chitin, which, in turn, is a major component of the shells of crustaceans and found commercially in the offal of marine food processing industry. In spite of its abundance and various biofunctionalities, utilization of chitosan is restricted, owing to its high molecular mass, high viscosity and, thus, low absorption for *in vivo* applications. Recent studies on chitosan depolymerization have drawn

considerable attention, since the products obtained are easily water-soluble and also possess versatile biofunctional properties such as antitumour, immuno-stimulating and antimicrobial activities and are being used for alleviating problems due to osteoarthritis–gastritis (Vishu Kumar *et al.*, 2005).

To our knowledge, no reports investigated the effects of chito-oligosaccharides on lysozyme activity levels of human cell cultures. Partially deacetylated chito-pentose DP5 is a chito-oligosaccharide investigated in our work concerning its possible influence on lysozyme activity of epithelial cells. No significant effects on the synthesis or secretion of lysozyme activity were found when HT-29 epithelial cells were treated with this chito-pentose.

#### **4.9. Effects of medicinal plants & phytochemicals**

##### **4.9.1. Selected herbal extracts & phytochemicals with immune-modulating, antibacterial, antiviral and/or anti-inflammatory activities**

Modulation of immune response and antibiotic activity by medicinal plant products as a possible therapeutic measure have become a subject of scientific investigations recently. Herbal medicines are being increasingly utilized to treat a wide variety of clinical diseases with relatively little knowledge regarding their mechanism of action. There are several medicinal plants and phytochemicals having well documented immunostimulatory, antibacterial, antiviral, anti-inflammatory and/or anti-tumor properties, such as *Echinacea purpurea* (Schoop *et al.*, 2006), *Rhodiola rosea* (Mishra *et al.*, 2006), *Humulus lupulus* (hop) (Zanoli & Zavatti, 2008), *Rosmarinus officinalis* (rosemary) (Luqman *et al.*, 2007; al-Sereiti *et al.*, 1999), *Glycyrrhiza uralensis* (Chinese liquorice) (Gao *et al.*, 2009), *Chamomilla recutita* (German chamomile) (McKay & Blumberg, 2006), *Aloe vera* (Grace *et al.*, 2008), *Scutellaria lateriflora* (skullcap) (Shang *et al.*, 2010) and green tea (Clarke & Mullin, 2008).

Since these medicinal plants are potential resources for identifying stimulators or inhibitors of lysozyme activity in human cells, we investigated their possible effects on the production and secretion of lysozyme activity of human monocytes and epithelial cells, which can elucidate a new mechanism of action underlying their therapeutic effects mentioned.

##### **4.9.1.1. *Echinacea purpurea* & its isolated phytochemical constituents**

The name *Echinacea* describes a genus of perennial plants native to the prairies of mid-western North America, which belongs to the *Asteraceae* family (Barrett 2003). Native American tribes discovered the potential of *Echinacea* in the treatment of cough, sore throat, snake bites, and analgesia (Schoop *et al.*, 2006). Currently the medicinal use of *Echinacea* is based on its immunomodulatory properties and it is mainly used to treat and prevent upper respiratory tract infections, such as the common cold and influenza (Barnes *et al.*, 2005; Kligler, 2003). Based on the available evidence, it is likely that *Echinacea* has a beneficial effect in diseases with a predominantly inflammatory component. The anti-inflammatory and

immune-modulatory effects of *Echinacea* observed *in vitro* could thus have direct ameliorating effect on virus-induced inflammation (Schoop *et al.*, 2006). The mechanisms leading to a beneficial effect of *Echinacea*, as well as the phytochemicals involved, are still largely unknown, but the literature shows an increasing number of studies mainly focus on its immunomodulatory activities. *Echinacea* appears to affect the non-specific immune response, activating innate immune cells such as macrophages, polymorphonuclear leukocytes and natural killer cells. Some studies have shown that various extracts have a stimulating effect on phagocytosis, both *in vitro* and *ex vivo*, and on other parameters of non-specific immunity such as cytokine release and cell proliferation (Barrett, 2003; Zhai *et al.*, 2007). However, it has also been proposed that *Echinacea* could have a role in inhibiting the production of inflammatory mediators by white blood cells, both in whole blood and isolated cells (Guiotto *et al.*, 2008; Woelkart & Bauer, 2007). Other studies have suggested that *Echinacea* preparations could also enhance adaptive immunity (Barrett, 2003; Zhai *et al.*, 2007). Taken together, these findings suggest that *Echinacea* might have more of a modulatory role on the innate immune system, able to both stimulate and inhibit the immune response. To date, no reports have yet studied the effects of *Echinacea* on the production and secretion of lysozyme activity, the important molecule of the innate immune response. Therefore, we studied the effect of *Echinacea purpurea* root extract (standardized to 7.84% polysaccharides) on the production and secretion of lysozyme activity in the human monocytic cell line THP1 and in the epithelial cell line HT-29. The results showed that *Echinacea* root extract induced significant increase in the secretion of lysozyme activity of epithelial cells after one hour exposure (Fig.42.B). The proven stimulatory effect of *Echinacea* root extract on lysozyme secretion of epithelial cells can present a novel mechanism of action for its therapeutic properties, and confirm the effectiveness of *Echinacea* in the treatment and prevention of common cold, influenza and other respiratory tract infections.

However, the biological activities of *Echinacea* appear to be the result of a combined action of its constituents, rather than of one single group. Limited number of studies has focused on the individual roles of the different phytochemical components in the immunomodulatory activity of *Echinacea*. Research has focused particularly on alkylamides, which have been the only *Echinacea* components shown to be bioavailable after oral dosing in humans (Matthias *et al.*, 2007, Matthias *et al.*, 2005; Woelkart *et al.*, 2005). In particular, *ex vivo* studies have found a specific agonistic activity on the cannabinoid-type 2 (CB2) receptor by the alkylamides dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*-dienoic acid isobutylamide (Raduner *et al.*, 2006).

Among the phytochemicals found in *Echinacea*, the alkylamides (also known as alkamides or isobutylamides) are thought to be the compounds responsible for its effects on the human

immune system (Toselli *et al.*, 2009). Hence, it was of importance to investigate the effect of *Echinacea* alkylamides on the secretion and production of lysozyme activity in the human monocytes and epithelial cells. The results demonstrated that *Echinacea* alkamide (Dodeca-2,4,8,10-tetraenoic acid isobutylamide) was able to enhance significantly the release of lysozyme activity in THP-1 monocytes with an increase of up to 92% after one hour exposure (Fig.43.A). The stimulation of lysozyme release in monocytes induced by the isolated alkamide of *Echinacea* was significantly higher than that induced by the polysaccharides-rich extract of *Echinacea* root mentioned above. The data obtained suggested the significant stimulatory effect of *Echinacea* alkamide on lysozyme secretion of monocytes as a novel mechanism of action for the immunomodulatory properties of this bioavailable phytochemical of *Echinacea*. Furthermore, the results support the concept that alkylamides are the most important components of *Echinacea* responsible for its effects on the human immune system, considering that above mentioned *Echinacea* extract enriched in polysaccharides had no significant stimulatory effects on lysozyme activity of monocytes.

#### 4.9.1.2. *Rhodiola rosea*

*Rhodiola rosea* (roseroot) is herbal supplement believed to possess adaptogenic and ergogenic properties and is used primarily to support the immune system and decrease the impact of fatigue on mental and physical performance. It has also been used to treat cold and flu-like symptoms. In numerous *in vitro* and *in vivo* studies on animals, immune-stimulating, antiviral, anti-inflammatory and antibacterial activity of *Rhodiola* has been demonstrated, among others (Panossian *et al.*, 2010). *Rhodiola* root has been clinically proven to be effective in enhancing human immune responses. The aqueous extract of *Rhodiola* rhizome was reported to have potent immunostimulatory and anti-cancer properties (Mishra *et al.*, 2006; Mishra *et al.*, 2008). However, the mechanism by which *Rhodiola* extract enhances immunostimulatory/anticancer activity remains largely unclear. The aqueous extract of *Rhodiola* rhizome was found to stimulate production of interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and nitric oxide (NO) in human peripheral blood mononuclear cells (PBMCs) as well as mouse macrophage cell line RAW 264.7 (Mishra *et al.*, 2006). *Rhodiola* was also reported to favor the proliferation of lymphocytes, and enhance the levels of IL-2, IL-4, granulocyte-macrophage colony-stimulating factor and the mRNA content of these cytokines (Loo *et al.*, 2010). It had reportedly adjuvant/immunopotentiating activity in terms of humoral (enhanced specific immunoglobulin levels) as well as cell-mediated immune response against antigens (Mishra *et al.*, 2010). Furthermore, *Rhodiola* was shown to stimulate human peripheral blood lymphocytes and its underlying immunomodulatory effects *in vitro* (Li *et al.*, 2009). Based on the demonstrated immune-stimulating, antiviral, anti-inflammatory and antibacterial properties of *Rhodiola*, we investigated the possible stimulatory properties of aqueous extract of *Rhodiola* root on lysozyme activity in human monocytic cell line THP-1

and epithelial cell line HT-29. Our results illustrated that *Rhodiola rosea* extract was able to induce statistically significant enhancement of lysozyme activity release in human monocytes by 55% after one hour exposure (Fig.41.A). This proven stimulatory activity of *Rhodiola* on lysozyme activity levels has elucidated a novel mechanism of action which can partly explain and demonstrate the immunostimulatory potential of *Rhodiola*, which can be used for the upregulation of immune response in patients with inadequate functioning of the immune system.

#### 4.9.1.3. Green tea

Several reports have demonstrated anti-inflammatory, anti-viral and anti-tumor activities of green tea and epigallocatechin gallate (EGCG), which is the most abundant, best-studied, and possibly most potent polyphenol found in tea (Saito *et al.*, 2009; Bode & Dong, 2009). Extracts of green tea and its polyphenols have been shown to inhibit the inflammatory responses *in vitro* in different cell types (Singh *et al.*, 2010). The effect of green tea on the production and secretion of lysozyme activity in human cells has not yet been studied. Above mentioned biological activities of green tea evoked our interest to investigate this possible effect. Our results showed that green tea extract (EGCG content >90%) was able to induce a statistically significant increase in the production of lysozyme activity in monocytes after 72 hours treatment with no cytotoxicity (Table 11). The stimulatory effect of EGCG-rich green tea extract on the production of lysozyme activity can suggest a new mechanism of action, which may explain some of its pharmacological properties.

#### 4.9.1.4. Benzyl isothiocyanate

Benzyl isothiocyanate (BITC) is an enzymatic hydrolysis product of the glucosinolate glucotropaeolin present in garden cress, papaya, and common Brassica vegetables (Higdon *et al.*, 2007). Several studies provided evidence that BITC has been a potential anti-inflammatory agent (Lee *et al.*, 2009; Cheenpracha *et al.*, 2010). BITC is also one of the most intensively studied isothiocyanates as a potential cancer chemopreventive agent (Hecht, 1995; von Weymarn *et al.*, 2006). It has been reportedly indicated that BITC can suppress the transcription and production of proinflammatory molecules including iNOS, COX-2, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in LPS-treated murine macrophages. In addition to these *in vitro* data, BITC demonstrated anti-inflammatory effects *in vivo* when tested in mice (Lee *et al.*, 2009).

Together, published results indicated that BITC has anti-inflammatory and anti-cancer activity. To the best of our knowledge, the effect of BITC on lysozyme activity of human cells has yet to be investigated. Our results showed that BITC exhibited significant inhibitory effects on lysozyme activity release in both THP-1 monocytes and HT-29 epithelial cells after one hour treatment with no cytotoxicity (Fig.43.B). This dose-dependent inhibitory effect

continued to exist only in monocytes after 24 hours treatment. The inhibitory influence of BITC on lysozyme activity of monocytes disappeared unexpectedly after further 48 hours incubation, whereas significant dose-dependent stimulatory effects on lysozyme activity production and release showed up after 72 hours treatment with no cytotoxicity (Table 14).

The obtained data suggested immunomodulatory properties of benzyl isothiocyanate through its effects on lysozyme activity especially in monocytes. The significant stimulatory effect of BITC on the production and release of lysozyme activity in monocytes upon long-term treatment can present a novel mechanism of action for the anti-inflammatory and anti-cancer activity of BITC.

#### **4.9.1.5. Other herbal extracts & phytochemicals**

CO<sub>2</sub>-fluid extracts of hop flowers, rosemary leaves, Chinese licorice root and chamomile flowers inhibited significantly the release and/or production of lysozyme activity in THP-1 monocytes and HT-29 epithelial cells after short- and long-term exposure (Fig.41; Table 11,12,13).

Though the illustrated inhibitory activity of these medicinal plants on lysozyme release suggested immunomodulatory properties of these extracts, the obtained data suggested no role of lysozyme activity in the mechanism of action by which they exert their antibacterial, antiviral and/or anti-inflammatory effectiveness.

On the other hand, *Aloe vera* and skullcap extracts pronounced no significant effects on the release and production of lysozyme activity either in monocytes or in epithelial cells upon short- and long-term treatment.

#### **4.9.2. Homeopathic mother tinctures & its isolated phytochemicals**

##### **4.9.2.1. Tomato extracts and its isolated compound tomatine**

The glycoalkaloid known as tomatine, first isolated by Fontaine *et al.* (1948), occurs naturally in tomatoes (*Lycopersicon esculentum*) and is known to be a primary toxicity-based defense mechanism against viral and bacterial pathogens (Rick *et al.*, 1994).

Tomatine consists of a mixture of two glycoalkaloids,  $\alpha$ -tomatine and dehydrotomatine (Friedman *et al.*, 1994). Both compounds are present in all parts of the tomato plant (Friedman & Levin, 1998). Because glycoalkaloids are reported to be involved in host-plant resistance, on the one hand, and to have a variety of pharmacological properties in animals and humans, on the other, a need exists to develop a better understanding of the role of these compounds both in plants and humans. Concurrent with the discovery of tomatine, studies were undertaken which showed that the molecule possessed antibiotic properties against a variety of fungi and human pathogens (Fontaine *et al.*, 1948; Kuhn *et al.*, 1956; 1957; Irving, 1947; Irving *et al.*, 1945;1946). These observations suggested that tomatine may play a major role in disease resistance in the tomato plant and may be biologically active in



animals and humans. The reported fundamental molecular–cellular mechanisms by which tomatine resists pathogens, including binding to cholesterol, disruption of cell membranes, inhibition of cholinesterases and perturbation of acid–base equilibria *in vivo*, may be involved in the biological effects of tomatine in fungi, bacteria, protozoa, insects, worms, animals and humans (Friedman, 2002).

Animal studies demonstrated that tomatine administered intramuscularly or subcutaneously offered protection against inflammation and allergies (Filderman & Kovacs, 1969; Calam & Callow, 1964; Wakkary *et al.*, 1970a,b). Experiments investigated the immune adjuvant effects of tomatine showed that it was a highly effective immunostimulator (Rajananthanan *et al.*, 1999b). Tomatine has been shown to have membrane disrupting qualities (Keukens *et al.*, 1992; 1995; 1996) similar in character to that of saponins which have long been established as potent immunostimulators (Espinet, 1951; Hyslop & Morrow, 1969; Kensil *et al.*, 1998). Several studies (Rajananthanan *et al.*, 1999a,b; Taylor-Robinson & Morrow, 2002) have found tomatine to induce cytokines, which are mediators of lymphoid cell function during an immune response produced mainly by T helper cells and macrophages. Further experiments demonstrated the potential utility of tomatine as a vaccine adjuvant in cancer (Morrow *et al.*, 2004). Based on these reports, tomatine showed remarkable potential as a vaccine adjuvant for infectious diseases as well as cancer immunotherapy. As well as being capable of inducing powerful humoral and cellular immune responses it was well tolerated and appeared non-toxic (Morrow *et al.*, 2004).

However, before tomatine achieves clinical application, a number of other basic issues need to be addressed including further animal studies, refinement and standardization of the formulation process as well as additional toxicity studies. Moreover, further studies need to be carried out on the mode of action of tomatine.

To investigate the possibility that the stimulation of lysozyme activity can serve as a new mechanism explaining immune-stimulating properties of tomatine, and to examine the relative contribution of this natural substance to lysozyme synthesis and release of monocytes and epithelial cells, lysozyme activity levels were measured in the culture fluids of these two cell types upon exposure to tomatine for various treatment periods. Tomatine was proved to stimulate significantly the release of lysozyme activity when incubated with THP-1 monocytes. The stimulation of lysozyme activity secretion of monocytes was statistically significant with all examined concentrations of tomatine after one hour treatment (Fig.46.A). Furthermore, a significant stimulatory effect on the production of active lysozyme of monocytes was observed upon exposure to high non-toxic concentration of tomatine for 72 hours (Table 16). Moreover, tomatine-containing natural extracts of fresh and dried tomato enhanced the secretion of lysozyme activity of monocytes and/or PMA-differentiated macrophages after one hour exposure (Fig.46.B,C).

The obtained results suggested tomatine and tomatine-containing tomato extracts as immunostimulant agents from natural origin through the stimulation of lysozyme activity release and production in monocytes and differentiated macrophages. Based on the ability of tomatine and tomatine-containing tomato extracts to stimulate lysozyme activity of monocytes, they can offer therapeutic effectiveness as natural immunostimulant agents in addition to the wide range of therapeutic activities for which lysozyme has been exploited.

#### 4.9.2.2. Isolated aconitine

Aconitine is a diterpenoidalkaloid derived from various species of *Aconitum* (Lampe & McCann, 1985). Aconitine and other phytochemical contents of the genus *Aconitum* have received special value in scientific society. These phytochemical contents have been reported to have antibacterial (Anwar *et al.*, 2003; Zhang *et al.*, 2009), antioxidant (Shaheen *et al.*, 2005; Mariani *et al.*, 2008), antiproliferative (Chodoeva *et al.*, 2005; Hazawa *et al.*, 2009) and enzyme inhibition activities (Shaheen *et al.*, 2005). Extracts of the *Aconitum* species have been used orally in traditional medicine to reduce fever associated with colds, pneumonia, laryngitis, croup and asthma; and for their analgesic, anti-inflammatory, hypotensive, diuretic, diaphoretic (cause sweating), cardiac depressant (slow heart rate) and sedative properties (Murayama *et al.*, 1991; Spoerke, 1980). Historically, aconite was most commonly used in Western cultures as a tincture. It was applied topically as a counter irritant liniment for neuralgia, rheumatism and sciatica (Fatovich, 1992). In homeopathy, aconite is used to treat fear, anxiety and restlessness; acute sudden fever; symptoms from exposure to dry, cold weather or very hot weather; tingling, coldness and numbness; influenza or colds with congestion; and heavy pulsating headaches (Boericke, 2010).

An example of homeopathic formulations contain *Aconitum* is 'Canova', which is a homeopathic complex medicine used as an immune modulator and anticancer (Sato *et al.*, 2005). The formula contains the ethanolic extract of *Aconitum napellus*, among others. Studies of its mechanism of action showed that it stimulated the immune system by activating macrophages. Activated macrophages stimulated the lymphocytes so that they increased their cytotoxic action in response to tumoral growth or infection. It had no genotoxic properties *in vitro* on lymphocytes (Seligmann *et al.*, 2003).

Furthermore, Shenfu injection, a traditional Chinese therapy that contains red ginseng and aconite root, has been reported to stimulate the immune system. Zhou *et al.* (2007) investigated the effect of Shenfu injection in postoperative recovery after abdominal surgery. They found that IgG and IgA levels in the group received Shenfu injection were significantly higher than that in control group after operation.

Traumeel S, another homeopathic formulation contains *Aconitum napellum*, is widely used in humans to relieve trauma, inflammation and degenerative processes. The effects of Traumeel were examined *in vitro* on human T cells, monocytes and gut epithelial cells.

Traumeel inhibited the secretion of IL-1 $\beta$ , TNF- $\alpha$  and IL-8 secretion by 50-70% in cells. Interestingly, the effect appeared to be inversely dose-related: maximal inhibition was seen with dilutions of 10<sup>-3</sup> to 10<sup>-6</sup> of the medicine stock material. This finding suggested that Traumeel did not inhibit immune cell functions by exerting toxic effect (Porozov *et al.*, 2004). Although additional *in vivo* studies are needed to clarify the mode of action of aconitine and aconitine-containing homeopathic formulations, the *in vitro* investigations may offer a mechanism for its pharmacological effects. The marked similarity in some biological properties between aconitine and lysozyme, such as the immune-modulatory, anti-inflammatory and anticancer activities, provoked our interest to investigate possible ability of aconitine to induce the expression and release of lysozyme in human cell lines.

Aconitine (100ng/ml) was found able to significantly stimulate the production and release of lysozyme activity in THP-1 monocytes upon long treatment periods up to 48 hours with no cytotoxicity (Table 17). On the other hand, aconitine was illustrated to stimulate significantly the release of lysozyme activity of HT-29 epithelial cells upon short-term treatment (1h) (Fig.48), while longer exposure periods up to 72 hours showed no stimulatory effect of aconitine.

Regarding toxicity to cells, aconitine pronounced no toxic effects on monocytes or epithelial cells at all concentrations and treatment periods used.

These *in vitro* data showed that aconitine was able to induce the production and release of lysozyme activity in both monocytes and epithelial cells, which suggested a novel mechanism of action for aconitine. Hence, the biological properties of aconitine mentioned above, such as the stimulation of immune system and the antibacterial activity, may be partially mediated by the stimulation of lysozyme activity in human cells.

#### 4.9.3. Saponins

Saponins are steroid or triterpenoid glycosides found in plants, lower marine animals and some bacteria (Riguera, 1997; Yoshiki *et al.*, 1998). Saponins contain a steroidal or triterpenoid aglycone to which one or more sugar chains are attached (Oda *et al.*, 2003). Experiments demonstrating the physiological, immunological and pharmacological properties of saponins have aroused considerable clinical interest in these substances. Various studies have shown the effect of saponins on the immune system. Saponins have been widely used as adjuvants for many years and have been included in several veterinary vaccines. In addition to the immunomodulating effects, saponins have been proved to have antitumoral, antiviral and antimicrobial activity (Plohmann *et al.*, 1997; Hostettmann & Marston, 1995).

Specific saponins not only have stimulatory effects on the components of specific immunity, but also present some non-specific immune reactions such as inflammation (de Oliveira *et al.*, 2001; Haridas *et al.*, 2001) and monocyte proliferation (Delmas *et al.*, 2000; Yui *et al.*, 2001). Some saponins were reported to have the ability to modulate the cell mediated

immune system as well as to enhance antibody production (Oda *et al.*, 2000). However, saponins are surface active agents and cause haemolysis of red blood cells *in vitro*, although haemolysis does not appear to be correlated with the adjuvant activity (Kensil, 1996).

The mechanisms of immune-stimulating action of saponins have not been clearly understood, but many explanations have been put forward. Saponins reportedly induce the production of cytokines such as interleukins and interferons that might mediate their immunostimulant effects (Jie *et al.*, 1984; Kensil, 1996).

To investigate the possibility that lysozyme might serve as a useful marker of the immune-stimulating properties of selected saponins and to examine the contribution of these natural substances to lysozyme production and release in monocytes and epithelial cells, lysozyme activity levels in culture fluids of these two cell types were measured after treatment with selected saponins. Majority of the examined saponins were demonstrated to stimulate significantly the release of lysozyme activity in monocytes (Fig.50,51) and/or epithelial cells (Fig.53,54) upon short-term treatment (1h) at non-toxic concentrations, including ginsenosid-Rd, primulic acid, glycyrrhizin, saponinum album (from *Gypsophila paniculata*), aescin, saponins of *Hydrocotyle vulgaris*, saponins of *Quillaja saponaria* bark, and saponins of *Helianthus annuus* marginal flowers. The obtained results suggested positive contribution of these saponins to lysozyme release of monocytes and/or epithelial cells especially upon short exposure.

Furthermore, demonstrated ability of saponins to enhance the release of lysozyme activity can present a new mechanism that contributes to explaining important biological characteristics of saponins, including antibacterial, antiviral, anti-inflammatory and immune-stimulating properties. The stimulation of immunological system, through elevation of lysozyme activity levels, introduced these saponins as immunostimulant agents from natural origin through lysozyme, the important defense molecule of the innate immune system.

#### **4.9.4. Essential oils**

Essential oils are volatile, natural, complex compounds characterized by a strong odour and formed by aromatic plants as secondary metabolites (Atta-ur-Rahman, 2000). There are several methods for extracting essential oils, including use of liquid carbon dioxide or microwaves, and mainly low or high pressure distillation employing boiling water or hot steam (Masotti *et al.*, 2003; Angioni *et al.*, 2006). Essential oils are very complex mixtures which can contain about 20–60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts. Generally, these major components determine the biological properties of the essential oils (Bakkali *et al.*, 2008).

In nature, essential oils are considered to be part of the preformed defense system of higher plants. They play an important role in the protection of plants as antibacterials, antivirals, antifungals and insecticides (Bakkali *et al.*, 2008; Reichling *et al.*, 2009).

Essential oils are used in aromatherapy as inhalations, orally, as gargles and mouthwashes and transdermally (Evans, 2008). Natural oils are extensively used in folk medicine for the treatment of a growing number of specific pathologies. Some essential oils exhibit particular medicinal properties that have been claimed to cure several organ dysfunctions and systemic disorders (Wabner & Beier, 2009; Silva *et al.*, 2003; Hajhashemi *et al.*, 2003; Perry *et al.*, 2003). Essential oils are used as antimicrobial, antiviral, anti-inflammatory, immunostimulant, antitumor and analgesic remedies, among others (Bakkali *et al.*, 2008; Atta-ur-Rahman, 2000; Wabner & Beier, 2009). Up to the present day, these characteristics have not changed much except that more is now known about some of their mechanisms of action, particularly at the antimicrobial level. Owing to the new attraction for natural products like essential oils, it is important to develop a better understanding of their mode of biological action for new applications in human health. Therefore we investigated the effect of some essential oils on the expression of lysozyme activity in the human monocytic cell line THP-1, and in the human epithelial cell line HT-29. The selection of these essential oils was based on their proven antibacterial, antiviral, anti-inflammatory or immune-stimulating properties.

#### **4.9.4.1. Secretion of lysozyme activity after short-term treatment with essential oils**

Our results showed that essential oils can exhibit different effects on the release of lysozyme activity in epithelial and monocytic cell lines after one hour exposure, depending on the dose and the plant species from which the essential oil was extracted. Both direct and reverse dose-dependent effects were observed.

After one hour treatment of the HT-29 epithelial cells, statistically significant stimulation of lysozyme activity release was demonstrated with tea tree, majoran and anise oil (Fig.62.A,B), suggesting lysozyme as a new mechanism of action for various pharmacological properties of these three oils, such as immune-stimulating, antibacterial and antiviral activity.

On the contrary, pine needle, cinnamon and *Melissa officinalis* oil exhibited significant inhibitory effect with no cytotoxicity on the lysozyme activity released in the culture medium of HT-29 epithelial cells depending on the dose used (Fig.62.A). *Melissa officinalis* oil had the same inhibitory effect with no cytotoxicity on AGS epithelial cells in addition to thyme and chamomile oil, while pine needle and cinnamon oil showed no significant influence when incubated with AGS cells for one hour (Fig.64; Fig.65).

Another group of selected essential oils showed no significant influences on the release of lysozyme activity in HT-29 epithelial cells after one hour exposure regardless of the used doses, including orange blossom, lavender, eucalyptus, rosemary, thyme, chamomile, clove, *Salvia officinalis*, Lippia dulcis and *Nigella sativa* seed oils.

After one hour treatment of the THP-1 monocytes, significant stimulatory influence on the secretion of lysozyme activity of monocytes was found with orange blossom oil (Fig.59.C). This stimulatory effect can introduce a novel mechanism of action for immune-stimulating effectiveness of orange blossom oil.

Cinnamon, anise, lavender, pine needle, chamomile, clove, *Melissa officinalis* and *Lippia dulcis* oil showed inhibitory effects on the lysozyme activity released in THP-1 culture medium, which were statistically significant with no toxicity to cells (Fig.58; Fig.59.A; Fig.60.C; Fig.61.A; Fig.66).

Thyme, *Salvia officinalis* and *Nigella sativa* seed oil were demonstrated to affect lysozyme activity release in monocytes either positively or negatively depending on the dose used (Fig.59.B; Fig.60.A,B).

On the other hand, majoran, tea tree, eucalyptus and rosemary oil showed no influences on lysozyme activity secreted in monocytic cell cultures after one hour incubation regardless of the concentration used.

#### **4.9.4.2. Synthesis & secretion of lysozyme activity after long-term treatment with essential oils**

All examined essential oils incubated with cells for 24 hours exhibited no tendency to cause increases in the secretion and expression of lysozyme activity in the tested cultures.

Most essential oils at non-toxic concentration exhibited no significant effects on lysozyme activity of epithelial and monocytic cells upon long-term exposure, whereas significant inhibitory effects on the expression and release of lysozyme activity were found at toxic concentrations of these oils.

Statistically significant inhibitory effects on lysozyme activity of HT-29 epithelial cells after 24 hours treatment with no cytotoxicity were observed with tea tree, anise, and thyme oil, whereas significant inhibitory effects on lysozyme activity of THP-1 monocytes were detected with thyme, clove, *Melissa*, *Nigella sativa* seed and *Lippia dulcis* oil (Fig.66; Table 22).

#### **4.9.5. Propolis**

Propolis (bee glue) is the generic name for a strongly adhesive resinous substance collected by honey bees from trees and leaf buds. Bees use the propolis along with bees wax to construct their hives. It originates as a gum secretion gathered by bees from a variety of plants, and can vary in color depending on the plant species of origin (Najafi *et al.*, 2007). Aptly named by the Greeks, 'pro' (for or in defense) and 'polis' (the city), propolis is used to protect the entrance of the hive against intrusion of animals and within the hive against a wide spectrum of microorganisms (Banskota *et al.*, 2001; Burdock, 1998). More than 250 individual compounds have been established as the constituents of propolis. Propolis contains mainly resins, balsams and phenolic aldehydes (polyphenols), waxes and fatty

acids, essential oils, pollen, other organics and minerals. Phenolic acids, esters, and flavonoids have been shown to account for most important of propolis composition. Bees modify propolis by glucodiases, enzymes from hypopharyngeal glands, during collection and processing. Results of this enzymatic modification are hydrolyzation of phenolic compounds like flavonoid heterosides to free flavonoid aglycones and sugars and enhancement of the pharmacological action of the resulting products (Marcucci *et al.*, 2001; Najafi *et al.*, 2007).

Propolis has remarkable therapeutic qualities, and is much sought after in some countries for the treatment of a range of human ailments. General medicinal uses of propolis include immune system support and improvement (Scheller *et al.*, 1989a), treatment of respiratory apparatus (for various infections), digestive tract disorders (ulcers and infections), dental care (Ikeno *et al.*, 1991), dermatology (tissue regeneration, ulcers, excema, wound healing-particularly burn wounds, mycosis, mucous membrane infections and lesions), cancer treatment (Grunberger *et al.*, 1988; Scheller *et al.*, 1989b; Hausen *et al.*, 1992; Búfalo *et al.*, 2009) and many others. Propolis compounds including flavonoids, phenolic acids and its esters have anti-inflammatory, antibacterial, antiviral, immunomodulatory, antioxidant and antiproliferative effects (Hu *et al.*, 2005; Noelker *et al.*, 2005; Orsi *et al.*, 2005; Kim *et al.*, 2006; Gekker *et al.*, 2005). Propolis has attracted researchers' interest in the last decades, because of its several biological and pharmacological properties. However the exact mechanisms underlying the biological effects are often not clearly described.

Since propolis shows several biological properties similar to those of lysozyme, we investigated the effects of the ethanolic extracts of propolis on the release of lysozyme activity of different human cells. Upon exposure to German propolis for one hour, THP-1 monocytes showed significantly enhanced lysozyme activity release with no cytotoxicity compared to control cultures (Fig.67.A). Significant stimulatory effect on lysozyme secretion was found with propolis from Syrian origin as well (Fig.67.B).

On the other hand, propolis from both origins exerted no significant effects when incubated with HT-29 epithelial cells, regardless of the concentration used.

The significant stimulatory effect of propolis on lysozyme release of monocytes can explain, at least in part, a novel mechanism for many propolis biological properties, including immune system support and immunomodulatory, antimicrobial, antitumor, anti-inflammatory, antiviral activities as well as treatment of respiratory infections, mucous membrane infections, skin and digestive ulcers and support of wound healing, among others.

#### **4.10. Therapeutic role of lysozyme in peptic ulcer**

##### **➤ Background**

Studies into the content of nonspecific defense factors in blood and biological fluids (saliva, gastric juice, duodenal contents) in patients suffering from peptic ulcer have demonstrated significant changes in local and general immunity (Kulyga *et al.*, 1992). In acute conditions,

nonspecific defence was depressed in peptic ulcer patients (Vinogradskiĭ *et al.*, 1987). Exacerbation of the disease was marked by significant decrease of lysozyme levels (Kulyga *et al.*, 1992; Upyrev *et al.*, 1982; Lobanova & Rudyk, 1980). An increase in lysozyme was recorded after treatment, although normal levels were never attained (Vinogradskiĭ *et al.*, 1987). Following vagotomy, the contents of lysozyme in the blood serum and gastric juice were reportedly higher than following the resection of the stomach. The investigation of factors of non-specific defense of the organism, lysozyme in particular, is thought to be necessary for the estimation of the postoperative period and healing of the ulcer (Upyrev *et al.*, 1982). Furthermore, relationship between the lysozyme concentration in gastric juice and the acidity of gastric contents has been shown. Decreased concentrations of lysozyme were more pronounced in patients with increased levels of hydrochloric acid (Lobanova & Rudyk, 1980). Ulcer-associated cell lineage (UACL), which is mucin-producing cells that form tubular structures and are induced in chronic intestinal ulcers in the human gut, has been proved to produce and secrete lysozyme. Both lysozyme mRNA and protein are found in abundance in the UACL (Stamp *et al.*, 1992). UACL is induced only in chronic inflammatory and ulcerative defence reaction, producing a cocktail of active peptides and proteins, including lysozyme, which would be expected to advance mucosal healing (Wright, 1998).

Moreover, clinical efficiency of lysozyme as a treatment for peptic ulcer has been reportedly proved. Results of Hung *et al.* report (2007) showed that daily intragastric lysozyme chloride can dose-dependently protect gastric mucosal hemorrhagic damage in the atherosclerotic rats. Lysozyme chloride elevated gastric mucus secretion, which contributed to ulcer healing. The results indicated that lysozyme-stimulated gastric mucus secretion was partly mediated by the release of prostaglandins, which possess potent gastric cytoprotective effects (Hung *et al.*, 2007). Another study reported the efficacy of lysozyme compound with molecular iodine and polyiodides as transendoscopic treatment for patients with gastroduodenal ulcers relapse. The relapse of peptic ulcer was accompanied by lysozyme decrease and helicobacter's sowing increase in stomach and duodenal mucosa, as well as mucosa flora increase, especially in periulcer's region. Significant microbicidal and sorptional effects to the revealed strains of microorganisms, increase of mucous membrane barrier properties and shortening of peptic ulcer's scarring time were demonstrated after transendoscopic treatment of peptic ulcer with iodine lysozyme 3-4 times (Bazlov *et al.*, 2001).

The data reported attest to the depression of nonspecific defense factors, lysozyme in particular, in peptic ulcer patients, giving rise to the consideration of these changes as one of potential mechanisms by which ulcer is formed. Therefore, we investigated the effect of herbal extracts and their identified active constituents on the release of lysozyme activity of AGS gastric epithelial cells, which were selected on the basis of proven effectiveness in the prevention and treatment of peptic ulcer.



➤ **Lysozyme and *Helicobacter pylori***

The Gram-negative bacterium *Helicobacter pylori*, which is linked to the majority of peptic ulcers and to some types of gastric cancer and known to be resistant to antibiotic treatment, has been found to be capable of inactivating lysozyme (Kirillov *et al.*, 2003; Lai *et al.*, 2008). Although most Gram-negative organisms are resistant to killing by lysozyme, several studies have demonstrated that lysozyme can synergistically kill Gram-negative bacteria in the presence of agents that damage the Gram-negative outer membrane of the bacteria. These include metal chelators such as EDTA and EGTA which remove stabilizing cations and cause release of LPS molecules; and polycations such as polymyxin B nonapeptide, polylysine, and a family of cationic defense proteins of neutrophils which appear to bind to the anionic core components of LPS molecules thereby altering their relationship within the membrane (Lehrer & Ganz, 1990).

A synergistic agent of special importance is lactoferrin, which has been demonstrated to have membrane permeabilizing activity, alter the Gram-negative outer membrane and consequently enhance bacterial susceptibility to lysozyme. While each protein alone is bacteriostatic, lysozyme and lactoferrin together can be bactericidal for Gram-negative strains such as *V. cholerae*, *S. typhimurium*, and *E. coli* (Ellison & Giehl, 1991).

As lactoferrin and lysozyme are present together in high levels in gastroduodenal secretions as well as in mucosal secretions and neutrophil granules, it is probable that their interaction contributes to host defense against Gram-negative organisms such as *Helicobacter pylori*.

➤ **Natural products with anti-ulcer activity**

Botanical compounds with anti-ulcer activity investigated in this work included various herbal extracts, flavonoids (i.e. quercetin, naringin, silymarin), saponins and essential oils.

The results obtained illustrated that the extract of *Echinacea purpurea* root had statistically significant stimulatory effect on the release of lysozyme activity in AGS gastric cells (Fig.45) as well as HT-29 colon cells (Fig.42.B) after one hour treatment. This stimulatory effectiveness may suggest potential mechanism of this herb in the prevention and treatment of peptic ulcer. The extract of *Echinacea purpurea* has been used traditionally in the treatment of peptic ulcer, but its clinical efficacy has not been documented, according to our knowledge.

On the contrary, extracts of Chinese licorice root and green tea ( $\geq 90\%$  EGCG) as well as the flavonoid curcumin showed dose-dependent inhibitory effects on the secretion of lysozyme activity in cultivated AGS cells (Fig.44). Supercritical CO<sub>2</sub>-fluid extract of chamomile flowers caused also decrease in the levels of released lysozyme activity of AGS gastric epithelial cells (Fig.45), which was also observed with HT-29 colon epithelial cells (Table 11; Fig.41.B).

The flavonoids quercetin, rutin, naringin and silymarin group as well as polysaccharides extracted from *Aloe vera* had no significant effects on the secretion of lysozyme activity when incubated with AGS cells regardless of the concentration examined.

*Quillaja saponaria* saponins showed statistically significant stimulatory effect on the secretion of lysozyme activity in AGS gastric cells (Fig.57.A) in addition to their stimulatory effect on lysozyme activity secretion in HT-29 colon cells (Fig.53.B). On the contrary, aescin, primulic acid and *Helianthus annuus* saponins exerted inhibitory effects on lysozyme activity release in AGS gastric cells (Fig.56; Fig.57.B,C). Glycyrrhizinic acid, ginsenosid-Rd, saponinum album (from *Gypsophila paniculata*) and *Hydrocotyle vulgaris* saponins showed no significant influences on lysozyme activity secreted in cell cultures after one hour treatment.

Among the essential oils tested, chamomile, thyme and *Melissa officinalis* oils exhibited statistically significant inhibitory effects on the release of lysozyme activity in AGS gastric cells (Fig.64; Fig.65). On the other hand, essential oils of majoran, lavender, pine needle, tea tree and *Nigella sativa* seeds showed no significant influences on the release of lysozyme activity of gastric cells.

Taken together, the data obtained suggested the extract of *Echinacea purpurea* root and saponins of *Quillaja saponaria* as herbal remedies for the prevention and treatment of peptic ulcer through their stimulatory effectiveness on lysozyme activity release in gastric and colon epithelial cells. Further investigations on the clinical efficacy of these two natural products in patients with peptic ulcer need to be done. The inhibitory effect or no effect of some natural products mentioned above which considered to have anti-ulcer properties illustrated that lysozyme play no role in the mechanism of action by which these products can prevent or heal peptic ulcers.

Concluding, all natural products investigated in this work and showed stimulatory effects on the production and release of lysozyme activity in AGS gastric epithelial cells as well as in HT-29 colon epithelial cells are suggested to be potential herbal remedies for peptic ulcer prophylaxis and treatment. Despite progress in the conventional chemistry and pharmacology in producing effective drugs, the plant kingdom may provide an important source of new anti-ulcer compounds for development of innovative pharmaceuticals or, alternatively, as simple dietary adjuncts to existing therapies.

#### **4.11. Lysozyme activity of human whole saliva**

It has long been suggested that the presence of lysozyme in biological fluids such as human saliva is an important factor in the nonspecific defense mechanisms of the body against microbial invasion and infections due to the antimicrobial, antiviral and antimycotic/ antifungal properties of this low molecular-weight enzyme (Jenzano *et al.*, 1986).

Many assay methods have been reported to determine lysozyme concentration in human salivary. Attempts to evaluate the results from any one study in terms of those obtained from

another study on the same pathophysiology have been frustrated by the lack of use of a single assay system and by the use of different standard lysozyme preparations. The interaction of lysozyme with components such as bacteria, bacterial cell wall fragments, and proteins, such as mucins, which are found in saliva, has been suggested to present a problem in the accurate determination of lysozyme concentration in saliva. In general, the turbidimetric assay depending on the lysis of *Micrococcus lysodeikticus* has been mostly used to determine lysozyme activity level in saliva and other body fluids. Other investigators have used an immunologically based assay which is not dependent on the expression of biological activity to determine the concentration of lysozyme in saliva. Because the level of active lysozyme may be more relevant for elucidating a defensive role for this protein, an assay method that can determine the active lysozyme in saliva, such as the turbidimetric assay, would provide more meaningful results (Jenzano *et al.*, 1986).

There has been a significant amount of effort on the part of various investigators to relate differences in the concentration of lysozyme in human saliva to various oral or systemic disease states. Lysozyme levels of human saliva have been proved to reduce in many diseases. For example, it was proved that the lysozyme values were low in the saliva of children suffering from chronic tonsillitis or sinusitis, and that they continued to decrease when antibiotics were administered (Hendrich & Pospišil, 1971). The level of salivary lysozyme derived from macrophages may play an important role in determining resistance or susceptibility to some diseases, such as acute bronchitis. These studies raise the interesting possibility that control of cell recruitment and activation in response to an antigenic challenge within the bronchus may be important in determining clinical outcome. Lysozyme may moderate the inflammatory response and pathogenesis of chronic bronchitis by providing an ancillary defence mechanism through both its antibacterial role and its influence on neutrophils. In the former case, lysozyme may aggregate bacteria, ensuring their rapid clearance from the oral cavity, whereby increased swallowing of aggregates may provide a constant antigenic stimulus for phagocytes, and in the case of the latter by inhibiting neutrophil chemotaxis and their toxic oxygen radicals at an inflammatory site and possibly limiting tissue injury and enzyme secretion. A clinical role for pharmacological or immunological products that can enhance lysozyme secretion should be envisaged (Taylor *et al.*, 1995).

Moreover, many studies have indicated that salivary lysozyme concentrations are sensitive to psychological stress and could be utilized as a potential marker in studies looking at the effects of stress on immunity. Salivary lysozyme levels were found to show a negative correlation with the perceived stress level (Perera *et al.*, 1997).

These reports have raised our interest to evaluate the sensitivity and convenience of the micro-plate turbidimetric assay to determine lysozyme activity levels in human saliva.

The microplate turbidimetric assay was proved in our experiments to provide sufficiently linear, specific, accurate, quick, inexpensive and convenient method for quantifying lysozyme activity in human saliva, especially when many samples have to be analyzed (Fig.5).

This should then make one step closer to achieving optimum aim of utilizing lysozyme activity levels in salivary, and in other body fluids as well, to diagnose, prevent and eliminate different diseases.

It is vitally important that the methods for saliva collection and analysis are more standardized to overcome the differences obtained in measuring saliva lysozyme activity.

The use of different assay conditions has complicated the evaluation of studies relating salivary lysozyme levels to oral or systemic disease. Therefore, the evaluation of the results of studies relating to changes in lysozyme level in whole saliva must involve the consideration of assay methods, standards used, and sample preparation.

Furthermore, we compared the levels of lysozyme activity in human whole saliva of 31 healthy non-medicated volunteers of different age groups in regard to sex and age (Fig.70; Table 27).

When the obtained results of lysozyme activity levels of the volunteers were analyzed in regard to sex, no clear correlation could be proved between saliva lysozyme activity levels and sex in the 20-30 years age group. Nevertheless, females older than 30 years showed higher saliva lysozyme activity in comparison with males of the same age group. These data may indicate that women in this age are more capable of fighting infections than men, taking into account that many of the viruses and bacteria find their way into the body through the mouth.

Concerning the age, our results suggested a negative correlation between saliva lysozyme activity levels and age with some exceptions. Normal aging has a marked effect on immunity, with the cumulative evidence indicating that immunity consistently shows age-related decrements in function (Hawkley & Cacioppo, 2004). This age-associated decrease in lysozyme activity levels of human salivary demonstrated in our results may partly contribute to the greater susceptibility of older people to some bacterial and viral infections and cancers, due to the antibacterial, antiviral and antitumor role of lysozyme and its influence on the immune system as well.

## 5. CONCLUSION & OUTLOOK

Plants are an important source for drug discovery, and investigations on biological actions of plant medicinal extracts and phytochemicals, as well as the understanding of the mechanisms underlying these actions, can support the search for novel drugs. Even with the growth in understanding of the human immune system and inflammatory mechanisms and increasing developments in antibiotics; there is a need to identify clinically useful and safe natural products from the plant kingdom which can support the immune system and alleviate or prevent bacterial, viral and even tumor diseases.

Lysozyme is one of the most important factors of innate immunity and a unique enzymatic in that it exerts not only antibacterial activity, but also antiviral, anti-inflammatory, anticancer and immunomodulatory activities. Modulation of lysozyme secretion and/or production may offer novel approaches in the treatment of a variety of diseases. One strategy in the modulation of lysozyme expression may be through the use of herbal medicines, which may offer an explanation of the effects of herbs on the immune system and other tissues.

The principal purpose of the present study was to investigate medicinal plants and phytochemicals as potential resources for identifying stimulators or inhibitors of lysozyme activity levels in human cells.

To achieve this aim it was essential to investigate rapid, sensitive and accurate methods to determine lysozyme levels of human cells. We evaluated, for the first time, a modern and sensitive fluorescence-based assay method of lysozyme activity in comparison to the classical turbidimetric assay of lysozyme activity, and the enzyme-linked immunosorbent assay (ELISA) of human lysozyme. The EnzChek<sup>®</sup> fluorescence-based lysozyme activity assay was proved to be good in regard to sensitivity and linearity. This method had detection limit of lysozyme concentration down to 4units/ml. On the other hand, the turbidimetric microplate lysozyme activity assay was proved to have good linearity but less sensitivity in comparison with the fluorometric assay. It had detection limit down to 12.5units/ml. Nevertheless, this detection limit of the turbidimetric microplate lysozyme assay was enough to provide a simple, accurate and inexpensive method to determine lysozyme levels in tissue or body fluids such as saliva and tears, which contain high lysozyme levels, whereas the EnzChek<sup>®</sup> fluorescence-based assay could provide a high-sensitivity method for detecting lysozyme activity at low levels e.g. in cell culture systems.

In order to measure lysozyme as a protein, we investigated the enzyme-linked immunosorbent assay (ELISA) of human lysozyme, which had detection limit down to 0.156ng/ml. Because the level of active lysozyme may be more relevant for elucidating a defensive role for this protein, a sensitive assay method that can determine the active lysozyme in cell culture supernatants, such as the EnzChek<sup>®</sup> fluorometric assay used mainly in our investigations, would provide more meaningful results.

Furthermore, it was important to identify human cell systems suitable for lysozyme-regulation studies, which can produce and release detectable amounts of lysozyme activity.

The monocytic system, as well as differentiated macrophages, was of special interest for our lysozyme-regulating studies, since they constitute the primary cellular effectors of the immune response, playing a pivotal role in the detection and elimination of foreign bodies such as pathogenic microorganisms. We investigated three monocytic cell lines, THP-1, HL-60 and U-937 regarding their ability to produce lysozyme activity in cell cultures. The human monocytic cell lines THP-1 and HL-60 were proved to produce and secrete detectable amounts of lysozyme activity in cell cultures. Contrary to previous reports, we couldn't find any evidence that the human leukemic monocyte lymphoma cell line U-937 was able to produce and secrete detectable levels of active lysozyme, neither with nor without addition of inducing factors such as PMA, taking into account that the used U-937 was fresh and low-passage cell line derived from the cell bank. In addition, this cell line was previously tested in our laboratory to have the typical characteristics of monocytes, such as differentiation and phagocytic activity (Weng *et al.*, 2008; Weng, 2009).

Another important system for our investigations was the epithelial system, considering that lysozyme is one of the most important host defense molecules in epithelial cells. Our results illustrated that the human colon cell line HT-29 and the gastric cell line AGS were able to produce lysozyme activity in cell cultures at levels markedly higher than those of the cultivated monocytic cells. On the other hand, no detectable amounts of lysozyme activity could be measured in the supernatants of A549 and BEAS-2B lung epithelial cell cultures even after 4 days incubation of cells in culture medium. No detectable levels of lysozyme activity could be measured in the culture medium of BHY human oral squamous cell line as well, whereas primary oral tumor cells were able to secrete active lysozyme in detectable concentrations.

We aimed also to identify natural or synthetic compound as positive control for the stimulatory effect on lysozyme release and production in human cells, which was not easy due to the limited and conflicting results reported in literature.

Differentiation-inducing agents, PMA in particular, have been stated in some reports to induce enhanced expression and release of lysozyme in monocytes even after one hour treatment. Our results suggested that PMA and other differentiation-inducing agents were not able to stimulate lysozyme activity of monocytes constantly in a regular manner. Therefore PMA was not suitable to be used as positive control for our experiments.

The immunosuppressant corticosteroid prednisolone was demonstrated to inhibit significantly the production and release of lysozyme activity measured fluorometrically in human monocytes, which have suggested a novel mechanism of action participates in the immunosuppressive effect of prednisolone, taking into consideration the surveillance

functions of monocytes/macrophages in the immune system as well as the important role of lysozyme as a part of the innate immune system.

Investigating inflammatory response inducers, thioglycolate was illustrated to activate slightly but significantly the microbicidal activity of monocytes through its stimulatory effect on lysozyme activity levels produced by monocytes especially after long-term treatment. Bacterial endotoxins have been reported to activate immunologic and inflammatory responses in the acute phase of bacterial infection, particularly in cells of immunologic system. The obtained results suggested lipopolysaccharides from *Salmonella enteritidis* (LPS) as immunostimulant agent through the stimulation of production and release of lysozyme activity in monocytes and differentiated macrophages. On the other hand, other bacterial products such as Leiber-Beta S and Auxoferm YGT Plus had no stimulatory effect on the secretion of lysozyme activity.

There is only very little data in the literature concerning the influence of medicinal plants and natural products on the production and release of lysozyme activity in human cells.

Several medicinal plants and phytochemicals have well documented immunostimulatory, antibacterial, antiviral anti-inflammatory and/or anti-tumor properties. Since these medicinal plants are potential resources for identifying stimulators or inhibitors of lysozyme activity in human cells, we investigated their possible effects on the production and secretion of lysozyme activity of human monocytes and epithelial cells, which can elucidate a new mechanism of action underlying their therapeutic effects.

Significant stimulatory effect on lysozyme secretion and production in monocytes and/or epithelial cells was observed with the root extract of *Echinacea purpurea* and its phytochemical constituents (alkamides in particular), root aqueous extract of *Rhodiola rosea*, tomatine and tomatine-containing tinctures, aconitine and less significantly with EGCG-rich green tea extract. On the contrary, CO<sub>2</sub>-fluid extracts of hop flowers, rosemary leaves, Chinese licorice root and chamomile flowers inhibited significantly the release and/or production of lysozyme activity in THP-1 monocytes and HT-29 epithelial cells, while extracts of *Aloe vera* leaves and skullcap herb pronounced no significant effects on the release of lysozyme activity.

Concerning saponins, majority of the examined saponins were demonstrated to stimulate significantly the release of lysozyme activity in monocytes and/or epithelial cells especially upon short-term treatment (1h) at non-toxic concentrations, including ginsenosid-Rd, primulic acid, glycyrrhizin, saponinum album (from *Gypsophila paniculata*), aescin, saponins of *Hydrocotyle vulgaris*, saponins of *Quillaja saponaria* bark and saponins of *Helianthus annuus* marginal flowers.

Among the essential oils examined, statistically significant stimulation of lysozyme activity release in HT-29 epithelial cells after short-term exposure was demonstrated with tea tree,

majoran and anise oil, while significant stimulation of lysozyme activity release in THP-1 monocytes was detected with orange blossom oil. On the other hand, pine needle, cinnamon and *Melissa officinalis* oil exhibited significant inhibitory effect with no cytotoxicity on lysozyme activity release in epithelial cells after short-term exposure, whereas significant inhibitory effect on monocytes was observed with cinnamon, anise, lavender, pine needle, chamomile, clove, *Melissa officinalis* and *Lippia dulcis* oil. Thyme, *Salvia officinalis* and *Nigella sativa* seed oils were demonstrated to affect lysozyme activity release in monocytes either positively or negatively depending on the dose used.

Since propolis shows several biological properties similar to those of lysozyme, we investigated the effects of propolis ethanolic extracts on the release of lysozyme activity in human cells. Propolis from different geographical origins was illustrated to have significant stimulatory effect on lysozyme release of monocytes, which can explain, at least in part, a novel mechanism for many propolis biological properties, including immune system support and immunomodulatory, antimicrobial, antitumor, anti-inflammatory and antiviral activities as well as treatment of respiratory infections, mucous membrane infections, digestive ulcers, skin ulcers and support of wound healing among others.

Due to the data reported attest to the depression of nonspecific defense factors, lysozyme in particular, in peptic ulcer patients, as well as the proven anti-ulcer activity of lysozyme, we investigated the effect of herbal extracts and their identified active constituents on the release of lysozyme activity in AGS gastric epithelial cells, which were selected on the basis of proven effectiveness in the prevention and treatment of peptic ulcer. Botanical compounds with anti-ulcer activity investigated in this work included various herbal extracts, flavonoids (i.e. quercetin, naringin, silymarin), saponins and essential oils.

Root extract of *Echinacea purpurea* and saponins of *Quillaja saponaria* were demonstrated to stimulate significantly lysozyme activity release in gastric as well as colon epithelial cells. Stimulation of lysozyme activity release suggested a novel mechanism of action for these natural products as herbal remedies for the prevention and treatment of peptic ulcer. Further investigations on the clinical efficacy in patients with peptic ulcer need to be done. Taken together, all natural products investigated in this work and showed stimulatory effects on the production and release of lysozyme activity in AGS gastric epithelial cells as well as in HT-29 colon epithelial cells are suggested to be potential herbal remedies for peptic ulcer prophylaxis and treatment.

To conclude, demonstrated ability of some herbal extracts and phytochemicals to enhance the release and/or production of lysozyme activity can present novel mechanism of action that contributes to explaining important biological characteristics of these natural products, including antibacterial, antiviral, anti-inflammatory, anti-tumor, anti-ulcer and immune-stimulating properties. The stimulation of immunological system, through elevation of



lysozyme activity levels, can introduce these natural products as immunostimulant agents from natural origin through lysozyme, the important defense molecule of the innate immune system, which is able to control the growth of susceptible bacteria and to modulate host immunity against infections and depressions of immune responses.

Though the illustrated inhibitory activity of some natural products on lysozyme release suggested immunomodulatory properties of these extracts, the obtained data suggested no role of lysozyme activity in the mechanism of action by which they exert their reported antibacterial, antiviral anti-inflammatory and/or anti-tumor effectiveness.

This work presented for the first time novel immune-stimulatory activity of tomatine, aconitine and many other natural products through stimulation of lysozyme activity, which warrants further future investigations.

The stimulatory effects of natural products on lysozyme release and production in human cells have to be further investigated through extensive studies on the molecular mechanisms of the regulation of lysozyme gene expression in cells.

Furthermore, the results obtained in the present study can provide the basis for further studies *in vivo* involve investigating lysozyme levels in blood and biological fluids (saliva, gastric juice, duodenal contents) in course of the treatment with herbal drugs. Elevated lysozyme levels in body fluids can suggest *in vivo* evidence for the immune-stimulatory activity of these natural products among many other biological activities of lysozyme.

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## 7. Publications

**Helal R, Melzig MF.**

Determination of lysozyme activity by a fluorescence technique in comparison with the classical turbidity assay. *Pharmazie* 2008; 63(6): 415-419.

**Helal R, Melzig MF.**

New aspects in the synthesis and secretion of lysozyme by cultured human monocyte cell lines. *In Vitro Cell Dev Biol Anim.* 2010; 46(6): 492-6.

**Helal R, Melzig MF.**

*In vitro* effects of selected saponins on the production and release of lysozyme activity of human monocytic and epithelial cell lines. *Sci Pharm.* 2011; in press. doi:10.3797/scipharm.1012-15.

### Poster

**Helal R, Melzig MF.**

Lysozyme activity determination using a fluorescence technique in comparison with the turbidity assay. Deutsche Pharmazeutische Gesellschaft DPhG Landesgruppentagung. Berlin, Germany, 4<sup>th</sup> July 2008.

**Helal R, Melzig MF.**

Effects of some essential oils and saponins on lysozyme activity in human monocytes and epithelial cells. Deutsche Pharmazeutische Gesellschaft DPhG Landesgruppentagung. Berlin, Germany, 2<sup>nd</sup> July 2010.

**Helal R, Melzig MF.**

Effects of some essential oils and saponins on lysozyme activity in human monocytes and epithelial cells. 58<sup>th</sup> International Congress and Annual Meeting of the Society for Medicinal Plant Research (GA). Berlin, Germany, 29<sup>th</sup> August – 2<sup>nd</sup> September 2010.

**8. Statutory Declaration**

Hereby, I testify that this thesis is the result of my own work and research, except of references given in the bibliography. This work contains material that is the copyright property of others, which cannot be reproduced without the permission of the copyright owner. Such material is clearly identified in the text.

Berlin, 21.03.2011

Racha Helal