Czech University of Life Sciences Prague

Faculty of Environmental Sciences

Department of Applied Ecology

Ph.D. Thesis

Antimicrobial Activity of Selected Alien Plants

Author: Ing. Sherif Taha Soliman Hassan
Specialization: Applied and Landscape Ecology
Supervisor: Doc. Ing. Kateřina Berchová-Bímová, Ph.D.

Prague 2017

Abstract

For decades, plants and their bioactive compounds have been shown to possess various biological activities including but not limited to antimicrobial activities. In this dissertation thesis, several alien plants were investigated for their antimicrobial inhibitory properties against various plant and human pathogens. The results showed that the ethanolic extracts of *Ecballium elaterium*, *Plumbago indica*, *Crisium arvense*, *Stellaria pallida* and *Atriplex sagittata* were the most effective plants against *Fusarium oxysporum* and *Blumeria graminis* at MIC value of 156 µg/mL, while against *Erwinia amylovora* and *Pseudomonas syringae* showed antimicrobial inhibitory properties at MIC value of 220 µg/mL. Plumbagin, the major active compound of *Plumbago indica* L. exerted inhibitory effect against all *Candida albicans* strains with MICs values ranging from 7.41 to 11.24 µg/mL. The additive effect of plumbagin when combined with amphotericin B at concentrations of (0.12, 0.13 and 0.19, 1.81 µg/mL, respectively) was obtained against five of seven strains tested with ΣFIC ranging from 0.62 to 0.91. In addition, plumbagin was found to be used safely for topical application when combined with amphotericin B at concentrations corresponding to the additive effect. Plumbagin exerted anti-HCV activity compared with that of telaprevir with IC_{50} values of 0.57 and 0.01 µM/L, respectively and selectivity indices SI= 53.7 and SI= 2127, respectively. Cucurbitacin B (Cuc B), an active compound of *Ecballium elaterium* exerted direct growth-inhibitory activity against all *Staphylococcus aureus* (*S. aureus*) strains tested with MICs values ranging from 0.15 to 0.44 µg/mL, as well as synergy effect with tetracycline or oxacillin against four of six *S. aureus* strains tested (ΣFIC ranging from 0.29 to 0.43). Cuc B showed remarkable anti-HSV-1 activity compared with that of acyclovir with IC_{50} values of 0.94 and 1.74 µM, respectively and selectivity indices SI= 127.7 and SI>132.2, respectively. The aqueous extract of *Hibiscus sabdariffa* (AEHS) exerted remarkable bacteriostatic effect against all *Helicobacter pylori* (HP) strains tested with MICs values ranging from 9.18 to

16.68 µg/mL. Synergy effect of AEHS with clarithromycin or metronidazole was obtained against four out of seven HP strains tested with ΣFIC ranging from 0.21 to 0.39. The additive effect of AEHS with amoxicillin was obtained against five out of seven HP strains tested with ΣFIC ranging from 0.61 to 0.91. Protocatechuic acid, an active substance of *Hibiscus sabdariffa* showed potent anti-HSV-2 activity compared with that of acyclovir, with EC_{50} values of 0.92 and 1.43 µg/mL, respectively, and selectivity indices > 217 and > 140, respectively. Based on these results, we can conclude that alien plants and their bioactive substances have potential application in the development of antimicrobial agents for combating diseases caused by plant and human pathogens, and could serve as safe and eco-friendly agents to environment and human health as well.

Keywords: antimicrobial activity; alien plant species; plant and human diseases; allelopathic effect; fungicides; bactericides; phytochemicals

Author's Declaration

I hereby declare that I am the sole author of this thesis with a help of literature listed in references.

Prague: …………………………......

Sherif Taha Soliman Hassan

ACKNOWLEDGEMENT

I would like to express my deepest gratitude and appreciation to my supervisor Doc. Ing. Kateřina Berchová-Bímová, Ph.D. for her guidance and support during this work and during my study, her help was greatly needed and I deeply appreciated that. I am grateful to Prof. MVDr. Alois Čížek, CSc. for giving me the opportunity to perform the antibacterial and antifungal activities in his laboratory at Department of Infectious Diseases and Microbiology, Faculty of Veterinary Medicine, University of veterinary and Pharmaceutical Science Brno, Czech Republic. I would like also to thank all colleagues at the Department of Natural Drugs, Faculty of Pharmacy, University of veterinary and Pharmaceutical Science Brno, Czech Republic, for their support and giving me the opportunity to perform the analytical part of this work. Special thanks to Department of Experimental Biology, Division of Microbiology, Masaryk University, Brno, Czech Republic, for the help of performing the antiviral activities.

I would like to express a sincere appreciation to my mother and brother for their unlimited support. My colleagues and friends have given me a great amount of support. This journey would not have been possible without their encouragement and contagious enthusiasm for science and friendship. I dedicate this dissertation thesis to the memory of my father.

The dissertation thesis was funded by Internal Grant Agency (IGA) of the Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Czech Republic. Projects No. 20134234/2013, 20144244/2014 and 20154247/2015.

Table of Contents

1. Introduction ... 9

2. The histroy of plant-derived chemicals in medicinal and agricultural practices ... 10

3. The role of alien plants as a source of new antimicrobial agents ... 12

4. Major groups of antimicrobial compounds from higher plants including alien plants ... 13

4.1. Phenolics and Polyphenols ... 13

4.1.1. Flavones, flavonoids, and flavonols ... 14

4.1.2. Tannins ... 15

4.1.3. Coumarins ... 16

4.2. Terpenoids and Essential Oils ... 16

4.3. Alkaloids ... 17

4.4. Quinones ... 17

5. Aim of the dissertation thesis ... 18

5.1. Objectives ... 18

6. Experimental section ... 19

6.1. Materials and methods ... 19

6.1.1. Plant Collection ... 19

6.1.2. Microorganisms ... 22

Evaluation of antimicrobial activities of selected alien plant species extracts against plant pathogens ... 23

Abstract ... 23

1. Introduction ... 23

1.1 Invasive taxa in the Czech Republic ... 23

2. Materials and methods ... 25

2.1 Extraction procedure ... 25

2.2. Antifungal activity ... 25

2.2.1. Fungi strains, cultures and antimycotics ... 25

2.3. Antibacterial activity ... 26

2.3.1. Bacterial strains, cultures, chemicals and antibiotics ... 26

3. Results and discussion ... 27

Plumbagin, a plant-derived compound, exhibits antifungal combinatory effect with amphotericin B against *candida albicans* clinical isolates and anti-hepatitis C virus activity ... 30

Abstract ... 30

1. Introduction ... 31

2. Materials and methods ... 33

2.1. Anticandidal activity ... 33

2.1.1. Fungi strains, cultures and antimycotics 33
2.1.2. Antifungal activity assay 33
2.1.3. Combinatory effect of plumbagin with amphotericin B 34
2.1.4. Cell toxicity assay 34
2.2. Antiviral activity 35
2.2.1. Cultures, cells and reagents 35
2.2.2. Cytotoxicity assay 36
2.2.3. Anti-HCV activity and immunoblot measurements 36
3. Results 37
4. Discussion 40
5. Conclusion 41
Cucurbitacin B interacts synergistically with antibiotics against *Staphylococcus aureus* clinical isolates and exhibits antiviral activity against HSV-1 42
Abstract 42
1. Introduction 43
2. Materials and methods 45
2.1. Anti-*Staphylococcus aureus* activity 45
2.1.1. Bacterial strains, cultures, chemicals and antibiotics 45
2.1.2. Antimicrobial assay 46
2.1.3. Combinatory effect of Cuc B with antibiotics 46
2.2. Anti-HSV-1 activity 47
2.2.1. Viral strains, cultures, cell lines and reagents 47
2.2.2. Determination of cytotoxicity 47
2.2.3. Antiviral assay 48
2.2.4. Statistical analysis 48
3. Results and discussion 48
4. Conclusion 52
***In vitro* synergistic effect of *Hibiscus sabdariffa* aqueous extract in combination with standard antibiotics against *Helicobacter pylori* clinical isolates 53**
Abstract 53
1. Introduction 54
2. Materials and methods 55
2.1 Bacterial strains, cultures and antibiotics 55
2.2 Preparation of plant material 55
2.2.1 Plant collection 55
2.2.2 Extraction procedure 56
2.2.3 Determination of total flavonoid content (TFC) in AEHS 56

2.2.4 Determination of total anthocyanins (TAC) in AEHS 56
2.3 Antimicrobial assay 57
2.4 Combination effect of AEHS with standard antibiotics 58
3. Results 58
4. Discussion 60
5. Conclusion 61
Protocatechuic acid from *Hibiscus sabdariffa* L. Exhibits Antiviral Activity against HSV-2 62
Abstract 62
1. Introduction 62
2. Materials and Methods 63
2.1. Plant Collection and Extraction Procedure 63
2.1.1. Determination of Concentration of PCA in Plant Material 63
2.2. Antiviral Activity against HSV-2 64
2.2.1. Viral Strains, Cultures, Cell lines and Reagents 64
2.2.2. Determination of Cytotoxicity 64
2.2.3. Anti-HSV-2 Activity 65
2.2.4. Statistical Analysis 65
3. Results 66
3.1. Determinations of Concentration of PCA in Plant Material 66
3.2. Anti-HSV-2 Activity and Cytotoxicity 66
4. Discussion 67
5. Conclusion 68
Overall of the importance of the results 69
Concluding remarks 69
References 71

1. Introduction

Plant diseases caused by pathogenic microorganisms significantly attribute to the overall loss in crop yield worldwide and many of them cause reduction of the shelf life and market values of food commodities and render them unfit for human consumption (Choi and Kim, 2008). Application of synthetic chemicals has long been the major choice to control variety of plant diseases. However, extensive use of agrochemicals has raised serious problems including appearance of resistant pathogens, chemical residues, and threats to human health and the environment (Mdee, et al 2009). There has been a growing interest in the research of the possible use of the plant-derived natural fungicides and bactericides such as plant extracts or pure isolated compounds, which can be relatively eco-friendly for disease control in agriculture or humans (Choi et al., 2008). Some of alien plants adversely affect on native ecosystem by unbalancing nutritional flows among the members, eventually threaten biodiversity. Extensive efforts for integrated management of harmful alien species have been being conducted including cataloging alien species and ecological risk assessment (Psysek et al., 2012). One of the main activities for management of alien plant species has been the physical eradication with little success due to their prolific nature. On the other hand, the successful habitation of the alien species has prompted intense interest in the mechanisms for the success (Pimentel et al., 2005). The prolific nature and successful invasion in new habitats suggest that the non-native species are hypothesized to be equipped with novel biochemistry that repels native species or unique compounds in the native flora. In this aspect, alien plant species can be useful sources for discovering new active compounds serving as antimicrobial agents (Cappuccino & Arnason, 2006). It has been reported that the leaf extracts of *Oxalis corniculata, Chromolaena odorata, Antigonon leptopus* have promising potential antifungal and antibacterial activities with a low MIC values renging from 0.3 to 1.9 µg/mL (Bajpai et al., 2011). The aim of this theis is to investigate selected alien plant species for their

antimicrobial activity against plant and human pathogens in order to produce an eco-friendly product, which could be useful for the treatment of plant diseases and human diseases as well.

2. The histroy of plant-derived chemicals in medicinal and agricultural practices

The history of plants and their ethnopharmacologic properties is rather old and dates back to the time when the early man became conscious of his environment. Plants have been used in virtually all cultures as a source of medicine (Lanfranco, 1999). The earliest record of human civilization and culture of China, Egypt, Assyria, and Indies valley reveals that the elders and wise men of those times used herbal medicines to treat various diseases. Information regarding these medicinal herbs is available in the old literature, epic poems and copper plates which are preserved even today. The excavation of Shanidar cave in Iraq in 1963 revealed the grave of Neanderthal man buried sixty thousand years ago along with many flowers of his time. The plants found in the grave were later identified having various medicinal properties (WHO, 2002).

One of the earliest records of the use of plants is that of Chaulmoogra oil from *Hydnocarpus gaertn*, which was known to be effective in the treatment of leprosy. Such a use was recorded in the pharmacopoeia of the Emperor of China between 2730 and 3000 B.C. Similarly, the seeds of the opium poppy (*Papaver somniferum*) and castor seeds (*Ricinus communis*) were excavated from some ancient Egyptian tombs, which indicated their use in that part of Africa as far back as 1500 B.C. The records available in “Ebers papyrus” also confirm that alien plants were used at that time in Egypt (Baquar, 1995). The ancient use of plants for healing purposes forms the origin of much of modern medicine. Many conventional drugs originate from plant sources, a century ago. Examples include aspirin (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine The

development of drugs from plants including alien plants continues, with drug companies engaged in large scale pharmaceutical screening of herbs (Tyler et al., 1976).

More than 800 million people in developing countries do not have adequate food supplies and at least 10% of food is lost due to plant diseases (Strange and Scott, 2005). Plant diseases are caused by pathogens such as fungi, bacteria, and viruses. Compared to other plant parasites, fungi cause the greatest impact with regard to diseases and crop production losses. This includes considerable foliage and post-harvest losses of fruits and vegetables which are brought about by decay due to fungal plant pathogens.

Common fungal diseases include powdery mildew, rust, leaf spot, blight, root and crown rots, damping-off, smut, anthracnose, and vascular wilts. Some notorious plant pathogenic fungi include *Pythium*, *Phytophthora*, *Fusarium* and *Rhizoctonia* spp, which cause root and crown rot, and seedling damping-off in many vegetables and ornamental plants. Apart from causing diseases in plants, many species of Fusarium, Aspergillus, Penicillium and Alternaria are also sources of important mycotoxins of concern in animal and human health (Robert and Richard, 1992; Eaton and Gallagher, 1994; Smith, 1997; Placinta et al., 1999). For example, aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* may cause liver cancer. The most important method of protecting plants against fungal attack is, the use of fungicides. However, many fungicidal agents in the market are toxic and have undesirable effects on other organisms in the environment. Furthermore, halogenated hydrocarbons such as methyl bromide, widely used to control soil-borne pathogens, have ozone-depleting potential (Abritton and Watson, 1992). Some synthetic fungicides are non-biodegradable, and hence can accumulate in soil, plants and water, and consequently effect humans through the food chain. The development of resistance of pathogenic fungi towards synthetic fungicides is of great concern. There is, therefore, a motivation to find safe, efficacious and environmentally

friendly fungicides. Plants have, and continue to be, sources of antifungal agents (Hostettmann et al., 2000).

3. The role of alien plants as a source of new antimicrobial agents

Many plant species contain vast quantity of antimicrobial compounds. To develop commercial products, a large quantity of the species has to be cultivated, raising an additional level of complication. If alien species contain good antifungal activity they may be a useful source of antifungl compounds or extracts because large quantities of material are available. (Vila and Weiner, 2004) considered whether alien plant species are better competitors than native plant species and concluded that this may be the case. There is another possibility that have not been addressed as far as we could ascertain. If fungal pathogens play an important role in the growth or establishment of plant species, alien species may have better resistance against plant pathogens. It has been found that the alien plant *Melianthus comosus* has excellent antifungal and antibacterial activities (Eloff et al., 2006). *Robinia pseudoacacia* has shown to possess antibacterial activity against *Escherichia coli* and *Proteus myxofaciens*. Its antibacterial activity was found to be related to its rich content of polyphenols (Lukasiewicz et al., 2015; Marutescu et al., 2017). Resveratrol, a stilbene-type phenolic compound found in *Fallopia* spp., reported to exert antimicrobial activity agaisnt wide range of microorganisms (Ferreira and Domingues, 2016). Ethanolic extract of *Fallopia japonica* showed antibacterial activity against *Acinetobacter baumanii* and antifungal activity against *Candida albicans* (Bardon et al., 2014). It is believed that the antimicrobial activities of alien plants are related to their allelopathy effect in a form of production of chemicals that inhibit the growth of other plants or/and microorganisms (Chengxu et al., 2011). Considering the importance of alien plants as a source of antimicrobial agents, it should be taken into consideration the risk of

their invasion and hence their impact on environment. Also, it should be taken into consideration the safe means of planting and harvesting these plants from wild populations in order to use them in medicinal or agricultural practices.

4. Major groups of antimicrobial compounds from higher plants including alien plants

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Georges et al., 2011). Most are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total (Schultes, 1978). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor (e.g., the terpenoid capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds (Piller, 1975). The major natural plant-derived compounds categorized into their classification are described below wtith emphasis on their medicinal and agricultural uses.

4.1. Phenolics and Polyphenols

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses (Wild, 1994), bacteria (Brantner et al., 1996 and Thomson, 1978), and fungi (Duke, 1985). Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two-OH

groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Geissman, 1963). In addition, some authors have found that more highly oxidized phenols are more inhibitory (Scalbert, 1991 and Urs et al., 1975). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason et al., 1987). Phenolic compounds possessing a C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well. Eugenol is a well-characterized representative found in clove oil. Eugenol is considered bacteriostatic against both fungi (Duke, 1985) and bacteria (Thomson, 1978).

4.1.1. Flavones, flavonoids, and flavonols

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). The addition of a 3-hydroxyl group yields a flavonol (Fessenden et al., 2005). Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection (Dixon et al., 2008), it should not be surprising that they have been found *in vitro* to be effective Catechins, the most reduced form of the C_3 unit in flavonoid (186, 224). The catechins inactivated cholera toxin in Vibrio (Thastrup et al., 2002) and inhibited isolated bacterial glucosyltransferases in *S. mutans* (Gallagher, 1994), possibly due to complexing activities described for quinones above. This latter activity was borne out *in vivo* tests of conventional rats. When the rats were fed a diet containing 0.1% tea catechins, fissure caries (caused by *S. mutans*) was reduced by 40%. compounds, deserve special mention. These flavonoids have been extensively researched due to their occurrence in oolong green

teas. It was noticed some time ago that teas exerted antimicrobial activity (Toda et al., 2007) and that they contain a mixture of catechin compounds. These compounds inhibited *in vitro Vibrio cholerae* O1, *Streptococcus mutans*, and Shigella spp (Hasegawa et al., 2009), and other bacteria and microorganisms antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described above for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Duke, 1985). Flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifrancheside (Hasegawa et al., 2009), glycyrrhizin (from licorice), and chrysin against wide range of plant fungal, bacterial, and viral diseases (Adams and Moss, 2000).

4.1.2. Tannins

"Tannin" is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Their molecular weights range from 500 to 3,000 (Fessenden et al., 2005), and they are found in almost every plant part: bark, wood, leaves, fruits, and roots (Gallagher, 1994). They are divided into two groups, hydrolyzable and condensed tannins. Hydrolyzable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Tanins were found to possess antimicrobial activity against wide range of plant and human pathogens. Scalbert (Stern et al., 1996) reviewed the antimicrobial properties of tannins in 1991. He listed 33 studies which had documented the inhibitory activities of tannins derived from several plants including alien plants up to that point. According to these studies, tannins can be toxic to filamentous fungi, yeasts, and bacteria. Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity

(Watanbe et al., 2006). Although this is still speculative, tannins are considered at least partially responsible for the antibiotic activity of methanolic extracts of the bark of *Terminalia alata* found in Nepal (Vohora et al., 2007). This activity was enhanced by UV light activation (320 to 400 nm at 5 W/m^2 for 2 h). At least two studies have shown tannins to be inhibitory to viral reverse transcriptases (Sato et al., 2002).

4.1.3. Coumarins

Coumarins are phenolic substances made of fused benzene and a-pyrone rings (161). They are responsible for the characteristic odor of hay. They exhibited a remarkable antimicrobial activity against plant and human pathogens.

4.2. Terpenoids and Essential Oils

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids. Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are methanol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids). Terpenenes or terpenoids are active against bacteria, fungi, viruses, and protozoa (Tsuchiya et al., 2004). In 1977, it was reported that 60% of essential oil derivatives examined were inhibitory to fungi while 30% inhibited bacteria (Zafriri et al., 2012). In addition, the inhibitory activity of essential oils against viruses has been reported (San-Blas et al., 2007).

4.3. Alkaloids

Heterocyclic nitrogen compounds are called alkaloids. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy Papaver somniferum (69); the name morphine comes from the Greek Morpheus, god of dreams. Codeine and heroin are both derivatives of morphine. Diterpenoid alkaloids, commonly isolated from the plants of the *Ranunculaceae*, or buttercup family (Vijaya at al., 2001), are commonly found to have antimicrobial properties (Addis et al, 2011). Berberine is an important representative of the alkaloid group, and found to have potent antifungal activity against plant fungal diseases (Tan et al., 2003).

4.4. Quinones

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. These compounds, being colored, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin (Schmidt, 1988). Their presence in henna gives that material its dyeing properties (Hu et al., 2010). The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxidation and reduction reactions. The individual redox potential of the particular quinone – hydroquinone pair is very important in many biological systems; witness the role of ubiquinone (coenzyme Q) in mammalian electron transport systems. Vitamin K is a complex naphthoquinone. Its antihemorrhagic activity may be related to its ease of oxidation in body tissues (Harris, 2006). Hydroxylated amino acids may be made into quinones in the presence of suitable enzymes, such as a polyphenoloxidase (Vamos-Vigyazo, 2011). In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern et al., 1996), often leading to inactivation of the protein and loss of function. For that reason, the potential range of quinone antimicrobial effects is great.

Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism. As with all plant-derived antimicrobials, the possible toxic effects of quinones must be thoroughly examined. Kazmi et al., 1994. described an anthraquinone from Cassia italica, a Pakistani tree, which was bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae*. Hypericin, an anthraquinone from St. John's wort *(Hypericum perforatum*), has received much attention in the popular press lately as an antidepressant, and Duke reported in 1985 that it had general antimicrobial properties (Duke, 1985).

5. Aim of the dissertation thesis

- Investigation and screening of selected alien plant species for antimicrobial activity against plant and human pathogens.
- Produce a useful product that can be used for the treatment of plant and human diseases, and to be relatively eco-friendly for disease control and to environment as well.

5.1. Objectives

- Evaluation and initial screening of the selected alien plants for antimicrobial activity *in vitro*.
- Selection of one or more plant species with promising activities for further study.
- Identification of the active compound(s) from the selected plant(s) species.
- Determination of antimicrobial activity of isolated compound(s) *in vitro*.

6. Experimental section

6.1. Materials and methods

6.1.1. Plant Collection

The alien plant species selected to the work have been collected from the Botanical Garden of Palacky University in Olomouc, Czech Republic, while *Hibiscus sabdariffa* calyces were collected from the Northern Part of Aswan, Egypt. The used plants are *Stellaria pallida*, *Convolvulus arvensis*, *Robinia pseudoacacia*, *Ecballium elaterium*, *Plumbago indica*, *Rumex alpinus*, *Conyza canadensis*, *Atriplex sagittata*, *Oxalis dillenii*, *Cirsium arvense*, *Echinochloa crus-galli*, *Acer negundo*, *Aillanthus altissima Solidago canadensis* and *Hibiscus sabdariffa.* The following table describes corresponding information regarding localities, collection duration, plant parts and taxonomic identification of plant materials used in this thesis.

Plant name and Type	Family and Place of origin	Plant parts	Locality	Collection duration
Stellaria pallida (archaeophyte)	Caryophyllaceae (North America)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Convolvulus arvensis (archaeophyte)	Convolvulaceae (Southern Europe and Asia)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014

Robinia pseudoacacia (neophyte)	Fabaceae (Central and Estern North America)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Ecballium elaterium (neophyte)	Cucurbitaceae (North Africa)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Rumex alpinus (neophyte)	Polygonaceae (The mountains of central and southern Europe)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Conyza canadensis (neophyte)	Asteraceae (North America)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Atriplex sagittata (archaeophyte)	Amaranthaceae (Central and southern Europe)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Oxalis dillenii (neophyte)	Oxalidaceae (Eastern and central North America)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Cirsium arvense	Asteraceae	Aerial parts	Botanical Garden of	Spring season of

(archaeophyte)	(Eastern Europe and Northern Asia)		Palacky University in Olomouc, Czech Republic	2014
Echinochloa crus-galli (archaeophyte)	Poaceae (Eastern Asia)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Acer negundo (neophyte)	Sapindaceae (Eastern and central North America)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Ailanthus altissima (neophyte)	Simaroubaceae (Eastern Asia)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Solidago canadensis (neophyte)	Asteraceae North America (Alaska)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014
Hibiscus sabdariffa (neophyte)	Malvaceae (West Africa)	Aerial parts and calyces*	Botanical Garden of Palacky University in Olomouc, Czech Republic, and Northern part of Aswan, Egypt	Spring and summer seasons of 2014

Plumbago indica (neophyte)	Plumbaginaceae (Southern Asia)	Aerial parts	Botanical Garden of Palacky University in Olomouc, Czech Republic	Spring season of 2014

* *Hibiscus sabdariffa* calyces were collected from Northern part of Aswan, Egypt.

6.1.2. Microorganisms

The following strains of bacteria were used: Methicillin-resistant *Staphylococcus aureus* (MRSA), *Erwinia amylovora*, *Pseudomonas syringae*, *Staphylococcus aureus* and *Helicobacter pylori*. The yeast strain used in this study was *Candida albicans* and the fungi *Fusarium oxysporum* and *Blumeria graminis*. The viral strains used in the study were Hepatitis C Virus (HCV) and Herpes Simplex Virus type-1 (HSV-1) and type-2 (HSV-2). All growth conditions and antibiotic references are described in experimantal sections.

Evaluation of antimicrobial activities of selected alien plant species extracts against plant pathogens

Abstract

In this study, 14 alien plant species extracts were evaluated for their antimicrobial activities against plant pathogens. Antimicrobial activity was determined by broth microdilution method. The results showed that the ethanolic extracts of *Ecballium elaterium*, *Plumbago indica*, *Crisium arvense*, *Stellaria pallida* and *Atriplex sagittata* were the most effective plants against *Fusarium oxysporum* and *Blumeria graminis* at MIC value of 156 µg/mL, while against *Erwinia amylovora* and *Pseudomonas syringae* showed inhibitory properties at MIC value of 220 µg/mL. This indicates that these plant extracts have good potential application in combating of diseases caused by plant and bacterial pathogens. Therefore, the search of the responsible bioactive compounds for such activities is greatly needed.

1. Introduction

1.1 Invasive taxa in the Czech Republic

According to Pyšek et al. (2012), the recent update of the alien plant checklist of the Czech Republic labelled 61 taxa as invasive. Invasive species are defined as one that forms self-replacing populations over many life cycles, produces reproductive offspring, often in very large numbers at considerable distances from the parent and/or site of introduction, and has the potential to spread over long distances (Blackburn et al. 2011; Richardson et al. 2000). It has been introduced another definition of invasive plants by Pyšek et al. (2012) that the metapopulation criterion to separate invasive species from naturalized, to account for the historical population dynamics of the respective taxa, and classified the invasion status based on the population history viewed from the current perspective, i.e. the state in which the populations of a given species exist at present. Therefore, some taxa previously considered

invasive are now classified as naturalized, reflecting the 'boom-and-bust phenomenon' (Williamson 1996, Blackburn et al. 2011). Another principle adopted was that of the highest stage achieved at the population level, reflecting that individual populations of alien species may occur in a region in different stages of the INIC (e.g. Essl et al. 2009; Saltonstall et al. 2010). Therefore, if some populations of a species reached the invasion stage, the species is classified as invasive. Among the taxa (till 2012) considered as invasive are 11 archaeophytes (*Angelica archangelica subsp. archangelica*, *Arrhenatherum elatius*, *Atriplex sagittata*, *Cirsium arvense*, *Conium maculatum*, *Digitaria ischaemum*, *Echinochloa crus-galli*, *Eragrostis minor*, *Portulaca oleracea subsp. oleracea*, *Prunus cerasifera*, and *Stellaria pallida*). and 50 neophytes species.

In this thesis, it has been used alien neophytes species occur in a wide range of habitats in the Czech Republic (*Robinia pseudoacacia*, *Ecballium elaterium*, *Plumbago indica*, *Rumex alpinus*, *Conyza canadensis*, *Oxalis dillenii*, *Acer negundo*, *Ailanthus altissima*, *Solidago canadensis* and *Hibiscus sabdariffa*), while archaeophytes species used in this study are (*Atriplex sagittata*, *Cirsium arvense*, *Echinochloa crus*-galli, Stellaria *pallida*, and *Convolvulus arvensis*). For decades, alien plant species did not attract the researchers' consideration to investigate their phytochemical profiles for medicinal uses (Blackburn et al. 2011). This is due to several reasons. Most importantly, alien plants in many regions all over the world are not used in traditional folk medicine because of their possible toxicity to humans. Moreover, in developing countries, the high costs and the lack of facilities or laboratory equipment for performing such experiments hurdle or limit the use of these plants (Saltonstall et al. 2010). Therefore, little information regarding the phytochemical profiles of alien plants is limited. In this thesis, we aim to shed light on the importance of alien plants as a source of antimicrobial agents that could be used to combating infections caused by plant

and human pathogens as well as to focus attention on their safety to environment as alternative to synthetic chemicals.

2. Materials and methods

2.1 Extraction procedure

250 g of each air-deried plant material extracted by Ethanol, Methanol and Ethyl Acetate via a maceration extraction method for 3 dayes. The mixtures were filtered, and the solutions evaporated to dryness. The extracts were stored at 4 ºC for further use.

2.2. Antifungal activity

2.2.1. Fungi strains, cultures and antimycotics

Fusarium oxysporum (CCM 4354) and *Blumeria graminis* (CCM 8325) strains were obtained form Czech Collection Microorganisms (CCM) from the Department of Experimental Biology, Faculty of Science, Masaryk University, Czech Republic, and amphotericin B was obtained from Sigma-Aldrich (Germany). All extracts were dissolved in Dimethyl Sulfoxide (0.05% DMSO; Sigma-Aldrich, Czech Republic) and concentrations that have been used the experiment were prepared by dilution with deionized water. Concentrations of DMSO in deionized water, which are equal to concentrations of DMSO in experimental solutions, were used as a blank control. For antifungal assay, the strains were grown in Mueller–Hinton broth (MHB; Oxoid, Basingstoke, UK) (CLSI, 2008 and Vijayarathna et al., 2012).

2.2.2. Antifungal activity assay

Fusarium oxysporum (CCM 4354) and *Blumeria graminis* (CCM 8325) strains were used as control strains. Amphotericin B was used as a reference antimycotic drug. DMSO and deionized water were used as a blank control that did not inhibit any strain tested. Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method

according to the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2008) using 96-well microtiter plates modified according to the recommendations that have been recommended for the more effective determination of anti-infective potential of natural products (Cos et al., 2006). Briefly, Ten twofold serial dilutions of each plant extract were prepared in the appropriate broth concentrations ranging from 2 to 520 µg/mL. Each well was inoculated with 5 µL of fungal suspension at a density of 10^7 CFU/mL, while microtiter plates were incubated at 37 ºC for 48 h, and fungal growth was determined as turbidity by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. MICs were determined as the lowest concentrations that inhibited the growth of the test fungi by ≥80% compared to that of the agent-free growth control. MICs were obtained from three independent experiments that performed in triplicate.

2.3. Antibacterial activity

2.3.1. Bacterial strains, cultures, chemicals and antibiotics

Ampicilin was purchased from Sigma-Aldrich (Prague, Czech Republic). *Erwinia amylovora* (CCM 8261) and *Pseudomonas syringae* (CCM 6531) obtained form Czech Collection Microorganisms (CCM) from the Department of Experimental Biology, Faculty of Science, Masaryk University, Czech Republic Dimethyl Sulfoxide (0.05% DMSO; Sigma-Adrich, Czech Republic) was used to dissolve each plant extract. For antimicrobial assay, the strains were grown in cation-adjusted Mueller–Hinton broth (MHB; Oxoid, Basingstoke, UK) equilibrated with Tris–buffered saline (Sigma-Aldrich, Prague, Czech Republic).

2.3.2. Antibacterial assay

For antibiotic susceptibility testing, *Erwinia amylovora* (CCM 8261) and *Pseudomonas syringae* (CCM 6531) were used as reference strains. Ampicilin was used as a reference antibiotic drug. Each plant extract at concentrations ranging from 2 to 520 µg/mL was used. 0.05% DMSO and deionized water were used as negative controls that did not inhibit any strain tested. The broth microdilution method using 96-well microtiter plates was performed to determine the minimum inhibitory concentrations (MICs) following the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2009). Briefly, the samples were two-fold diluted in MHB (100 µL), and inoculated with bacterial suspension to reach the density of 5×10^5 CFU/mL. Microtiter plates were incubated at 37 °C for 24 h, and bacterial growth was determined as turbidity by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. MICs were subjected as the lowest concentrations that inhibited the growth of the test bacteria by ≥80% compared with that of negative controls. MICs obtained from three parallel experiments, each performed in triplicate.

3. Results and discussion

The results showed that no inhibitory activities of methanolic and ethyl acetate extracts of all tested plants were observed, while the ethanolic extracts of *Ecballium elaterium*, *Plumbago indica*, *Crisium arvense*, *Stellaria pallida* and *Atriplex sagittata* were the most effective plants against *Fusarium oxysporum* and *Blumeria graminis* at MIC value of 156 µg/mL (Table 1), while against *Erwinia amylovora* and *Pseudomonas syringae* showed antibacterial inhibitory properties at MIC value of 220 µg/mL (Table 2).

Table 1: *In vitro* antifungal activity of ethanolic extracts of *Ecballium elaterium*, *Plumbago indica*, *Crisium arvense*, *Stellaria pallida* and *Atriplex sagittata* and amphotericin B against *Fusarium oxysporum and Blumeria graminis* strains

Strains	MIC (µg/mL)					
	Amphotericin B	*Ecballium elaterium*	*Plumbago indica*	*Crisium arvense*	*Stellaria pallida*	*Atriplex sagittata*
Fusarium oxysporum (CCM)	52 ± 0.12	156 ± 0.22	156 ± 0.31	156 ± 0.34	156 ± 0.16	156 ± 0.18
Blumeria graminis (CCM)	35 ± 0.20	156 ± 0.24	156 ± 0.22	156 ± 0.24	156 ± 0.22	156 ± 0.25

MIC─ Minimum inhibitory concentration; the values are expressed as an average from three independent experiments that performed in triplicate
Values presented are means ±S.D. of three independent experiments
CCM: Czech Collection Microorganisms

Table 2: *In vitro* antibacterial activity of ethanolic extracts of *Ecballium elaterium*, *Plumbago indica*, *Crisium arvense*, *Stellaria pallida* and *Atriplex sagittata* and ampicilin against *Erwinia amylovora and Pseudomonas syringae* strains

Strains	MIC (µg/mL)					
	Ampicilin	*Ecballium elaterium*	*Plumbago indica*	*Crisium arvense*	*Stellaria pallida*	*Atriplex sagittata*
Erwinia amylovora (CCM)	57 ± 0.16	220 ± 0.34	220 ± 0.30	220 ± 0.29	220 ± 0.23	220 ± 0.14
Pseudomonas syringae (CCM)	87± 0.23	220 ± 0.21	220 ± 0.28	220 ± 0.23	220 ± 0.20	220 ± 0.19

MIC─ Minimum inhibitory concentration; the values are expressed as an average from three independent experiments that performed in triplicate
Values presented are means ±S.D. of three independent experiments
CCM: Czech Collection Microorganisms

From the above obtained results, it is clear that the solvent plays an important role of the inhibitory properties of tested plants against the fungal and bacterial pathogens used in this study. For instance, the methanolic and ethyl acetate extracts did not show any inhibition activities; this is due to the bioactive compounds, which are resposible for antimicrobial activities have not been extracted by those solvents. It is known that the solvents used in extraction procedures play an important role of the extracted content of biologically active compounds (Laghari et al., 2010; Rahman et al., 2011). Therefore, it is very crucial to choose the most suitable solvent in extractive method to obtain the higher content of bioactive compounds. The extensive use of synthetic fungicides or bacericides in the treatment of plant deseases caused by fungal and bacterial pathogens, has led to the developement of drug resistant strains (Atta and El-Sooud, 2004). It is very important to search for alternative sources that provide bioavailability, less resistance and relatively friendly to eco-system. It is

known that alien plant species have negative impact on eco-system through their highly invasion in environment. Several studies have revealed that the allelopathic effect of alien plants is responsible for their agressive invasion into the environment that mainly suppress the growth of other plants surrounding them (Tiley, 2010; Cripps et al., 2011; Zia et al., 2011). In this context, we may suggest that the inhibition properties of *Ecballium elaterium*, *Plumbago indica*, *Crisium arvense*, *Stellaria pallida* and *Atriplex sagittata* against fungal and bacterial pathogens is related to their content of antimicrbial agents. Therefore, we can conclude that these plants showed excellent ability to inhibit the growth of *Fusarium oxysporum*, *Blumeria graminis, Erwinia amylovora* and *Pseudomonas syringae*. Thus, have potential application in the treatment of diseases caused by fungal and bacterial pathogens or could be used as a preventive agents against these infections. In addition, these plants provide a cheap solution to treat plant diseases in comparison with using commercial antifungal and antibacterial agents with providing safety to environments.

In the next chapters of this thesis, *Ecballium elaterium*, *Plumbago indica* and *Hibiscus sabdariffa* have been chosen for further investigations to identify the bioactive compounds and examine them against human pathogens as well as investigate their cytotoxicity.

Plumbagin, a plant-derived compound, exhibits antifungal combinatory effect with amphotericin B against *candida albicans* clinical isolates and anti-hepatitis C virus activity

Abstract

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), the major active constituent of *Plumbago indica* L. has been shown to be effective against a wide range of infectious microbes. In this study, plumbagin has been evaluated *in vitro* for its antifungal combinatory effect with amphotericin B against *Candida albicans* (*C. albicans*) clinical isolates, and anti-hepatitis C virus (HCV) activity. Antifungal activity was determined by broth microdilution method and combinatory effect was evaluated by checkerboard assay according to ΣFIC indices, while cytotoxicity was determined by MTT assay. Anti-HCV activity was determined in infected Huh7.5 cells using qRT-PCR and cytotoxicity was evaluated by MTT assay. Plumbagin exerted inhibitory effect against all *C. albicans* strains with MICs values ranging from 7.41 to 11.24 μg/mL. The additive effect of plumbagin when combined with amphotericin B at concentrations of (0.12, 0.13 and 0.19, 1.81 μg/mL, respectively) was obtained against five of seven strains tested with ΣFIC ranging from 0.62 to 0.91. In addition, plumbagin was found to be used safely for topical application when combined with amphotericin B at concentrations corresponding to the additive effect. Plumbagin exerted anti-HCV activity compared with that of telaprevir with IC_{50} values of 0.57 and 0.01 μM/L, respectively and selectivity indices SI= 53.7 and SI= 2127, respectively. Our results present plumbagin as a potential therapeutic agent in the treatment of *C. albicans* and HCV infections.

Keywords: Plumbagin; Antimicrobial agents; fungal infection; HCV infection; natural products

1. Introduction

In the last two decades, *Candida* species have become the most common cause of systemic fungal infections worldwide (Kim et al., 2011; Miceli et al., 2011). *Candida albicans* (*C. albicans*) is the main cause of candiduria and candidemi with high morbidity and mortality (40–60%). In addition, it is the most frequently isolated pathogenic fungi in immunocompromised patients that cause localized invasive mucosal mycosis or life-threatening disseminated and deep-seated organ infections (Lilic and Haynes, 2007; Staniszewska et al., 2013; Shirtliff et al., 2009). In recent years, the increasing use of antibiotics has led to the problem of drug-resistant strains, and thus has led to the failure of current treatment regimens of fungal infections (Dai et al., 2009). Therefore, a new treatment strategy has been applied to overcome the problem by using plant-derived products in combination with antimycotics to enhance the treatment efficacy of fungal infections as well as reducing toxicity and side effects (Shirtliff et al., 2009; Hassan et al., 2015; Aiyegoro and Okoh, 2009; Wagner, 2011).

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus belonging to the family Flaviviridae. Approximately 170 million people worldwide are infected with HCV, and many of them died from HCV-related liver diseases (Lindenbach and Rice, 2005; Penin et al., 2004). To date, there is no prophylactic vaccine available to prevent HCV infection. Recently, a new generation of Direct-acting antiviral agents (DAA) has become available and currently the interferon-free combination therapy of sofosbuvir (NS5B polymerase inhibitor), ledipasvir (NS5A replication complex inhibitor) and telaprevir has resulted in a cure rate up to 100% among patients infected with HCV (Sofia, 2014; Summers et al., 2014). However, this regimen has an unfavorable side effect profile (including flu-like symptoms, hemolytic anemia, and depression), which often leads to discontinuance of therapy (Stankiewicz-Drogoń et al., 2010; Kayali and Schmidt, 2014; Gentile et al., 2014). Thus, there is a strong medical

need to discover novel agents with a high therapeutic index and few side effects to treat chronic HCV infection.

Plumbago indica L. (Plumbaginaceae) is a medicinal plant that is well known for its traditional uses as a thermogenic, antimicrobial, anti-inflammatory, abortifacient, anti-periodic, carminative, digestive, nerve stimulatory and rejuvenating drug (Joy et al., 2001). It is commercially important as a major source of plumbagin (Fig. 1). Plumbagin (5-hydroxy-2 methyl-1,4-naphthoquinone), naturally occurring yellow pigment accumulating mainly in roots of the plant *Plumbago indica*, showed anticancer, antimalarial, antifertility, hyperglycemic, and hypolipidemic properties and possesses antimicrobial activity against a wide spectrum of microorganisms including anticandidal activity (Mossa et al., 2004; de Paiva et al., 2003; Singh and Udupa, 1997). This study was designed to evaluate *in vitro* antimicrobial and combination effect of plumbagin with amphotericin B against *C. albicans* clinical isolates, and evaluate its safety for topical administration as well as antiviral activity against HCV.

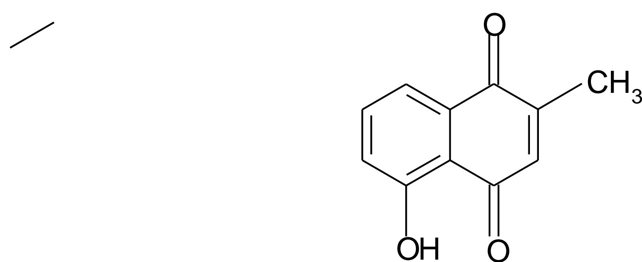

Fig 1. Chemical structure of plumbagin.

2. Materials and methods

2.1. Anticandidal activity

2.1.1. Fungi strains, cultures and antimycotics

Plumbagin, *C. albicans* (ATCC 10231) strain and amphotericin B were obtained from Sigma-Aldrich (Germany). Plumbagin in all experiments was dissolved in Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Czech Republic) and concentrations that have been used in each experiment were prepared by dilution with deionized water. Concentrations of DMSO in deionized water, which are equal to concentrations of DMSO in experimental solutions, were used as a blank control. In addition, 0.1% of DMSO has been found to be the highest concentration that can be safely used for dissolving plumbagin. Six clinical isolates of *C. albicans* (CA1, CA2, CA3, CA4, CA5 and CA6; isolated from patients with vaginal yeast infection) were obtained from The Motol Univesity Hospital, Prague, Czech Republic. For antifungal assay, the strains were grown in Mueller–Hinton broth (MHB; Oxoid, Basingstoke, UK) (CLSI, 2008 and Vijayarathna et al., 2012).

2.1.2. Antifungal activity assay

C. albicans (ATCC 10231) was used as a control strain. Amphotericin B was used as a reference antimycotic drug. DMSO and deionized water were used as a blank control that did not inhibit any strain tested. Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method according to the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2008) using 96-well microtiter plates modified according to the recommendations that have been recommended for the more effective determination of anti-infective potential of natural products (Cos et al., 2006). Briefly, Ten twofold serial dilutions of plumbagin were prepared in the appropriate broth concentrations ranging from 5 to 412 µg/mL. Each well was inoculated with 5 µL of fungal suspension at a density of 10^7 CFU/mL, while microtiter plates were incubated at 37 ºC for 48 h, and fungal growth was determined as

turbidity by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. MICs were determined as the lowest concentrations that inhibited the growth of the test fungi by ≥80% compared to that of the agent-free growth control. MICs were obtained from three independent experiments that performed in triplicate.

2.1.3. Combinatory effect of plumbagin with amphotericin B

Checkerboard assay was used to determine the combination effect of amphotericin B with plumbagin. The combination effect was evaluated algebraically based on the sum of the fractional inhibitory concentration (ΣFIC) indices as previously described (Rondevaldova et al., 2015; Vuuren and Viljoen, 2011). In brief, twofold serial dilutions of amphotericin B prepared in horizontal rows of microtiter plate were subsequently cross-diluted vertically by twofold serial dilutions of plumbagin. The one-half MIC of plumbagin and amphotericin B were used as a starting concentration in combinations. For evaluation of combinatory effect of plumbagin (A) with antimycotic drug tested (B), the ΣFIC was calculated based on the following equation: $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_A$ (in the presence of B) / MIC_A (alone), and $FIC_B = MIC_B$ (in the presence of A) / MIC_B (alone). The MICs used in this equation are the averages of MICs obtained from three independent experiments that performed in triplicate. The interpretation of the *in vitro* antifungal interactions was determined as follows: synergistic effect if $\Sigma FIC \leq 0.5$; additive if $\Sigma FIC > 0.5$ and ≤ 1; no interaction if $\Sigma FIC > 1$ and ≤ 4; and antagonistic if $\Sigma FIC > 4$ (Novy et al., 2013).

2.1.4. Cell toxicity assay

The toxic effect of plumbagin in combination with amphotericin B on HaCaT keratinocytes (HaCaT cell line, ATCC, USA; obtained from The Motol Univesity Hospital, Prague, Czech Republic) was evaluated by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Di Grazia et al., 2014). Briefly, HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat

inactivated Fetal Bovine Serum (FBS; Gibco, USA), L-glutamine (4 mM) and 0.05 mg/mL gentamicin, at 37 °C and 5% CO_2, in 25 cm^2 flasks. 10^4 cells in their culture medium supplemented with 2% FBS were seeded in each well of the microtiter plate. After overnight incubation at 37 °C in 5% CO_2 atmosphere, the plumbagin in combination with amphotericin B at concentrations that exhibited the antifungal additive effect (0.12, 0.13 and 0.19, 1.81 µg/mL, respectively) were added to HaCaT cells in their culture medium supplemented with 2% FBS. After 24 h of treatment, the tested compounds were removed and the cells were incubated with Hank's buffer containing 0.5 mg/mL MTT solution (Islas-Rodrìguez et al., 2009). After 4 h incubation at 37 °C, formazan crystals were solubilized with acidified isopropanol and the absorbance of the samples was measured at 570 nm using a microplate reader (Infinite M200, Tecan, Salzburg, Austria). Cell viability was calculated with respect to the control (untreated cells).

2.2. Antiviral activity

2.2.1. Cultures, cells and reagents

Huh7.5 human liver cells were obtained from The Motol Univesity Hospital, Prague, Czech Republic, and were cultured in Dulbecco's Modified Eagle's Medium (DMEM), which was supplemented with 10% inactivated Fetal Bovine Serum (FBS; Gibco, USA) and 1% penicillin-streptomycin. The cells were cultured at 37 ºC in 5% CO_2, released with 0.05% trypsin-EDTA and split twice a week. The plasmid pFL-J6/JFH/JC1, which contains the full-length chimeric HCV cDNA was obtained from The Motol Univesity Hospital, Prague, Czech Republic. Vero cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS and antibiotics (100 U/mL penicillin G and 100 mg/mol streptomycin).

2.2.2. Cytotoxicity assay

The Huh7.5 cells were used in the assay; Huh7.5 cells (1×10^4 cells/well) were planted into 96-microwell plates. After 6 h, the culture media was replaced with fresh medium containing the tested compound at various concentrations. Cytotoxicity was evaluated with the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Reed and Muench, 1983) at 96 h, and the 50% cytotoxic concentration (CC_{50}) was calculated.

2.2.3. Anti-HCV activity and immunoblot measurements

The antiviral activity of plumbagin against HCV was tested in infected Huh7.5 cells using the real-time reverse transcription PCR (qRT-PCR) and evaluated as previously described by Peng et al. (2011). Telaprevir (VX-950), a NS3/4A protease inhibitor (Sigma-Aldrich, USA), was used as a positive control. PCR master-mix (as per manufacturer's instructions, Applied Biosystems) contains primer pairs of 5'-CGGGAGAGCCATAGTGGTCTGCG-3' and 5' CTCGCAAGCACCCTATCAGGCAGTA-3', and TaqMan probe 5'-FAM-AGGCC TTGTGGTACTGCCT-TAMRA-3' was used. Fluorescent signals were detected with 7500-fast real time PCR system (Applied Biosystems) using the following conditions: 30 minutes at 48°C followed by 10 minutes at 95 °C, 40 cycles of 15 seconds at 95°C, and 1 minute at 60 °C. Briefly, The Huh7.5 cells were seeded into six-well plates (Costar) at a density of 3×10^4 cells/cm^2. The cells were infected with HCV viral stock at an infective dose of 45 IU/cell after 6 h incubation, and treated simultaneously with plumbagin, telaprevir, or the solvent control. After 96 h inoculation, the culture medium was removed and the total intracellular RNA and total intercellular protein were extracted with RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The intracellular HCV RNA was quantified using a one-step RT-PCR kit (Invitrogen). The half maximal inhibitory concentration (IC_{50}) was calculated according to the

method (Reed and Muench, 1983). Selectivity index (SI) value was calculated as the ratio of CC_{50}/IC_{50}. Immunoblot experiments were similar to those previously described by Peng et al. (2011).

3. Results

The results demonstrated that plumbagin exhibited remarkable inhibitory activity against all *C. albicans* strains tested with MICs values ranging from 7.41 to 11.24 µg/mL (Table 1). The additive antifungal interaction of plumbagin in combination with amphotericin B at concentrations of (0.12, 0.13 and 0.19, 1.81 µg/mL, respectively) was obtained against CA (ATCC 10231), CA3, CA4, CA5, and CA6 strains with ΣFIC ranging from 0.62 to 0.91 (Table 2). In few cases ΣFIC values were close to 0.62, which can be considered as strong additive effect (close to the border of synergy). It has been found that no additive effect was observed when plumbagin was combined with amphotericin B at concentration of 0.5 µg/mL (Plumbagin at 0.28 and amphotericin B at 0.22 µg/mL) and the additive effect was observed at combined concentrations of 2 µg/mL (Plumbagin at 0.19 and amphotericin B at 1.81 µg/mL) and 0.25 µg/mL (Plumbagin at 0.12 and amphotericin B at 0.13 µg/mL). Based on these results, we may suggest that the ratio of plumbagin to amphotericin B (lower than one) plays an important role in the additive antifungal interaction. Therefore, for the combination effect, it should be taken into consideration the concentration and ratio between both compounds in combination. Moreover, no synergy or antagonistic effects have been observed. Plumbagin when combined with amphotericin B, showed weak cytotoxicity against HaCaT keratinocytes cells (98.85 and 96.77% viable cells at 0.12, 0.13 and 0.19, 1.81 µg/mL, respectively) (Table 3). In addition, Plumbagin showed antiviral activity against HCV compared with that of telaprevir with IC_{50} values of 0.57 and 0.01 µM/L, respectively and selectivity indices SI= 53.7 and SI= 2127, respectively (Table 4). For evaluating the effect of plumbagin on HCV replication, the expression of intracellular hA3G protein and HCV NS3

protein were evaluated in the Huh7.5 cells with or without using the test compound. Plumbagin treatment exhibited higher intracellular hA3G protein levels in a dose-dependent manner (Fig. 2), while showed lower HCV NS3 protein levels (Fig. 3) in a dose-dependent manner. Thus, plumbagin has been shown to inhibit HCV replication.

Table 1: *In vitro* anticandidal activity of amphotericin B and plumbagin against *C. albicans* strains

C. albicans strains	MIC (µg/mL)	
	Amphotericin B	Plumbagin
CA (ATCC 10231)	8.28 ± 0.38	10.14 ± 0.35
CA1*	7.26 ± 0.30	10.68 ± 0.38
CA2*	9.15 ± 0. 35	11.24 ± 0.36
CA3*	11.21 ± 0.32	9.32 ± 0.31
CA4*	8.38 ± 0.32	7.41 ± 0.33
CA5*	10.24 ± 0.35	10.47 ± 0.31
CA6*	7.43 ± 0.33	10.35 ± 0.34

MIC─ Minimum inhibitory concentration; the values are expressed as an average from three independent experiments that performed in triplicate
Values presented are means ±S.D. of three independent experiments
ATCC: American Type Culture Collection
*: Clinical isolates

Table 2: *In vitro* combinatory anticandidal effect of plumbagin with amphotericin B against *C. albicans* strains

C. albicans strains	Concentrations of the antifungal compounds in combination (µg/mL)							
	Plumbagin at 1 and amphotericin B at 2		Plumbagin at 0.19 and amphotericin B at 1.81		Plumbagin at 0.28 and amphotericin B at 0.22		Plumbagin at 0.12 and amphotericin B at 0.13	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
CA (ATCC 10231)	9.14 ± 0.39	2.91 ± 0.03	9.38 ± 0.32	**0.66 ± 0.03**	10.32 ± 0.38	2.47 ± 0.04	9.73 ± 0.36	**0.69 ± 0.03**
CA1*	10.14 ± 0.37	1.14 ± 0.01	10.17 ± 0.35	1.76 ± 0.04	7.17 ± 0.37	2.74 ± 0.04	7.64 ± 0.33	2.24 ± 0.04
CA2*	10.36 ± 0.30	1.21 ± 0.02	11.12 ± 0.37	1.22 ± 0.04	8.78 ± 0.32	1.41 ± 0.03	7.79 ± 0.32	2.21 ± 0.03
CA3*	11.45 ± 0.33	2.73 ± 0.01	10.78 ± 0.36	**0.88 ± 0.03**	9.14 ± 0.34	1.31 ± 0.02	8.74 ± 0.31	**0.84 ± 0.02**
CA4*	13.41 ± 0.34	3.25 ± 0.03	7.78 ± 0.31	**0.82 ± 0.02**	11.75 ± 0.31	2.17 ± 0.03	9.69 ± 0.34	**0.78 ± 0.02**
CA5*	8.37 ± 0.36	1.27 ± 0.04	6.46 ± 0.36	**0.89 ± 0.03**	9.47 ± 0.34	1.99 ± 0.02	10.78 ± 0.35	**0.91 ± 0.03**
CA6*	7.17 ± 0.35	3.57 ± 0.03	8.14 ± 0.33	**0.62 ± 0.02**	6.12 ± 0.37	1.47 ± 0.02	11.17 ± 0.34	**0.63 ± 0.02**

ΣFIC: sum of fractional inhibitory concentrations—the combination interaction is evaluated as follows: synergy ΣFIC ≤ 0.5; additive ΣFIC >0.5 and ≤ 1 (bold font indicates additive acting combinations); no interaction ΣFIC>1 and ≤ 4; antagonistic if ΣFIC>4
MIC─ Minimum inhibitory concentration; the values are expressed as an average from three independent experiments that performed in triplicate
Values presented are means ± S.D. of three independent experiments
ATCC: American Type Culture Collection
*: Clinical isolates

Table 3: Cytotoxic effect of plumbagin in combination with amphotericin B on HaCaT cells

Compound	Concentration µg/mL	% Cell viability
Plumbagin plus amphotericin B	0.12 and 0.13	98.85
Plumbagin plus amphotericin B	0.19 and 1.81	96.77

% Cell viability; all data were average values from three independent experiments

Table 4: Anti-HCV activity and cytotoxicity of plumbagin

Compound	IC_{50} (µM/L)	CC_{50} (µM/L)	SI
Plumbagin	0.57 ± 0.20	30.65 ± 1.25	53.7
VX-950	0.01 ± 0.40	21.27 ± 4.70	2127

All data were average values from three independent experiments
Values presented are means ± S.D. of three independent experiments
CC_{50}: 50% cytotoxic concentration
IC_{50}: Half maximal inhibitory concentration
SI: Selectivity index (CC_{50}/IC_{50})
VX-950: Telaprevir (positive control)

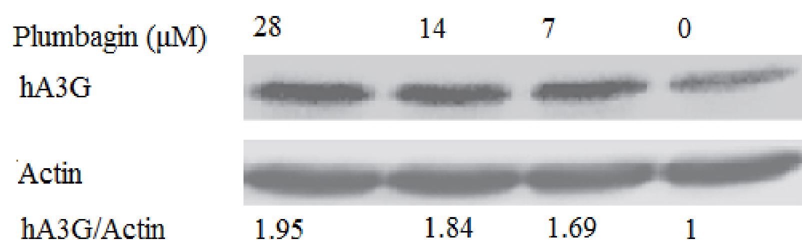

Fig 2. Effect of plumbagin on HCV replication; after 24 h of plumbagin treatment in naïve Huh7.5 cells, intracellular hA3G protein level has been increased dose-dependently.

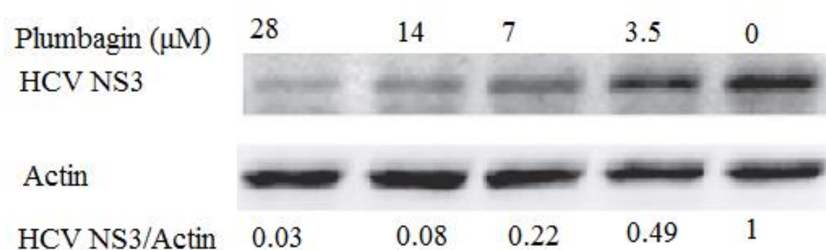

Fig 3. Effect of plumbagin on HCV replication; after 72 h of plumbagin treatment in HCV infected Huh7.5 cells, HCV NS3 protein synthesis has been decreased dose-dependently.

4. Discussion

Infectious diseases remain the main cause of morbidity and mortality among humans, especially in developing countries. Plants remain the main source of biologically active substances with great bioavailability, low toxicity, less resistance and various mechanisms of action (Hassan at al., 2015; Gibbons, 2008; Cavero et al., 2013). Therefore, their application in the treatment of microbial infections has gained much interest in research field. Despite limited studies have reported the direct anticandidal growth-inhibitory activity of plumbagin, these studies did not focus on the possibility of its combination effect with commercial standard antimycotics. Therefore, we report for the first time the additive effect of plumbagin with amphotericin B as well as the direct inhibitory activity against *C. albicans* strains, and anti-HCV activity. Although plant extracts containing plumbagin have been used in folk medicine for decades, there is much evidence to suggest that it may have potential value as a chemotherapeutic agent; however, concerns have been raised about its safety, perhaps due to reports on its vesicant and abortifacient properties. These have been adequately dealt with in several reports that have been previously discussed (Talcott et al., 1985; Babula et al., 2007; Ogihara et al., 1997). Plumbagin is not only potentially beneficial for the treatment of various diseases, it has also been reported to have many side effects in a dose–dependent manner such as diarrhea, increase in white blood cell counts, skin rashes, increase in serum phosphate and acid phosphate level, and reproductive toxicity in male and female animals (Chandrasekaran and Nagarajan, 1981; Singh and Udupa, 1997; Premakumari et al., 1977; Bhargava, 1984; Kini et al., 1997). It has been reported that cytotoxic action of plumbagin and related quinones is mainly due to two different mechanisms, namely, redox cycling and reaction with glutathione (GSH). In addition, topical preparations containing plumbagin should be used with care, and taking into consideration the non-toxic dosses (Inbaraj and Chignell, 2004). It has been reported that cytotoxicity of antimicrobial compounds can be improved by

combining them, at low concentrations, with non-toxic compounds (Anantharaman et al., 2010; Mangoni et al., 2000). Based on these facts, we tried to evaluate the cytotoxic effect of plumbagin on mammalian cells and we found that plumbagin in combination with amphotericin B could be used safely for topical administration at concentrations of 0.12, 0.13 and 0.19, 1.81 µg/mL, respectively) against *C. albicans* infection. Plumbagin is very efficient in treating infectious diseases caused by *C. albicans*. However, to explain the mechanism of action against *C. albicans* have to be determined by further studies *in vivo* and in clinical trials. Plumbagin exhibited anti-HCV activity at a non-cytotoxic concentration (CC_{50}= 30.65 µM/L), and thus can play an important role in pharmaceutical industry as a natural antiviral agent.

5. Conclusion

In summary, there is growing interest in natural products as they provide health benefits. Plant-derived products are believed to contain many bioactive compounds with various biological activities. The present findings indicated that plumbagin exhibited *in vitro* notable antifungal and additive effect with amphotericin B against *C. albicans* clinical isolates. This combinatory effect offers the possibility to decrease the toxicity of plumbagin and to improve its therapeutic efficacy against *C. albicans* infection. Moreover, plumbagin exhibited anti-HCV activity, and thus has promising application in the treatment of HCV infection. However, further studies should be carried out to eliminate the possible adverse effects of this compound by using improved delivery techniques prior its possible practical application. In addition, further research focused on validation of its activity *in vivo* and in clinical trials will be necessary.

Cucurbitacin B interacts synergistically with antibiotics against *Staphylococcus aureus* clinical isolates and exhibits antiviral activity against HSV-1

Abstract

The search for biologically promising compounds from natural sources against microbial diseases remains an important theme in drug discovery to overcome problems with drug-resistant strains. In this study, Cucurbitacin B (Cuc B), an active constituent of *Ecballium elaterium* L., has been investigated *in vitro* for its synergy effect with antibiotics against clinical isolates of *Staphylococcus aureus* (*S. aureus*) and anti-HSV-1 activity. Broth microdilution method was used to determine the antibacterial activity, while checkerboard assay was used to evaluate the synergy effect according to ΣFIC indices. The anti-HSV-1 activity was determined by the plaque number reduction assay, while cytotoxicity was evaluated by MTT assay. In this study, Cuc B exerted direct growth-inhibitory activity against all *S. aureus* strains tested with MICs values ranging from 0.15 to 0.44 µg/mL, as well as synergy effect with tetracycline or oxacillin against four of six *S. aureus* strains tested (ΣFIC ranging from 0.29 to 0.43). Cuc B showed remarkable anti-HSV-1 activity compared with that of acyclovir with IC_{50} values of 0.94 and 1.74 µM, respectively and selectivity indices SI= 127.7 and SI>132.2, respectively. This study presents Cuc B as a promising therapeutic agent in the development of anti-staphylococcal and anti-HSV-1 drugs.

Keywords: *Ecballium elatrium*; cucurbitacin B; HSV; *Staphylococcus aureus*; synergy

1. Introduction

Ecballium elaterium L. (*E. elaterium*, squirting cucumber, Cucurbitacae) is a wild-growing medicinal plant found abundantly in the Mediterranean region. The interior of the fruits contains black seeds and juice. In traditional folk medicine, *E. elaterium* fruits have been used as antimicrobial, analgesic, antipyretic and antiphlogistic agents. Moreover, *E. elaterium* is of interest in Mediterranean region due to the use of its fruits extracts in various medicinal uses (Raikhlin-Eisenkraft and Bentur, 2000; Bohlooli et al., 2012; Khalil and Qaoud, 1993). It has been reported that the main active compounds from the fruits that are responsible for the biological activities including antimicrobial properties were found to be fatty acids, proteins, cucurbitacins (B, D, E, I and L) and cucurbitacin derivatives such as glycosylcucurbitacins and triterpenoids glycosides (Chen et al., 2005; Rao et al., 1974; Attardet al., 2005). Cucurbitacin B (Cuc B, Fig 1), an active constituent of *E. elaterium*, is a natural tetracyclic triterpene compound that belongs to Cucurbitacins (CUs) compounds, which are widely distributed in the family of Cucurbitaceae. Structurally, CUs are characterized by a tetracyclic cucurbitane nucleus skeleton, namely, 9β-methyl-19-nor lanosta-5-enea, which is traditionally divided arbitrarily into twelve categories, incorporating CUs A-T (Chen et al., 2005). Numerous studies demonstrated that Cuc B possesses a variety of bioactivities, such as antibacterial, antifungal, anti-inflammatory, hepatoprotective and anticancer activities (Chen et al., 2005; Chen et al., 2012; Liu et al., 2008). For decades, plants remain the main source of biologically active compounds that have been used for the treatment of various diseases, including infectious diseases with reduced side effects, bioavailability, less resistance, low toxicity and various mechanisms of action (Hassan et al., 2015; Cowan, 1999; Mabona et al., 2013). In recent years, plant-derived chemicals are increasingly used in pharmaceutical industry (Gibbons, 2008; Fischer et al., 2013). *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium has become one of the most serious human pathogens that cause serious

infections, such as bacteraemia, severe pneumonia and skin infections, while methicillin-resistant *S. aureus* (MRSA) is considered one of the most main causes of antibiotic-resistant healthcare-associated infections worldwide (Pandey, 2007; Sharifi-Rad et al., 2014; Cutler and Wilson, 2004; Appelbaum, 2007). The intensive use of antibiotics has led to the problem of drug-resistant strains, and thus has led to the failure of current treatment regimens of staphylococcal infections in humans (Drago et al., 2014; Farrell et al., 2014). Therefore, a new treatment strategy has been devoted to overcome the problem by using plant-derived products in combination with antibiotics to enhance the treatment efficacy (Aiyegoro and Okoh, 2009; Wagner, 2011). Herpes simplex virus (HSV) infections are quite common in humans. HSV is a member of Herpesviridae, a wide family of enveloped-DNA viruses that cause several clinically significant syndromes in both adults and neonates. HSV-1 is mainly connected with oral or facial infection and encephalitis (Hassan et al., 2015; Paludan et al., 2011; Field, 2011). Treatment of HSV infection remains a main target for many researchers worldwide, where it cannot be managed by vaccination. Acyclovir and related nucleoside analogs have been widely used in the treatment of HSV, but the intensive use of such drugs has led to several undesirable effects including drug-resistant strains (Piret and Boivin, 2011; Evans et al., 2013). Despite few studies have reported that crude extracts of *E. elatrium* and various fractions of cucurbitacins have been shown to possess direct anti-staphylococcal growth inhibitory activity (Adwan et al., 2011; Oskay et al., 2009; Dogruoz et al., 2008), these studies did not evaluate the possibility of antimicrobial combinatory effect of Cuc B with standard antibiotics against *S. aureus*. Therefore, in this study, we report the synergistic effect of Cuc B with tetracycline (TET) or oxacillin (OX) as well as the direct inhibitory effect against *S. aureus*. In addition, we report for the first time the antiviral activity of Cuc B against HSV-1.

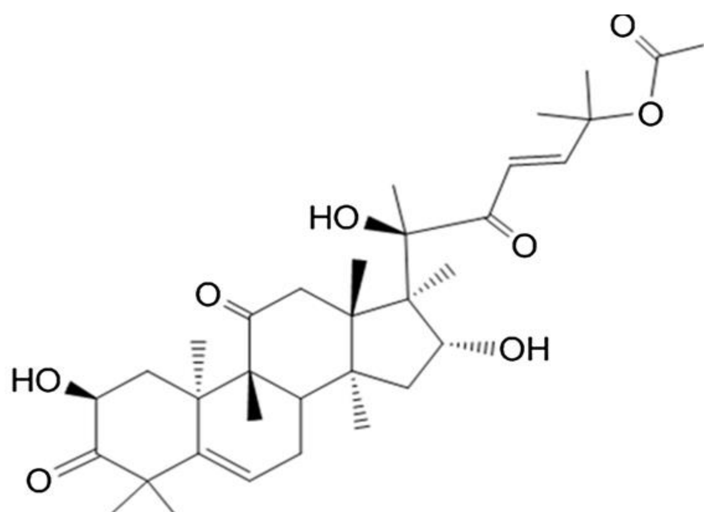

Fig. 1: Chemical structure of cucurbitacin B

2. Materials and methods

2.1. Anti-Staphylococcus aureus activity

2.1.1. Bacterial strains, cultures, chemicals and antibiotics

Tetracycline, oxacillin and Cuc B were purchased from Sigma-Aldrich (Prague, Czech Republic). *S. aureus* (ATCC 29213) and methicillin-resistant *S. aureus* (MRSA-ATCC 43300) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Four clinical isolates of *S. aureus* (SA1, SA2, SA3, and SA4; isolated from patients with skin infection) were obtained from U Sv. Anny Univesity Hospital, Brno, Czech Republic. Dimethyl sulfoxide (0.05% DMSO; Sigma-Adrich, Czech Republic) was used to dissolve Cuc B. For antimicrobial assay, the strains were grown in cation-adjusted Mueller–Hinton broth (MHB; Oxoid, Basingstoke, UK) equilibrated with Tris–buffered saline (Sigma-Aldrich, Prague, Czech Republic).

2.1.2. Antimicrobial assay

For antibiotic susceptibility testing, *S. aureus* (ATCC 29213) was used as a reference strain. Cuc B at concentrations ranging from 0.25 to 3 µg/ml was used. Oxacillin-supplemented with 2% NaCl and tetracycline were used as standard antibiotics at concentrations ranging from 0.25 to 3 µg/mL. 0.05% DMSO and deionized water were used as negative controls that did not inhibit any strain tested. The broth microdilution method using 96-well microtiter plates was performed to determine the minimum inhibitory concentrations (MICs) following the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2009). Briefly, the samples were two-fold diluted in MHB (100 µL), and inoculated with bacterial suspension to reach the density of 5×10^5 CFU/mL. Microtiter plates were incubated at 37 °C for 24 h, and bacterial growth was determined as turbidity by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. MICs were subjected as the lowest concentrations that inhibited the growth of the test bacteria by ≥80% compared with that of negative controls. MICs obtained from three parallel experiments, each performed in triplicate.

2.1.3. Combinatory effect of Cuc B with antibiotics

The checkerboard assay was used to evaluate the combination effect of antibiotics with Cuc B, and the sum of the fractional inhibitory concentration (ΣFIC) indices have been evaluated as previously described (Vuuren and Viljoen, 2011; Hassan et al., 2016). Briefly, two-fold serial dilutions of oxacillin-supplemented with 2% NaCl or tetracycline prepared in horizontal rows of microtiter plate (at concentrations ranging from 0.25 to 3 µg/mL) were subsequently cross-diluted vertically by two-fold serial dilutions of Cuc B (at concentrations ranging from 0.25 to 3 µg/mL). The one-half MIC of Cuc B, oxacillin and tetracycline was used as a starting concentration in combinations. For evaluation of antibacterial combination effect of

Cuc B (A) with antibiotic tested (B), the following equation $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_A$ (in the presence of B) / MIC_A (alone), and $FIC_B = MIC_B$ (in the presence of A) / MIC_B (alone), was used to calculate ΣFIC. The MICs used in this equation are the averages of MICs obtained from three parallel experiments, each performed in triplicate. The interpretation of the *in vitro* antibacterial interactions was determined as follows: synergistic effect if $\Sigma FIC \leq 0.5$; additive if $\Sigma FIC > 0.5$ and ≤ 1; no interaction if $\Sigma FIC > 1$ and ≤ 4; and antagonistic if $\Sigma FIC > 4$.

2.2. Anti-HSV-1 activity

2.2.1. Viral strains, cultures, cell lines and reagents

For antiviral activity, Vero cells (ATCC: CCL 81, UK; were obtained from the Motol University Hospital, Prague, Czech Republic) were grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, UK), 100 U/ml penicillin G, 100 μg/ml streptomycin and 25 μg/ml amphotericin B (Sigma-Aldrich, Germany) and maintained at 37 ◦C in a humidified incubator with 5% CO_2. HSV-1 [KOS strain] was obtained from The Motol Univesity Hospital, Prague, Czech Republic, and propagated in Vero cells. Viral stocks were stored at –80 ◦C and titrated based on plaque forming units (PFU) count by plaque assay as previously described (Burleso et al., 1992).

2.2.2. Determination of cytotoxicity

MTT [3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide] assay was used to determine the cytotoxic effect as previously described (Mosmann, 1983). In brief, confluent Vero cells were exposed to different concentrations of Cuc B for 72 h, and after incubation, the 50% cytotoxic concentration (CC_{50}) of Cuc B was calculated as the concentration that reduces cell viability by 50%, when compared to the untreated controls.

2.2.3. Antiviral assay

For antiherpetic activity, acyclovir was used as a positive control and the plaque number reduction assay was performed to evaluate the anti-HSV-1 activity as previously described (Silva et al., 2010). Briefly, cell monolayers were infected with 100 PFU of the virus for 1 h at 37 ◦C, and then were overlaid with MEM containing 1.5% carboxymethylcellulose (CMC; Sigma-Aldrich, Germany) either in the presence or absence of different concentrations of Cuc B. The cells were incubated for 72 h at 37 ◦C, and then were fixed and stained with naphtol blue-black (Sigma-Aldrich, Germany), and the plaques were counted. The concentration of Cuc B required to reduce the plaque number by 50% (IC_{50}) was calculated, when compared to the untreated controls. Selectivity index (SI) value was calculated as the ratio of CC_{50}/IC_{50}.

2.2.4. Statistical analysis

The mean values ± standard deviations are representative of three to five independent experiments. Nonlinear regressions of concentration– response curves were used to determine CC_{50} and IC_{50} values. Anova ⁄ Dunett ⁄ SNK tests were used to evaluate the significance between Cuc B and control. In addition, the selectivity index (SI= CC_{50}/IC_{50}) was determined.

3. Results and discussion

The results showed that Cuc B exerted inhibitory activity against all *S. aureus* strains tested with MICs values ranging from 0.15 to 0.44 µg/mL (Table 1). The synergistic effect of Cuc B in combination with OX was obtained at a concentration of 0.25 µg/ml (Cuc B at concentration of 0.11 µg/mL and OX at concentration of 0.14 µg/mL) against MRSA-ATCC 43300 (ΣFIC=0.43) and SA2 (ΣFIC=0.39) strains (Table 2). In addition, the synergistic effect of Cuc B when combined with TET was determined at a concentration of 0.5 µg/mL (Cuc B at concentration of 0.21 µg/mL and TET at concentration of 0.29 µg/mL) against MRSA-

ATCC 43300 (ΣFIC= 0.29), and SA4 (ΣFIC= 0.36) strains. Moreover, no additive and antagonistic interactions have been observed. Cuc B exhibited remarkable anti-HSV-1 activity compared with that of acyclovir with IC_{50} values of 0.94 and 1.74 µM, respectively and selectivity indices SI= 127.7 and SI>132.2, respectively. Furthermore, cytotoxicity study revealed that Cuc B could be used safely at a non-cytotoxic concentration of 120 µM. This may give us an indication that Cuc B could be used safely in topical application. However, further studies should be carried out to determine its safety as well as the possible of adverse effects *in vivo* (Table 3).

Table 1: *In vitro* antibacterial activity of OX, TET and Cuc B against *S. aureus* strains

S. aureus strains	MIC (µg/mL)		
	OX	TET	Cuc B
S. aureus (ATCC 29213)	0.19	0.18	0.20
MRSA (ATCC 43300)	0.16	0.20	0.15
SA1*	0.40	0.17	0.20
SA2*	0.42	0.43	0.27
SA3*	0.19	0.22	0.36
SA4*	0.12	0.17	0.44

MIC─ Minimum inhibitory concentration; the values are presented as an average from three parallel experiments, each performed in triplicate
OX: Oxacillin
TET: Tetracycline
ATCC: American Type Culture Collection
MRSA: methicillin-resistant *Staphylococcus aureus*
Cuc B: Cucurbitacin B
*: Clinical isolates

Table 2: *In vitro* combinatory antibacterial effect of Cuc B with OX or TET against *S. aureus* strains

S. aureus strains	Cuc B with OX at following concentrations (µg/mL)				Cuc B with TET at following concentrations (µg/mL)			
	Cuc B at 0.9 and OX at 2.1	Cuc B at 0.4 and OX at 0.6	Cuc B at 0.21 and OX at 0.29	Cuc B at 0.11 and OX at 0.14	Cuc B at 0.9 and TET at 2.1	Cuc B at 0.4 and TET at 0.6	Cuc B at 0.21 and TET at 0.29	Cuc B at 0.11 and TET at 0.14
	ΣFIC	ΣFIC	ΣFIC	ΣFIC	ΣFIC	ΣFIC	ΣFIC	ΣFIC
S. aureus ATCC (29213)	1.49	2.14	2.41	1.39	2.73	3.24	2.14	2.36
MRSA (ATCC 43300)	1.39	2.47	1.98	**0.43**	3.14	2.97	**0.29**	2.38
SA1*	2.34	1.73	2.35	1.35	2.97	1.17	2.54	1.58
SA2*	3.13	2.47	3.23	**0.39**	2.47	1.89	1.48	2.00
SA3*	2.73	2.74	1.36	1.79	2.36	2.47	1.78	2.79
SA4*	2.67	1.79	2.36	1.84	2.73	3.65	**0.36**	3.12

ΣFIC: sum of fractional inhibitory concentrations—the combination interaction is evaluated as follows: synergy ΣFIC ≤ 0.5 (bold font indicates synergistically acting combinations); additive ΣFIC >0.5 and ≤ 1; no interaction ΣFIC>1 and ≤ 4; antagonistic if ΣFIC>4; MIC— bold font indicates fold reductions
OX: Oxacillin
TET: Tetracycline
ATCC: American Type Culture Collection
MRSA: methicillin-resistant *Staphylococcus aureus*
Cuc B: Cucurbitacin B
*: Clinical isolate

Table 3: Cytotoxicity and anti-HSV-1 activity of Cuc B

Compound	CC_{50} (µM)	IC_{50} (µM)	SI
Cuc B	120 ± 1.5	0.94 ± 0.2	127.7
Acyclovir	>230	1.74 ± 0.25	>132.2

Values represent the mean ± standard deviations of three to five independent experiments
CC_{50}: 50% cytotoxic concentration that was determined in Vero cells
IC_{50}: Half maximal inhibitory concentration
SI: Selectivity index (CC_{50}/IC_{50})
Cuc B: Cucurbitacin B

The increasing problem of drug-resistant strains has led to the failure of current treatment strategies of bacterial and viral infections in humans. Therefore, it was an urgent issue to search for new antimicrobial agents derived from natural sources to overcome the problem and to enhance the treatment efficacy (Jenssen et al., 2006). It is well known that tetracycline and oxacillin are standard antibiotics for the treatment of staphylococcal infections (Macheboeuf et al., 2006). Therefore, their combination with Cuc B seems to be very perspective, since the synergy effect was determined effectively in our study. It has been reported that the mechanism contributing to tetracycline resistance in *S. aureus* is mainly due to the decrease in the intracellular accumulation of the antibiotic that can be caused be bacterial efflux proteins, which pump antibiotics from the periplasm or cytosol to the extracellular medium (Speer et al., 1992). For instance, it has been described that ferruginol

has been shown to suppress the tetracycline resistance pump in *S. aureus* (Aiyegoro and Okoh, 2009). Based on these facts, we may suggest that Cuc B could act as an efflux pump inhibitor. The problem with staphylococcal oxacillin resistance is associated with production of exogenous class B of penicillin-binding protein (PBP), especially (PBP2), which has deformed active site unable to bind β-lactams (Macheboeuf et al., 2006). Cucurbitacins are highly bioactive compounds, which can make a complex with nucleophilic amino acids of proteins (Hassan et al. 2015; Hassan et al., 2016). The antibacterial mechanism of β-lactams is mainly related to inactivation of (PBPs) by their covalent binding. In the view of those facts, we may also suggest that Cuc B could act as inhibitor of PBPs. However, the mode of action of Cuc B is still poorly understood, we suggest that Cuc B is useful for the development of new synergistically acting drugs with the potential to extend the pharmacological action of tetracycline and oxacillin. On the other hand, Cuc B is known to possess cytotoxic effect on cancer cells with various mechanisms of action (Guo et al., 2014; Gupta and Srivastava, 2014; Shukla et al., 2015) Therefore, it should be taken into consideration the non-toxic doses, especially with topical preparations containing Cuc B. HSV infections remain a serious threat to human health. To date, no prophylactic HSV vaccine has been found to be entirely effective in the treatment of HSV infections. This is due to the establishment of viral latency and reactivation that occurs in the presence of humoral and cell mediated immunity. Therefore, the use of bioactive natural substances, such as Cuc B, which has been shown to exert notable inhibitory effect against HSV-1 in Vero cells at a non-cytotoxic concentration of 120 µM will open new options for the development of anti-HSV drugs. Although, several studies have reported that the mode of action of plant-derived terpenes toward HSV-1 are mainly related to their ability to inhibit viral protein synthesis, or by impeding nuclear factor κB activation in HSV-1 (Cheng et al., 2004; Kurokawa et al., 1999; Kim et al., 1999), further studies are needed to be carried out to investigate the mechanism of action of Cuc B as

potential anti HSV-1 agent *in vivo* and in clinical trials. It is known that anti-HSV drugs do not cure the disease, while modifying the clinical course of the infection by inhibiting viral replication and subsequent epithelial damage (Hassan et al., 2015). Therefore, the combination effect with acyclovir and related nucleoside analogs will open new options to promote the treatment course of the disease.

4. Conclusion

Plant-derived antimicrobial agents have played an essential role in the treatment of bacterial and viral diseases. Plant-derived antimicrobial agents may not serve directly as drugs, but they provide leads for the development of potential antimicrobial drugs. In the present study, Cuc B exhibited *in vitro* synergy effect with TET or OX against *S. aureus* strains. Thus, Cuc B can be used as a useful agent acting in combination with antibiotics to improve the treatment efficacy of staphylococcal infections. Moreover, Cuc B showed remarkable inhibition of HSV-1, and thus will play an important role in the development of antiherpetic drugs. Based on these findings, we suggest that Cuc B provides potential therapeutic applications as a promising natural candidate for the development of anti-staphylococcal and anti-HSV-1 drugs. In addition, further studies need to be performed to evaluate its mechanism of action and structure-activity relationship *in vivo* and in clinical trials.

In vitro synergistic effect of *Hibiscus sabdariffa* aqueous extract in combination with standard antibiotics against *Helicobacter pylori* clinical isolates

Abstract

Context: The increasing problem of drug-resistant strains has led to the failure of current treatment regimens of *Helicobacter pylori* (HP) infection. Recently, a new treatment strategy has been developed to overcome the problem by using natural products in combination with antibiotics to enhance the treatment efficacy.

Objective: The antimicrobial combinatory effect of the aqueous extract of *Hibiscus sabdariffa* L. (Malvaceae) (AEHS) with antibiotics (clarithromycin, amoxicillin, and metronidazole) has been evaluated *in vitro* against HP strains.

Materials and methods: *Hibiscus* calyces (35 g) were brewed in 250 mL of boiled water for 30 min, and minimum inhibitory concentrations (MICs) were determined by agar dilution method. The checkerboard assay was used to evaluate the antimicrobial combinatory effect according to the sum of fractional inhibitory concentration (ΣFIC) indices.

Results: In this study, AEHS exerted remarkable bacteriostatic effect against all HP strains tested with MICs values ranging from 9.18 to 16.68 µg/mL. Synergy effect of AEHS with clarithromycin or metronidazole was obtained against four out of seven HP strains tested with ΣFIC ranging from 0.21 to 0.39. The additive effect of AEHS with amoxicillin was obtained against five out of seven HP strains tested with ΣFIC ranging from 0.61 to 0.91.

Conclusion: This study presents AEHS as a potent therapeutic candidate alone, or in combination with antibiotics for the treatment of HP infection.

Keywords: drug-resistant strains; *H. pylori* infection; antimicrobial agents; combinatory effect; natural products

1. Introduction

Helicobacter pylori (HP) infection is one of the most common causes of many diseases of the gastrointestinal tract, including non-ulcer dyspepsia, peptic ulcer, gastritis, and gastric cancer (Jung et al., 2015; Paydas, 2015; Mansour-Ghanaei et al., 2015). Numerous treatment strategies containing a proton pump inhibitor (PPI) and combination of two or more antibiotics such as clarithromycin (CLA), amoxicillin (AMX), and metronidazole (MTZ) or tetracycline (TET) have been successfully used to eradicate HP infection (Chaabane & Al-Adhba, 2015; Nagahara et al., 2000). Although the efficacy of such strategies in the therapy, the increasing use of antibiotics has led to the problem of drug-resistant strains (Essa et al., 2009; Gisbert & Calvet, 2011). Resistance rates have been reported vary from 0-45% for clarithromycin, from 0-33% for amoxicillin, from 10-90% for metronidazole, from 6-21% for levofloxacin, and from 5-59% for tetracycline (Karamanolis et al., 2014; Song et al., 2014; O'Connor et al., 2014). In recent years, the combination effect of common antibiotics with natural products has been applied as a new strategy to enhance the treatment of bacterial infections and overcome the complications of drug-resistant strains (Nostro et al., 2006; Hassan et al., 2015). For decades, plants play an essential role in drug discovery development as a rich source of biologically active compounds that exhibited significant antimicrobial properties (Castillo-Juárez et al., 2009; Njume et al., 2011). *Hibiscus sabdariffa* L. (Malvaceae) (HS; roselle) is a medicinal plant, which has a long history of herbal and edible uses worldwide (Wang et al., 2014; Alshami & Alharbi, 2014). Numerous studies have reported the antibacterial activity of *Hibiscus sabdariffa*, and its phytochemicals against Gram negative and Gram positive bacteria (Chao & Yin, 2009; Liu et al., 2005). This study aims to evaluate the *in vitro* antimicrobial combinatory effect of AEHS with standard antibiotics (CLA, AMX, and MTZ) against HP strains (six clinical isolates and one standard control of HP).

2. Materials and methods

2.1 Bacterial strains, cultures and antibiotics

HP (ATCC 6583) standard strain was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) as well as 6 clinical isolates of HP (HP01, HP02, HP03, HP04, HP05, and HP06; isolated from patients with duodenal ulcers) were obtained from The Motol University Hospital, Prague, Czech Republic. For antimicrobial assay, the strains were grown in Mueller-Hinton agar-7% horse blood (Sigma Aldrich, Czech Republic), and incubated under micro-aerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37 $^{\circ}$C for 3 days. The identification was based on microaerophilic growth requirement, morphology, Gram's stain, and oxidase, catalase, and urease activities. In addition, the effects of aging, temperature, aerobiosis, starvation, and antibiotics on the morphologic conversion rate to coccoid forms, and culturability of HP were determined. HP strains were kept in trypticase soy broth supplemented with 20% glycerol at -80 $^{\circ}$C until further use. Clarithromycin, amoxicillin, and metronidazole were purchased from Sigma-Aldrich (Prague, Czech Republic).

2.2 Preparation of plant material

2.2.1 Plant collection

Hibiscus sabdariffa was collected from the northern part of Aswan, Egypt in June 2014, and identified by the authors in the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic. A voucher specimen of the plant was deposited with the number EGHS5 at the herbarium of the department.

2.2.2 Extraction procedure

Air dried hibiscus calyces were prepared as a form of tea extract, which is in common use in traditional folk medicine, especially in tropical and subtropical regions. *Hibiscus* calyces (35 g) were brewed in 250 mL of boiled water and allowed to stand for 30 min. The mixture was filtered, and the solution evaporated to dryness. The extract was stored at 4 °C.

2.2.3 Determination of total flavonoid content (TFC) in AEHS

Total flavonoid content (TFC) of AEHS was determined as previously described (Fernandes et al., 2012). Briefly, 5 g of air dried *hibiscus* calyces were extracted in 100 mL of boiled water for 1 hour by sonication extraction. The mixture was filtered by Sartorius 388 filter paper. 1 mL of the extract was transferred to a 25 mL volumetric flask, and 2 ml of $AlCl_3$ (5% w/v) was added, and the volume was completed with distilled water (probe solution, PS). The same procedure was repeated without the addition of $AlCl_3$ for preparation of contrast solution (CS). The absorbance of PS against CS was determined in spectrophotometer at 410 nm. The percentage of TFC was calculated as rutin according to the following equation: TFC $= \frac{A \times DF}{A_{1\,cm}^{1\%} \times (w - ld)}$, where A = absorbance (AU); DF = dilution factor; w = mass of plant material (g); ld = loss on drying plant material (8%, w/w); $A_{1\,cm}^{1\%}$ = specific absorption for rutin-$AlCl_3$ complex (259.4).

2.2.4 Determination of total anthocyanins (TAC) in AEHS

Total anthocyanins (TAC) of AEHS were determined by a pH-differential method as previously described (Jakobek et al., 2007). In brief, 5 g of air dried *hibiscus* calyces were extracted in 100 mL of boiled water for 1 hour by sonication extraction. The mixture was filtered by Sartorius 388 filter paper. 0.4 mL of the extract was transferred to a 25 mL volumetric flask, and two dilutions of the extract were prepared (ratio = 1:62.5), one with

potassium chloride buffer (pH 1.0) (1.86g KCI in 1 L of distilled water, pH value adjusted to 1.0 with concentrated HCI), and the other with sodium acetate buffer (pH 4.5) (54.43 g $CH_3CO_2Na.3H_2O$ in 1 L of distilled water, pH value adjusted to 4.5 with concentrated HCI). After 15 min of incubation at room temperature, absorbance was measured simultaneously at 520 and 700 nm. The content of total anthocyanins was calculated in mg of cyaniding-3-O-glucoside equivalent (CGE) per 100 g of dry weight using a molar extinction coefficient (ε) of cyaniding-3-O-glucoside of 26900 L/mol/cm and molecular weight (MW) (449.4 g/mol).

2.3 Antimicrobial assay

For antibiotic susceptibility testing, HP (ATCC 6583) was used as a control strain. Clarithromycin, amoxicillin, and metronidazole were used as positive control drugs. Minimum inhibitory concentrations (MICs) were determined by agar dilution method according to the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2009) using Mueller–Hinton agar supplemented with 7% horse blood. Briefly, serial dilutions of CLA, AMX, and MTZ ranging from 0.5-5 µg/mL and AEHS at concentrations ranging from 0.5-5 µg/mL were added to Mueller-Hinton agar supplemented with 7% horse blood in a 5% O_2, 10% CO_2, and 85% N_2. The bacteria were sub-cultured on Mueller–Hinton agar supplemented with 7% horse blood under the same micro-aerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37 °C for 3 days. The bacterial suspension in Mueller–Hinton broth was adjusted to a final concentration of a McFarland No. 0.5 standard, 2 µL of the adjusted inoculum was delivered to the agar plates. After 72 h of incubation under the micro-aerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37 °C, the MICs of CLA, AMX, MTZ, and AEHS were determined. MICs were considered as the lowest concentration of drugs inhibiting visible growth. MICs were obtained from three independent experiments that performed in triplicate.

2.4 Combination effect of AEHS with standard antibiotics

The combination effect of antibiotics and AEHS was determined by checkerboard assay and evaluated algebraically based on the sum of the fractional inhibitory concentration (ΣFIC) indices as previously described (Vuuren & Viljoen, 2011; Rondevaldova et al., 2015). In brief, two-fold serial dilutions of each CLA, AMX, and MTZ prepared in horizontal rows of microtiter plate were subsequently cross-diluted vertically by 2-fold serial dilutions of AEHS. The one-half MIC of each tested compound was used as a starting concentration in combinations. For evaluation of synergistic effect of AEHS (A) with antibiotic tested (B), the ΣFIC was calculated based on the following equation: $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_A$ (in the presence of B) / MIC_A (alone), and $FIC_B = MIC_B$ (in the presence of A) / MIC_B (alone). The MICs used in this equation are the averages of MICs obtained from three independent experiments performed in triplicate. The interpretation of the *in vitro* antibacterial interactions was determined as follows: synergistic effect if $\Sigma FIC \leq 0.5$; additive if $\Sigma FIC > 0.5$ and ≤ 1; no interaction if $\Sigma FIC > 1$ and ≤ 4; and antagonistic if $\Sigma FIC > 4$.

3. Results

The results indicated that AEHS showed remarkable inhibition of the growth of all HP strains tested (bacteriostatic effect) with MICs values ranging from 9.18 to 16.68 µg/mL (Table 1), while demonstrated synergistic effects when combined with CLA or MTZ against four out of seven HP strains tested (Table 2). The synergistic effect of AEHS in combination with CLA was obtained at a concentration of 1µg/mL against HP (ATCC 6583) (ΣFIC = 0.28) and HP06 (ΣFIC = 0.21) strains, causing 46- and 29-fold reductions in CLA MICs, respectively. The synergistic effect of AEHS when combined with MTZ was determined (at a concentration of 0.5 µg/mL) against HP01 (ΣFIC = 0.34), and HP03 (ΣFIC = 0.39) strains, and caused 40- and

30-fold reductions in MTZ MICs, respectively. The additive antibacterial effect of AEHS with AMX (at concentrations of 0.5, 1, and 3 µg/mL) was obtained against HP (ATCC 6583), HP02, HP04, HP05, and HP06 strains (Table 3), while in few cases ΣFIC values were close to 0.61, which can be considered as strong additive effect. The percentage of total flavonoid content (TFC) in AEHS was determined as rutin (0.247%), while the content of total anthocyanins (TAC) was estimated as mg of cyaniding-3-O-glucoside equivalent (1465.8 mg/100 g dry weight of hibiscus calyces) (Table 4).

Table 1: *In vitro* antibacterial activity of CLA, AMX, MTZ, and AEHS against HP strains

HP strains	MIC (µg/mL)			
	CLA	AMX	MTZ	AEHS
HP (ATCC 6583)	16.21	13.50	2.24	12.25
HP01*	12.56	12.41	13.54	16.68
HP02*	9.15	13.85	4.59	15.24
HP03*	10.56	16.89	11.99	9.18
HP04*	10.98	13.69	10.17	12.81
HP05*	14.23	12.88	10.97	13.56
HP06*	10.49	14.73	13.12	14.17

MIC: Minimum inhibitory concentration; the values are expressed as an average from three independent experiments, each performed in triplicate
HP: *Helicobacter pylori*
*: Clinical isolates
ATCC: American Type Culture Collection
AEHS: The aqueous extract of *Hibiscus sabdariffa*
CLA: Clarithromycin
AMX: Amoxicillin
MTZ: Metronidazole

Table 2: *In vitro* combinatory antibacterial effect of AEHS with CLA or MTZ against HP strains

HP strains	AEHS with CLA at following concentrations (µg/mL)								AEHS with MTZ at following concentrations (µg/mL)							
	5		3		1		0.5		5		3		1		0.5	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
HP (ATCC 6583)	22.23	1.27	19.23	3.13	**0.35**	**0.28**	9.45	2.93	13.56	3.12	16.34	2.12	12.34	1.99	12.23	1.27
HP01*	21.45	2.13	16.24	3.78	12.34	1.72	9.23	3.15	13.32	3.18	12.43	2.23	13.12	3.15	**0.34**	**0.34**
HP02*	17.34	1.98	19.34	2.45	16.34	2.14	15.63	1.98	14.26	2.15	7.43	1.16	9.32	2.45	9,45	1.36
HP03*	18.56	3.24	20.23	2.16	16.23	3.45	14.65	2.43	17.23	2.87	8.45	1.13	17.32	3.14	**0.40**	**0.39**
HP04*	19.34	2.89	18.45	2.13	13.78	2.45	18.45	3.15	8.34	1.98	8.34	2.84	17.32	2.36	9.45	2.26
HP05*	17.43	3.14	17.34	1.13	14.67	3.19	19.43	3.48	7.23	1.92	17.43	2.36	16.43	2.73	10.21	1.99
HP06*	18.34	2.75	18.45	1.78	**0.36**	**0.21**	12.45	2.41	8.98	1.23	21.34	2.73	12.98	2.83	21.32	1.78

ΣFIC: sum of fractional inhibitory concentrations—the combination interaction is evaluated as follows: synergy ΣFIC ≤ 0.5 (bold font indicates synergistically acting combinations); additive ΣFIC >0.5 and ≤ 1; no interaction ΣFIC>1 and ≤ 4; antagonistic if ΣFIC>4; MIC— bold font indicates fold reductions
MIC: Minimum inhibitory concentration
HP: *Helicobacter pylori*
*: Clinical isolates
ATCC: American Type Culture Collection
AEHS: The aqueous extract of *Hibiscus sabdariffa*
CLA: Clarithromycin
MTZ: Metronidazole

Table 3: *In vitro* combinatory antibacterial effect of AEHS with AMX against HP strains

HP strains	AEHS with AMX at following concentrations (µg/mL)							
	5		3		1		0.5	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
HP (ATCC 6583)	12.43	1.36	13.12	**0.73**	13.23	**0.62**	15.32	**0.71**
HP01*	14.25	1.36	12.34	2.13	14.23	2.13	13.24	3.13
HP02*	13.65	2.36	18.34	**0.81**	17.44	**0.79**	14.56	**0.92**
HP03*	13.45	2.34	17.34	1.68	17.34	1.95	10.89	1.98
HP04*	10.89	3.15	15.34	**0.78**	18.34	**0.96**	12.34	**0.82**
HP05*	15.25	3.98	14.28	**0.91**	17.36	**0.84**	8.45	**0.87**
HP06*	16.34	3.57	9.78	**0.63**	16.98	**0.61**	7.14	**0.82**

ΣFIC: sum of fractional inhibitory concentrations—the combination interaction is evaluated as follows: synergy ΣFIC ≤ 0.5; additive ΣFIC >0.5 and ≤ 1 (bold font indicates additive acting combinations); no interaction ΣFIC>1 and ≤ 4; antagonistic if ΣFIC>4.
MIC: Minimum inhibitory concentration
HP: *Helicobacter pylori*
*: Clinical isolates
AEHS: The aqueous extract of *Hibiscus sabdariffa*
ATCC: American Type Culture Collection
AMX: Amoxicillin

Table 4: Chemical characterization of total flavonoid content (TFC) and total anthocyanins (TAC) in AEHS

TFC (%) calculated as rutin	TAC as mg of cyaniding-3-O-glucoside equivalent (mg/100 g DW)
0.247%	1465.8 mg/100 g DW

TFC: Total flavonoid content
TAC: Total anthocyanins
DW: Dry weight of *Hibiscus* calyces
AEHS: The aqueous extract of *Hibiscus sabdariffa*

4. Discussion

Hibiscus sabdariffa, consumed by people worldwide in the form of tea extract, has a wide range of antimicrobial activities (Rukayadi et al., 2008; Jung et al., 2013). Beside this fact, the present study suggests that AEHS could be a useful agent acting in combination with antibiotics to enhance the treatment efficacy of HP infection. Numerous studies have reported that compounds such as flavonoids and anthocyanins are responsible for the antimicrobial properties of *Hibiscus sabdariffa* (Yin & Chao, 2008; Camelo-Méndez et al., 2013; Alarcón-Alonso et al., 2012). The antibacterial activity of AEHS may be related to its ability to inhibit bacterial protein synthesis (Higginbotham et al., 2014). The bacteriostatic activity of clarithromycin depends on its capacity to inhibit protein synthesis by binding to the 50S bacterial ribosomal subunit. Clarithromycin resistance is mainly due to point mutations in the 23S ribosomal RNA (rRNA) gene, and nucleotides A2142G and A2143G are the most

frequent mutations (Gerrits et al., 2006; Dolapcioglu et al., 2014; Ferreira & Moss, 2014). Metronidazole resistance among HP strains has been related to alterations in gene products having metronidazole nitroreductase activities, mainly including oxygen-insensitive NAD(P)H nitroreductase (RdxA) and NAD(P)H flavin oxidoreductase (FrxA) (Jenks et al., 1999, 2002). Based on these facts, we suggest that AEHS in combination with CLA or MTZ inhibit bacterial protein synthesis, and thus can be considered as a useful alternative therapeutic agent in the development of anti-HP drugs.

5. Conclusion

New antimicrobial combination drugs which include plant products combinations have recently gained a great attention in research field. This approach has financial implications as reformulation of existing drugs or combinations may prove to be a more viable option, rather than developing a new drug which will require extensive clinical trials for verification. In this study, the susceptibility of HP strains to AEHS in combination with antibiotics (CLA, AMX, and MTZ) was examined. In addition, the chemical characterization of TFC and TAC in AEHS was determined. The results demonstrated that AEHS can enhance the growth inhibitory activity of CLA, AMX, and MTZ against HP strains tested. Furthermore, no antagonistic interactions were observed. Although the synergistic effect of AEHS in combination with CLA or MTZ against four out of seven HP strains tested as well as the additive effect of AEHS in combination with AMX against five out of seven HP strains tested were investigated, further studies should be carried out to confirm these activities as well as the mechanisms of action *in vivo* and in clinical trials.

Protocatechuic acid from *Hibiscus sabdariffa* L. Exhibits Antiviral Activity against HSV-2

Abstract

Protocatechuic acid, an active substance of *Hibiscus sabdariffa* showed potent anti-HSV-2 activity compared with that of acyclovir, with EC_{50} values of 0.92 and 1.43 $\mu g \cdot mL^{-1}$, respectively, and selectivity indices > 217 and > 140, respectively.

1. Introduction

Herpes simplex virus (HSV) infections are quite common in humans, affecting about 90% of the world population. HSV is a member of Herpesviridae, a wide family of enveloped-DNA viruses that cause several clinically significant syndromes in both adults and neonates (Einsenberg et al., 2012). HSV-2 is mainly connected with genital infection, and has been recorded as a risk factor of HIV infection in humans (Gescher et al., 2011). Currently, treatment of HSV-2 infection mainly relies on the use of acyclovir and related nucleoside analogues that target viral DNA polymerase. Unfortunately, although several strategies have shown high efficacy results, HSV-2 infection treatment fails in about 30%–45% of infected adults (Khan et al., 2005; Keller et al., 2005). This is due to the extensive use of acyclovir and related nucleoside analogues, which has created drug resistance, associated with other adverse effects, alongside with the establishment of viral latency and reactivation that occurs in the presence of humoral- and cell-mediated immunity (Wang et al., 2016). To date, no prophylactic HSV vaccine has been found to be entirely effective in the prevention of HSV infections (Hassan et al., 2017; Hassan et al., 2015). Therefore, it is crucial to find alternative strategies to combat this viral resistance, and increase treatment efficacy results.

Protocatechuic acid (PCA), an active compound in *H. sabdariffa*, has been also shown to exert various pharmacologic properties, including but not limited to antimicrobial and antioxidant activities (Da-Costa-Rocha et al., 2014). This study was designed to evaluate *in vitro* antiviral activity of AEHS and PCA against HSV-2 clinical isolates and to evaluate their safety for topical administration.

2. Materials and Methods

2.1. Plant Collection and Extraction Procedure

Hibiscus sabdariffa L. was collected from the northern part of Aswan, Egypt in June 2014, and identified by Sherif T. S. Hassan in the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences (Brno, Czech Republic). A voucher specimen of the plant was deposited with the number EGHS5 at the herbarium of the department. One-gram air dried Hibiscus calyces were extracted in 10 mL of boiled water by sonication extraction for 30 min. The extract was stored at 4 °C for further use. A 1 mL of aliquot of crude extract was evaporated to dryness and then weighted to yield a final amount of 0.0243 g of dry extract.

2.1.1. Determination of Concentration of PCA in Plant Material

Air dried *Hibiscus* calyces (0.51 g) were extracted in 10 mL of boiled water by sonication extraction for 30 min. HPLC-MS (Agilent 1200 HPLC system, Böblingen, Germany) coupled with a mass spectrometer (Sciex-3200QTRAP-hybrid triple quadrupole/linear ion trap, Toronto, ON, Canada) fitted with Electrospray Ionization (ESI) were used for the analysis. For the quantification, an external calibration method with standard PCA (Sigma Aldrich, Prague, Czech Republic) was used as previously described (Carvalho et al., 2015).

2.2. Antiviral Activity against HSV-2

2.2.1. Viral Strains, Cultures, Cell lines and Reagents

For antiviral activity, Vero cells (ATCC: CCL 81, UK; were obtained from the Motol University Hospital, Prague, Czech Republic) were grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, UK), 100 U/mL penicillin G, 100 μg·mL⁻1 streptomycin and 25 μg·mL⁻1 amphotericin B (Sigma-Aldrich, Berlin, Germany) and maintained at 37 °C in 5% CO_2 atmosphere. Ten clinical isolates of HSV-2 (isolated from patients with HSV-2 infection) were kindly obtained from the Motol University Hospital, Prague, Czech Republic). All clinical isolates were typed by quantitative real-time reverse transcription PCR using primers pairs H_2M_{40} 5'-GTACAGACCTTCGGAGG-3' and H_2P_4 5'-CGCTTC ATCATGGGC-3' for identification as previously described (Markoulatos et al., 2001) and then were propagated in Vero cells. The cytopathic end-point assay was used to determine the titers which were expressed as 50% tissue culture infective dose ($TCID_{50}$/mL) as previously described (Reed and Muench, 1938). Viral stocks were stored at −80 °C.

2.2.2. Determination of Cytotoxicity

Cytotoxicity was evaluated by the neutral red dye-uptake method as previously described (Walker et al., 1971). Briefly, AEHS, protocatechuic acid (PCA; Sigma Aldrich, Prague, Czech Republic) and acyclovir (positive control; Sigma-Aldrich, Berlin, Germany) were prepared in 0.1% dimethyl sulfoxide (DMSO). Stock solutions were prepared in deionized water at concentration of 400 μg·mL⁻1 and sterilized. Vero cell monolayers were cultivated in 96-well microtiter plates with two-fold serial dilutions of AEHS, PCA and acyclovir, and incubated for 48 h at 37 °C in 5% CO_2 atmosphere. After incubation, the morphological

alterations of the treated cells were determined using an inverted optical microscope (Leitz, Berlin, Germany) and the maximum non-toxic concentrations (MNTC) were determined. The 50% cytotoxic concentrations (CC_{50}) of AEHS, PCA and acyclovir were calculated as the concentration that reduces cell viability by 50%, when compared to the untreated controls as previously described (Borenfreund and Puerner, 1985).

2.2.3. Anti-HSV-2 Activity

Antiherpetic activity was determined by the titer reduction assay as previously described (Nishimura et al., 1977). Briefly, acyclovir was used as a positive control and Vero cell monolayers were treated with AEHS, PCA and acyclovir at concentrations at which no change was observed in cell morphology, and 80% cell viability were determined. 100 $TCID_{50}$/mL of HSV-2 acyclovir-sensitive suspensions were added to treated and untreated cell cultures and incubated at 37 °C for 48 h in a 5% CO_2 atmosphere. After incubation, the virus titers in treated and untreated cells, were determined. Anti-HSV-2 activity was evaluated as percentage inhibition (PI) using antilogarithmic $TCID_{50}$ values as follows: PI = [1–(antilogarithmic test value/antilogarithmic control value)] × 100. The dose response curve was determined from the MNTC, and the 50% effective concentration (EC_{50}) was determined as the concentration required for 50% protection against virus-induced cytopathic effects. Selectivity index (SI) value was calculated as the ratio of CC_{50}/EC_{50}.

2.2.4. Statistical Analysis

Experiments were generated in duplicate in at least three independent experiments. PRISM software version 5.0 (GraphPad Software, Inc., CA, USA) was used for statistical analysis (one-way ANOVA test) and to calculate EC_{50} and CC_{50} parameters.

3. Results

3.1. Determinations of Concentration of PCA in Plant Material

The results demonstrated that the concentration of PCA in AEHS was found to be 94.1 µg/g dry weight of *Hibiscus* calyces (Figure 1).

3.2. Anti-HSV-2 Activity and Cytotoxicity

AEHS and PCA were evaluated with respect to their inhibitory effect on HSV-2 replication. Before performing the antiherpetic assay, we assessed the cytotoxicity of each sample in Vero cells by the neutral red dye-uptake method. The CC_{50} values for PCA and acyclovir were found to be higher than 200 $\mu g \cdot mL^{-1}$ (Table 1). Antiherpetic activity was determined by the titer reduction assay in infected Vero cells using quantitative real-time reverse transcription PCR. Ten acyclovir-sensitive strains of HSV-2 (clinical isolates) were used and typed by quantitative real-time reverse transcription PCR using primers pairs H_2M_{40} 5’-GTACAGACCTTCGGAGG-3’ and H_2P_4 5’-CGCTTCATCATGG GC-3’ for identification. AEHS was not active against HSV-2. This could be related to the low concentrations of antiherpetic compounds in the crude extract. PCA showed potent anti-HSV-2 activity compared with that of acyclovir with EC_{50} values of 0.92 and 1.43 $\mu g \cdot mL^{-1}$, respectively, and selectivity indices > 217 and > 140, respectively. PCA exhibited cytotoxic effect on Vero cells at concentration higher than its EC_{50}. The selectivity index (SI) is fundamental to determine any possible toxic effect of any compound on the cells that could be confused with an antiviral activity. Based on our results, PCA demonstrated anti-HSV-2 activity with SI > 217 higher than acyclovir (> 140). Thus, the SI verifies the safety index of PCA.

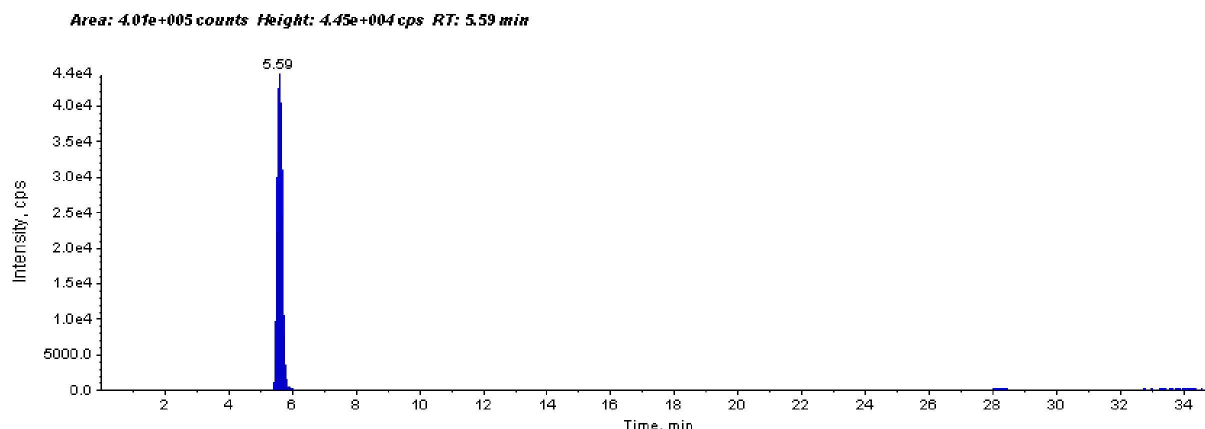

Figure 1. HPLC-MS chromatogram shows identification (two MRM transitions *m/z* 153→109 and 153→91) and determination of concentration of protocatechuic acid (PCA) in aqueous extract of *Hibiscus sabdariffa* (AEHS). PCA was detected at retention time (RT): 5.59 min and quantified using an external calibration method with standard PCA (94.1 μg/g dry weight of *Hibiscus* calyces).

Table 1. Anti-HSV-2 activity and cytotoxicity of PCA and AEHS.

Compound	EC_{50} (μg·mL^{-1})	CC_{50} (μg·mL^{-1})	SI
AEHS	n.d	n.d	n.d
PCA	0.92 ± 0.21	>200	>217
Acyclovir	1.43 ± 0.43	>200	>140

All data were average values from three independent experiments; Values presented are means ± S.D. of three independent experiments performed in duplicate; CC_{50}: 50% cytotoxic concentration; EC_{50}: 50% Effective concentration; SI: Selectivity index (CC_{50}/EC_{50}); n.d: Not determined; PCA: Protocatechuic acid; AEHS: Aqueous extract of *Hibiscus sabdariffa*

4. Discussion

For decades, treatment of infectious diseases has been a focus of interest for both researchers and healthcare providers, as the arising issue of drug resistant strains has become a serious problem worldwide. This study adds to the growing body of knowledge on the antiviral activity of polyphenol-enriched extracts derived from plants. We show for the first time that PCA, an active compound of *H. sabdariffa* L. exerts potent antiviral activity against clinical isolates of HSV-2, the mechanism of which involves the inhibition of viral replication. Plant-derived phytochemicals, such as polyphenols, phenolics, terpenes, alkaloids and other substances have been reported to possess inhibitory properties on HSV replication (hassan et

al., 2017; Firdous et al., 2015). For instance, polyphenols have been found to interfere with the early phases of the HSV replicative cycle and/or with viral particles directly (Yang et al., 2007). In this study, PCA showed excellent ability to inhibit HSV-2 replication and hence might open new gates for the development of anti-HSV-2 drugs. In addition, PCA is known to exhibit cytotoxic effect on cancer cells with various mechanisms of action (Tseng et al., 1998; Tseng et al., 2000). Therefore, it should be taken into consideration the non-toxic doses, especially with topical preparations containing PCA. Considering the significant global incidence, morbidity, and mortality rates of viral sexually transmitted infections (STIs), the development of new, safe, topically applied antiviral agents for their prevention is of high priority (Charlton et al., 2002). Therefore, in recent years, a new approach has been under focus of interest to maximize the treatment efficacy by combining natural products with nucleoside analogues, resulting in reduction of cytotoxicity (Friend, 2010). Thus, this approach will reduce the cytotoxicity of PCA. However, further studies should be carried out to determine its safety as well as the possibility of adverse effects *in vivo*.

5. Conclusion

Plant-derived phytochemicals provide an excellent option for the treatment of infectious diseases. The present findings indicated that PCA exhibited notable inhibitory properties on HSV-2 replication and thus has promising application in the development of anti-HSV-2 drugs. However, further studies should be carried out to evaluate its safety, validate the activity *in vivo*, and to eliminate the possible adverse effects of this compound by using improved delivery techniques prior its possible practical application.

Overall of the importance of the results

The results confirmed the beneficial effects of the crude extracts of alien plant species and their active phytochemicals against plant and human pathogens. The crude extracts may be purified for the isolation of compounds with antimicrobial activity. These results also validate the possibility of using these plants for the treatment of infections caused by plant and human pathogens.

Concluding remarks

The European Union defines alien plant species as those that are introduced accidentally or deliberately into a natural environment where they are not normally found, with serious negative consequences for their new environment. They represent a major threat to native plants in Europe, causing damage worth billions of euros to the European economy every year. Different plant pathogens threaten food security worldwide, therefore the most important method of protecting plant against pathogens attack is, the use of synthetic fungicides and bactericides (as well as useful for the treatment of human bacterial, fungal pathogens) but the development of resistance towards synthetic fungicides and bacreicides is a great concern. Moreover, the health risk and hazardous effects on environments associated with the use of chemical fungicides and bactericides increase the need to search for safe, efficacious and environmentally friendly fungicides and bactericides. Many alien plant species release chemical compounds into the environment, which are not generally harmful to them, but those chemicals suppress the growth of other plant species growing in close proximity of such alien species. Prime importance can be given for the bioprospecting of novel active compound which can be utilized for the management of several plant diseases.

This negative effect (often referred to as allelopathic effect) of invaders on the native species confers a tremendous competitive advantage on the former.

In summary, alien plant species can be considered as a rich source of bioactive compounds with promising activity against various plant and human pathogens. Despite relatively few of isolated antimicrobial compounds from alien plant species advance to become practically effective drugs in their own right, these substances can be used as models for the preparation of analogues using chemical methodology such as total or combinatorial synthesis, or manipulation of biosynthetic pathways. Thus, plant-derived natural substances may open new options for the development of new antimicrobial agents and may play an essential role in agriculture, environmental protection and pharmaceutical industy as well.

References

Abritton D.L., Watson R.T., 1992. Methyl bromide interim scientific assessment. Montreal Protocol Assessment Supplementary. J Ethnopharmacol. 2, 235-238.

Adams M. R. and Moss, M. O. 2000. *Food Microbiology*. The Royal Society of Chemistry, London. 56, 3225-3226.

Addis M., Pal M, Kyule, M. N. 2011. Isolation and identification of *Staphylococcus* species from Ethiopian cottede cheese (Ayib) in Debre Zeit, Ethiopia. *Veterinary research* 4: 13-17.

Adwan G, Salameh Y, Adwan K. 2011. Effect of ethanolic extract of Ecballium elaterium against Staphylococcus aureus and Candida albicans. Asian Pac J Trop Biomed 1, 456-460.

Aiyegoro OA, Okoh AI. 2009. Review: Use of bioactive plant products in combination with Standard antibiotics: implications in antimicrobial chemotherapy. J Med Plants Res 3:1147–1152.

Alarcón-Alonso J, Zamilpa A, Aguilar FA, et al. 2012. Pharmacological characterization of the diuretic effect of *Hibiscus sabdariffa* Linn (Malvaceae) extract. J Ethnopharmacol. 139: 751-756.

Alshami I, Alharbi AE. 2014. Antimicrobial activity of *Hibiscus sabdariffa* extract against uropathogenic strains isolated from recurrent urinary tract infections. Asian Pac J Trop Dis 4: 317-322.

Anantharaman A, Rizvi MS, Sahal D. 2010. Synergy with rifampin and kanamycin enhances potency, kill kinetics, and selectivity of de Novo-designed antimicrobial peptides. Antimicrob Agents Chemother 54: 1693–1699.

Appelbaum, P.C. 2007. Reduced glycopeptide susceptibility in methicillin-resistant Staphylococcus aureus (MRSA). Int J Antimicrob Agents 30, 398-408.

Atta, AH a El-Sooud, KA. 2004. The antinociceptive effect of some Egyptian medicinal plant. J Ethnopharmacol. 95, 235-8.

Attard E., Brincat M.P, Cuschieri A., 2005. Immunomodulatory activity of cucurbitacin E isolated from Ecballium elaterium. Fitoterapia.76, 439-441.

Babula P, Adam V, Havel L, et al. 2007 Naphthoquinones and their pharmacological properties. Ceska Slov Farm 56:114–120.

Badei A.Z.M., El-Akel A.T.M., Morsi,H.H., Baruah P., Sharma R.K., Singh R.S., Ghosh A., 1996. Fungicidal activity of some naturally occurring essential oils against *Fusarium moniliforme*. J. Essen. Oil Res. 8, 411-412.

Bajpai V. K., Kwang-Hyun Baek, Eun Sil Kim, et al. 2011. Preliminary Phytochemical Analysis and *in Vitro* Evaluation of Antifungal Activity of Five Invasive Plant Species against *Macrophomina Phaseolina* (Tassi) Goid, International Journal of Plant Research 2011; 1(1): 11-15.

Baquar S. R. 1995. The role of traditional medicine in rural environment. In: *Traditional Medicine in Africa*, pp. (56-79, Issaq, S. ed.). East Africa Educational Publishers Ltd., Nairobi.

Bardon C, Piola F, Bellvert F. Et al. 2014. Evidence for biological denitrification inhibition (BDI) by plant secondary metabolites. New Phytol. 204 :620-630.

Bashir A., Mujahid T. and Jehan N. 2007. Antibiotic resistance profile: Isolation and characterization of clinical isolates of *Staphylococci* from patients with community acquired skin infections. *Pakistan Journal of Pharmaceutical Science* 20(4): 295-299.

Bhargava SK. 1984. Effects of plumbagin on reproductive function of male dog. Indian J Exp Biol 22: 153–156.

Blackburn T. M., Pyšek P., Bacher S., Carlton J. T., Duncan R. P., Jarošík V., Wilson J. R. U. & Richardson D. M. 2011) A proposed unified framework for biological invasions. – Trends Ecol. Evol. 26: 333–339.

Bohlooli S., Jafari N., Jahed S., 2012. Cytotoxic effect of freeze-dried extract of Ecballium elaterium fruit on gastric adenocarcinoma (AGS) and esophageal squamous cell carcinoma (KYSE30) cell lines. J Gastrointest Cancer. 43, 579-583.

Borenfreund E.; Puerner J.A. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 1985, 24, 119–124.

Brantner A., Z. Males,S. Pepeljnjak, et al.,. 1996. Antimicrobial activity of Paliurus spina-christi mill. J. Ethnopharmacol. 52:119–122.

Burleson F.G., Chamberts T.M., Wiedbrauk D.L. 1992. Virology: a laboratory manual. SanDiego: Academic Press. p. 250.

Camelo-Méndez GA, Ragazzo-Sánchez JA, Jiménez-Aparicio AR, et al. (2013). Comparative study of anthocyanin and volatile compounds content of four varieties of Mexican roselle (*Hibiscus sabdariffa* L.) by multivariable analysis. Plant Foods Hum Nutr. 68 :229-234.

Cappuccino, N. and Arnason, J. T. 2006. Novel chemistry of invasive exotic plants. Biol. Lett. 2:189−193.

Carvalho, D.O.; Curto, A.F.; Guido, L.F. Determination of phenolic content in different barley varieties and corresponding malts by liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry. Antioxidants 2015, 4, 563–576.

Castillo-Juárez I, González V, Jaime-Aguilar H, et al. (2009). Anti-*Helicobacter pylori* activity of plants used in Mexican traditional medicine for gastrointestinal disorders. J Ethnopharmacol 122: 402-405.

Cavero RY, Akerreta S, Calvo MI. 2013. Medicinal plants used for dermatological affections in Navarra and their pharmacological validation. J Ethnopharmacol 149: 533-542.

Clinical and Laboratory Standards Institute. (2009). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically—Eighth Edition: Approved Standard M07-A8. CLSI, Wayne, PA, USA.

CLSI. 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-third edition; CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Cos P, Vlietinck AJ, Vanden Berghe D, Maes L. 2006. Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. J Ethnopharmacol 106 :290-302.

Cowan, M.M. 1999. Plant products as antimicrobial agents. Clin Microbiol Rev12, 564–582.

Cripps, MG, et al.,. 2011. Classical biological control of Cirsium arvense: Lesson from the past. Biological Control. 57, 165-174.

Cutler, R.R., Wilson, P. 2004. Antibacterial activity of a new, stable, aqueous extract of allicin against methicillin-resistant Staphylococcus aureus. Br J Biomed Sci 61, 71-74.

Da-Costa-Rocha, I.; Bonnlaender, B.; Sievers, H.; Pischel, I.; Heinrich, M. Hibiscus sabdariffa L.—A phytochemical and pharmacological review. Food Chem. 2014, 165, 424–443.

Dai BD, Cao YY, Huang S, et al. 2009. Baicakein induces programmed cell death in Candida albicans. J Microbiol Biotechnol 19:803-809.

de Paiva SR, Figueiredo MR, Aragao TV, et al. 2003. Antimicrobial activity in vitro of plumbagin isolated from Plumbago species. Mem I Oswaldo Cruz 98: 959–961.

Di Grazia A, Luca V, Segev-Zarko LA, et al. 2014. Temporins A and B stimulate migration of HaCaT keratinocytes and kill intracellular Staphylococcus aureus. Antimicrob Agents Chemother 58: 2520–2527.

Dixon, R. A., P. M. Dey, and C. J. Lamb. 2008. Phytoalexins: enzymology and molecular biology. Adv. Enzymol. 55:1–69.

Dogruoz, N., Zeybek. Z., Karagoz. A. Antibacterial activity of some plant extracts. IUFS J Biol 67, 17-21.

Dolapcioglu C, Koc-Yesiltoprak A, Ahishali E, et al. 2014. Sequential therapy versus standard triple therapy in *Helicobacter pylori* eradication in a high clarithromycin resistance setting. Int J Clin Exp Med. 7: 2324-2328.

Drago, L., De Vecchi, E., Cappelletti, L., Mattina, R., Vassena, C., Romanò, C.L. 2014. Role and antimicrobial resistance of staphylococci involved in prosthetic joint infections. Int J Artif Organs 37, 414-421.

Duke, J. A. 1985. Handbook of medicinal herbs. CRC Press, Inc., Boca Raton, Fla.

Eaton, D.L., Gallagher, E.P., 1994. Mechanism of aflatoxin carcinogenesis. Annual Review Pharmacology and Toxicology 34, 135–172.

Einsenberg, R.; Atanasiu, D.; Cairns, T.M.; Gallagher, J.R.; Krummenacher, C.; Cohen, G.H. Herpes virus fusion and entry: A story with many characters. Viruses. 2012, 4, 800–832.

Eloff, J.N., Angeh, I., McGaw, L.J., 2006. A plant antifungal product from Melianthus comosus (Melianthaceae) leaf extracts. GA Conference Helsinki September 2006. Planta Medica 72, 982.

Essa AS, Kramer JR, Graham DY, et al. 2009. Meta-analysis: Four-drug, three-antibiotic, non-bismuth-containing "concomitant therapy" versus triple therapy for *Helicobacter pylori* eradication. Helicobacter. 14: 109-118.

Essl F., Dullinger D. & Kleinbauer I. 2009: Changes in the spatio-temporal patterns and habitat preferences of Ambrosia artemisiifoliaduring the invasion of Austria. – Preslia 81: 119–133.

Evans, C.M., Kudesia, G., McKendrick, M. 2013. Management of herpesvirus infections. Int J Antimicrob Agents 42,119-128.

Farrell, D.J., Flamm, R.K., Sader, H.S., Jones, R.N.2014. Activity of ceftobiprole against methicillin-resistant Staphylococcus aureus strains with reduced susceptibility to daptomycin, linezolid or vancomycin, and strains with defined SCCmec types. Int J Antimicrob Agents 43, 323-327.

Fernandes AJ, Ferreira MR, Randau KP, et al. (2012). Total flavonoids content in the raw material and aqueous extractives from *Bauhinia monandra* Kurz (Caesalpiniaceae). ScientificWorldJournal. 2012: 923462.

Ferreira J, Moss SF. (2014). Current paradigm and future directions for treatment of *Helicobacter pylori* infection. Curr Treat Options Gastroenterol. 12: 373-384.

Ferreira S, Domingues F. 2016. The antimicrobial action of resveratrol against Listeria monocytogenes in food-based models and its antibiofilm properties. J Sci Food Agric. 96 :4531-4535.

Fessenden, R. J., and J. S. Fessenden. 2005. Organic chemistry, 2nd ed. Willard Grant Press, Boston, Mass.

Field, H.J. 1989. Persistent herpes simplex virus infection and mechanisms of virus drug resistance. Eur J Clin Microbiol Infect Dis 8, 671–680.

Firdous, S.; Ansari, N.H.; Fatima, I.; Malik, A.; Afza, N.; Iqbal, L.; Lateef, M. Ophiamides A-B, new potent urease inhibitory sphingolipids from Heliotropium ophioglossum. Arch. Pharm. Res. 2012, 35, 1133–1137.

Fischer, R., Schillberg, S., Buyel, J.F., Twyman, R.M. 2013. Commercial aspects of pharmaceutical protein production in plants. Curr Pharm Des 19, 5471-5447.

Friend D.R. 2010. Pharmaceutical development of microbicide drug products. Pharm. Dev. Technol. 15, 562–581.

Geissman, T. A. 1963. Flavonoid compounds, tannins, lignins and related compounds, p. 265. In M. Florkin and E. H. Stotz (ed.), Pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol. 9. Elsevier, New York, N.Y.

Gentile I, Buonomo AR, Borgia F, et al. 2014. Ledipasvir: a novel synthetic antiviral for the treatment of HCV infection. Expert Opin Investig Drugs 23:561–571.

Georges, M., and K. M. Pandelai. 1949. Investigations on plant antibiotics. IV. Further search for antibiotic substances in Indian medicinal plants.Indian J. Med. Res. 37:169–181.

Gerrits MM, Van Vliet AH, Kuipers EJ, et al. (2006). *Helicobacter pylori* and antimicrobial resistance: Molecular mechanisms and clinical implications. Lancet Infect Dis. 6: 699-709.

Gescher, K.; Hensel, A.; Hafezi, W.; Derksen, A.; Kühn, J. Oligomeric proanthocyanidins from Rumex acetosa L inhibit the attachment of herpes simplex virus type-1. Antivir. Res. 2011, 89, 9–18.

Gibbons S. 2008. Phytochemicals for bacterial resistance—strengths, weaknesses and opportunities. Planta Med 74: 594-602.

Gisbert JP, Calvet X. (2011). Review article: Non-bismuth quadruple (concomitant) therapy for eradication of *Helicobater pylori*. Aliment Pharmacol Ther. 34: 604-617.

Guo, J., Zhao, W., Hao, W., Ren, G., Lu, J., Chen, X. 2014. Cucurbitacin B induces DNA damage, G2/M phase arrest, and apoptosis mediated by reactive oxygen species (ROS) in leukemia K562 cells.Anticancer Agents Med Chem 14, 1146-1153.

Gupta, P., Srivastava, S.K. 2014. Inhibition of Integrin-HER2 signaling by Cucurbitacin B leads to in vitro and in vivo breast tumor growth suppression. Oncotarget 5, 1812-1828.

Harborne, J.B. 1973. Phytochemical Methods: A guide to Modern Techniques of Plant Analysis, Chapman and Hall Ltd., London.

Harris, R. S. 2006. Vitamins K, p. 192–198. In M. Florkin and E. Stotz (ed.), Pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol. 9. Elsevier, New York, N.Y.

Hasegawa, H., S. Matsumiya, M. Uchiyama, T. Kurokawa, Y. Inouye, R Kasai, S. Ishibashi, and K. Yamasaki. 2009. Inhibitory effect of some triterpenoid saponins on glucose transport in tumor cells and its application to in vitro cytotoxic and antiviral activities. Planta Med. 6:240–243.

Hassan STS, Masarčíková R, Berchová K. 2015. Bioactive natural products with anti-herpes simplex virus properties. J Pharm Pharmacol 67:1325-1336.

Hassan STS, Švajdlenka E, Berchová-Bímová K. 2017. Hibiscus sabdariffa L. and Its Bioactive Constituents Exhibit Antiviral Activity against HSV-2 and Anti-enzymatic Properties against Urease by an ESI-MS Based Assay. Molecules. 22. pii: E722

Hassan STS., Žemlička, M. 2016. Plant-Derived Urease Inhibitors as Alternative Chemotherapeutic Agents. Arch Pharm (Weinheim) 349, 507-522.

Hassan, S.T.S.; Berchová-Bímová, K.; Petráš, J.; Hassan, K.T.S. Cucurbitacin B interacts synergistically with antibiotics against Staphylococcus aureus clinical isolates and exhibits antiviral activity against HSV-1. S. Afr. J. Bot. 2017, 108, 90–94.

Hassan, STS., Berchová-Bímová, K., Petráš, J. 2016. Plumbagin, a Plant-Derived Compound, Exhibits Antifungal Combinatory Effect with Amphotericin B against Candida albicans Clinical Isolates and Anti-hepatitis C Virus Activity. Phytother Res. 30:1487-1492.

Higginbotham KL, Burris KP, Zivanovic S, et al. (2014). Antimicrobial activity of *Hibiscus sabdariffa* aqueous extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus* in a microbiological medium and milk of various fat concentrations. J Food Prot. 77: 262-268.

Hostettman, K., Marston, A., Ndjoko, K., Wolfender J.L., 2000. The potential of African plants as a source of drugs. *Current Organic Chemistry* 4, 973-1010.

Hu, C. Q., K. Chen, Q. Shi, R. E. Kilkuskie, Y. C. Cheng, and K. H. Lee. 2010. Anti-AIDS agents. 10. Acacetin-7-O-beta-D-galactopyranoside, and anti-HIV principle from Chrysanthemum morifolium and a structure-activity correlation with some related flavonoids. J. Nat. Prod. 57:42–51.

Chaabane NB, Al-Adhba HS. (2015). Ciprofloxacin-containing versus clarithromycin-containing sequential therapy for *Helicobacter pylori* eradication: A randomized trial. Indian J Gastroenterol. 34: 68-72.

Chandrasekaran B, Nagarajan B. 1981. Metabolism of ethylamine and plumbagin in rats. Journal of Biological Science 3: 395–400.

Chao CY, Yin MC. 2009. Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Pathog Dis. 6: 201-206.

Charlton, A.J.; Baxter, N.J.; Khan, M.L.; Moir, A.J.; Haslam, E.; Davies, A.P.; Williamson, M.P. 2002. Polyphenol/peptide binding and precipitation. J. Agric. Food Chem. 50, 1593–1601.

Chen, J.C., Chiu, M.H., Nie, R.L., Cordell, G.A., Qiu, S.X. 2005. Cucurbitacinsand cucurbitane glycosides: structures and biologicalactivities. Nat Prod Rep 22, 386–399.

Chen, X., Bao, J., Guo, J., Ding, Q., Lu, J., Huang, M., Wang, Y. 2012. Biological activities and potential molecular targets of cucurbitacins: a focus on cancer. Anticancer Drugs 23, 777–787.

Cheng, H.Y., Lin, T.C., Yang, C.M., Wang, K.C., Lin, L.T., Lin, C.C. 2004. Putranjivain A from Euphorbia jolkini inhibits both virus entry and late stage replication of herpes simplex virus type 2 in vitro. J Antimicrob Chemother 53, 577–583.

Chengxu W, Zhu Mingxing Z, Xuhui C, Bo Q. 2011. Review on Allelopathy of Exotic Invasive Plants. Procedia Engineering. 18 240 – 246.

Choi, N. H., Choi, G. J., Jang, K. S., Choi, Y. H., Lee, S. O., Choi, J. E. and Kim, J. C. 2008. Antifungal activity of the methanol extract of Myristica malabarica fruit rinds and the active ingredients malabaricones against phytopathogenic fungi. Plant Pathol. J. 24:317−321.

Inbaraj JJ, Chignell CF. 2004. Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes. Chem Res Toxicol 17:55-62.

Islas-Rodrìguez AE, Marcellini L, Orioni B, et al. 2009. Esculentin 1–21: a linear antimicrobial peptide from frog skin with inhibitory effect on bovine mastitis-causing bacteria. J Pept Sci 15: 607–614.

Jack, I.R. and Orubike, O.K. 2008. Phytochemical analysis and antimicrobial activity of the extract of leaves of fleabane (Conyza sumatrensis). Journal of applied science and environmental management, 12(4): 63-65.

Jakobek L, Seruga M, Medvidovic-Kosanovic M, et al. 2007. Anthocyanin content and antioxidant activity of various red fruit juices. Dtsch Lebensmitt Rundsch. 103: 58–64.

Jenks PJ, Edwards DI. 2002. Metronidazole resistance in *Helicobacter pylori*. Int J Antimicrob Agents. 19: 1-7.

Jenks PJ, Ferrero RL, Labigne A. 1999. The role of the rdxA gene in the evolution of metronidazole resistance in *Helicobacter pylori*. J Antimicrob Chemother. 43: 753-758.

Jenssen, H., Hamill, P., Hancock, R.E. 2006. Peptide Antimicrobial Agents. Clin Microbiol Rev19, 491-511.

Joy PP, Thomas J, Mathew S, et al. 2001. Medicinal plants. Tropical Horticulture Vol. 2. Calcutta: Naya Prokash 104.

Jung E, Kim Y, Joo N. (2013). Physicochemical properties and antimicrobial activity of Roselle (*Hibiscus sabdariffa* L.). J Sci Food Agric. 93: 3769-3776.

Jung SW, Thamphiwatana S, Zhang L, et al. (2015). Mechanism of antibacterial activity of liposomal linolenic acid against *Helicobacter pylori*. PLoS One. 10: e0116519.

Karamanolis GP, Daikos GL, Xouris D, et al. 2014. The evolution of *Helicobacter pylori* antibiotics resistance over 10 years in Greece. Digestion. 90: 229-231.

Kayali Z, Schmidt WN. 2014. Finally sofosbuvir: an oral anti-HCV drug with wide performance capability. Pharmgenomice Pers Med 7: 387–398.

Kazmi M. H., A. Malik S. Hameed N. Akhtar, Noor Ali. 1994. An anthraquinone derivative from Cassia italica. Phytochemistry 36:761–763.

Keller M.J.; Tujama A.; Carlucci M.J.; Herold B.C. 2005. Topical microbicides for prevention of genital herpes infection. J. Antimicrob. Chemother. 55, 420–423.

Khalil A.M., Qaoud K.M. 1993. Toxicity and partial characterization of Ecballium elaterium fruit juice. Int. J. Pharmacogn. 3, 135–141.

Khan M.T.; Alter A.; Thompson K.D.; Gambari, R. 2005. Extracts and molecules from medicinal plants against herpes simplex viruses. Antivir. Res. 67, 107–119.

Kim J, Sudbery P. 2011. Candida albicans, a major human fungal pathogen. J Microbiol 49: 171-177.

Kim M., Kim S.K., Park B.N., Lee K.H., Min G.H., Seoh J.Y., et al. 1999. Antiviral effects of 28 deacetylsendanin on herpes simplex virus-1 replication. Antiviral Res 43, 103–112.

Kini DP, Pandey S, Shenoy BD, et al. 1997. Antitumor and antifertility activities of plumbagin controlled release formulations. Indian J Exp Biol 35: 374–379.

Kurokawa, M., Basnet, P., Ohsugi, M., Hozumi, T., Kadota, S., Namba, T., et al. 1999. Anti-herpesimplex virus activity of moronic acid purified from Rhus javanica in vitro and in vivo. J Pharmacol Exp Ther 289, 72–78.

Laghari AH et al. 2010. A new flavanenol with urease-inhibition activity isolated from roots of manna plant camelthorn (Alhagi maurorum). J Mol Struct., 965, 1, 65-67.

Lanfranco G. 1999. Invited review article on Traditional Medicine. *Electronic Journal of Biotechnology* 2: 1-3.

Lilic D, Haynes K. Candida. in Immunology of Fungal Infections. In: Brown,G.D., Netea, M.G. (Eds.). Springer, The Netherlands. 2007; 361–382: Chapter 16.

Lindenbach BD, Rice CM. 2005. Unravelling hepatitis C virus replication from genome to function. Nature 436: 933–938.

Liu KS, Tsao SM, Yin MC. 2005. *In vitro* antibacterial activity of roselle calyx and protocatechuic acid. Phytother Res. 19: 942-945.

Liu T., Zhang M., Zhang H., Sun C., Deng, Y. 2008. Inhibitory effects of cucurbitacin B on laryngeal squamous cell carcinoma. Eur Arch Otorhinolaryngol 265, 1225–1232.

Mabona U., Viljoen A., Shikanga E., Marston A., Van Vuuren, S. 2013. Antimicrobial activity of southern African medicinal plants with dermatological relevance: from an ethnopharmacological screening approach, to combination studies and the isolation of a bioactive compound. J Ethnopharmacol 148, 45–55.

Macheboeuf, P., Contreras-Martel, C., Job, V., Dideberg, O., Dessen, A. 2006. Penicillin Binding Proteins: key players in bacterial cell cycle and drug resistance processes. FEMS Microbiol Rev 30, 673–691.

Mangoni ML, Rinaldi AC, Di Giulio A, et al. 2000. Structure-function relationship of temporins, small antimicrobial peptides from amphibian skin. Eur J Biochem 267:1447–1454.

Mansour-Ghanaei F, Joukar F, Mojtahedi K, et al. (2015). Does treatment of *Helicobacter pylori* infection reduce gastric precancerous lesions?. Asian Pac J Cancer Prev. 16: 1571-1574.

Markoulatos P.; Georgopoulou A.; Siafakas N, Plakokefalos E.; Tzanakaki G.; Kourea-Kremastinou, J. 2001. Laboratory diagnosis of common herpesvirus infections of the central nervous system by a multiplex PCR assay. J. Clin. Microbiol. 39, 4426–4432.

Mason, T. L., and B. P. Wasserman. 1987. Inactivation of red beet betaglucan synthase by native and oxidized phenolic compounds. Phytochemistry, 26:2197–2202.

Mdee, L.K., Masoko, P. and Eloff, J.N. 2009. The activity of extracts of seven common invasive plant species on fungal phytopathogens. S.Afr.J. B. 75; 375-379.

Miceli MH, Diaz JA, Lee SA. 2011. Emerging opportunistic yeast infections. Lancet Infect Dis 11: 142-151.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival:application to proliferation and cytotoxicity assays. J Immunol Methods 65, 55–63.

Mossa JS, El-Feraly FS, Muhammad I. 2004. Antimycobacterial constituents from Juniperus procera, Ferula communis and Plumbago zeylanica and their in vitro synergistic activity with isonicotinic acid hydrazide. Phytother Res 18: 934–937.

Murray, P. R. 1999. *Manual of Clinical Microbiology*. ASM Press, Washington D.C.

Murray, P.R., et al.: *Medical microbiology*. Philadelphia: Elsevier Inc., 2008, 6th edition, 947 pp. ISBN 978-0-323-05470-6. pp. 848 – 849 (Trypanosoma), 269 – 272 (Nocardia), 277 – 289 (*Mycobacterium*), 303 – 306 (*Escherichia*).

Nagahara A, Miwa H, Ogawa K, et al. 2000. Addition of metronidazole to rabeprazole-amoxicillin-clarithromycin regimen for *Helicobacter pylori* infection provides an excellent cure rate with five-day therapy. Helicobacter. 5: 88-93.

Nishimura T.; Toku H.; Fukuyasu.; H. 1977. Antiviral compounds. XII. Antiviral activity of amidinohydrazones of alkoxyphenyl-substituted carbonyl compounds against influenza virus in eggs and in mice. Kitasato Arch. Exp. Med. 50, 39–46.

Njume C, Afolayan AJ, Samie A, et al. 2011. Inhibitory and bactericidal potential of crude acetone extracts of *Combretum molle* (Combretaceae) on drug-resistant strains of *Helicobacter pylori*. J Health Popul Nutr. 29: 438-445.

Nostro A, Cellini L, Di Bartolomeo S, et al. 2006. Effects of combining extracts (from propolis or *Zingiber officinale*) with clarithromycin on *Helicobacter pylori*. Phytother Res. 20: 187-190.

Novy P, Rondevaldova J, Kourimska L, Kokoska L. 2013. Synergistic interactions of epigallocatechin gallate and oxytetracycline against various drug resistant Staphylococcus aureus strains in vitro. Phytomedicine 20:432-435.

O'Connor A, Vaira D, Gisbert JP, et al. 2014. Treatment of *Helicobacter pylori* infection 2014. Helicobacter. 19: 38-45.

Ogihara K, Yamashiro R, Higa M, et al. 1997. Preparation of naphthoquinone derivatives from plumbagin and their ichthyotoxicity. Chem Pharm Bull 45:437–445.

Oskay, M., Oskay, D., Kalyoncu, F. 2009. Activity of some plant extracts against multi-drug resistant human pathogens. Iran J Pharm Res 8, 293-300.

Paludan, S.R., Bowie, A.G., Horan, K.A., Fitzgerald, K.A. 2011. Recognition of herpesviruses by the innate immune system. Nat Rev Immunol 11, 143–54.

Pandey, A.K. 2007. Anti-staphylococcal activity of a pan-tropical aggressive and obnoxious weed parihenium histerophorus: an in vitro study. Nat. Acad. Sci. Lett 30, 383–386.

Paydas S. 2015. *Helicobacter pylori* eradication in gastric diffuse large B cell lymphoma. World J Gastroenterol. 21: 3773-3776.

Peng ZG, Zhao ZY, Li YP, et al. 2011. Host apolipoprotein B messenger RNA-editing enzyme catalytic polypeptide-like 3G is an innate defensive factor and drug target against hepatitis C virus. Hepatology 53: 1080-1089.

Penin F, Dubuisson J, Rey FA, et al. 2004. Structural biology of hepatitis C virus. Hepatology 39: 5–19.

Piller, N. B. 1975. A comparison of the effectiveness of some anti-inflammatory drugs on thermal oedema. Br. J. Exp. Pathol. 56:554–559.

Pimentel D., Zuniga R. and Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol. Economic. 52:273−288.

Piret J., Boivin G. 2011. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. Antimicrob Agents Chemother 55, 459–472.

Placinta C.M., D'Mello, J.P.F., Macdonald, A.M.C., 1999. A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. Animal Feed Science Technology 78, 21–37.

Premakumari P, Rathinam K, Santhakumari G. 1977. Antifertility activity of plumbagin. Indian J Med Res 65: 829–838.

Pyšek P, Chytry M, Pergl J, Sadlo J, Wild J. 2012. Plant invasions in the Czech Republic: current state, introduction dynamics, invasive species and invaded habitats. Preslia 84: 575–629.

Rahman SMA, Abd-Ellatif SA, Deraz SF, Khalil AA. 2011. Antibacterial activity of some wild medicinal plants collected from western Mediterranean coast, Egypt: Natural alternatives for infectious disease treatment . African J Biotechnol. 52, 10733-10743.

Raikhlin-Eisenkraft B., Bentur Y. 2000. Ecballium elaterium (squirting cucumber) – remedy or poison? J Toxicol Clin Toxicol. 38, 305-308.

Rao M.M., Meshulam, H., Lavie, D., 1974. The constituents of Ecballium elaterium L. Part XXIII. Cucurbitacins and hexanorcucurbitacins. J Chem Soc Perkin Trans. 1, 2552-2556.

Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. Am J Hyg 27: 493-497.

Richardson D. M., Pyšek P., Rejmánek M., Barbour M. G., Panetta F. D. & West C. J. 2000: Naturalization and invasion of alien plants: concepts and definitions. – Diversity Distrib. 6: 93–107.

Robert, J.F., Richard, J.L., 1992. Aflatoxins in animal and human health. Review Environmental Contamination and Toxicology 127, 69–94.

Rondevaldova J, Novy P, Kokoska L. 2015. *In vitro* combinatory antimicrobial effect of plumbagin with oxacillin and tetracycline against *Staphylococcus aureus*. Phytother Res. 29: 144-147.

Rukayadi Y J, Shim S, Hwang JK. (2008). Screening of Thai medicinal plants for anticandidal activity. Mycoses. 51: 308-312.

Saltonstall K., Lambert A. & Meyerson L. A. 2010: Genetics and reproduction of common Phragmites australis) and giant reed (Arundo donax). – Inv. Plant Sci. Manage. 3: 495–505.

San-Blas, G., L. Marino, F. San-Blas, and R. Apitz-Castro. 1993. Effect of ajoene on dimorphism of Paracoccidioides brasiliensis. J. Med. Vet. Mycol. 31:133–141.

Sato, Y., H. Odetani, K. Singyouchi, T. Ohtsubo, M. Kihara, H. Shibata, Scalbert, A. 2007. Antimicrobial properties of tannins. Phytochemistry 30: 3875–3883.

Sharifi-Rad, J., Hoseini Alfatemi, S., Sharifi Rad, M., Iriti M. 2014. Antimicrobial Synergic Effect of Allicin and Silver Nanoparticles on Skin Infection Caused by Methicillin-Resistant Staphylococcus aureus spp. Ann Med Health Sci Res 4, 863-868.

Sharma, N., 1998. Control of postharvest diseases with natural plant products. In: Sharma, N., Alam, M.M. (Eds.), Postharvest Diseases of Horticultural Perishables. International Book Distributing Company, Lucknow, 226004 (India).

Sharma, N., Tripathi, A., 2006. Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. Microbiological Research Singh, 77, 161-167.

Shirtliff ME, Krom BP, Meijering RA, et al. 2009. Farnesolinduced apoptosis in Candida albicans. Antimicrob Agents Chemother 53: 2392- 2401.

Shukla, S., Khan, S., Kumar, S., Sinha, S., Farhan, M., Bora, H.K., Maurya, R., Meeran, S.M. 2015. Cucurbitacin B Alters the Expression of Tumor-Related Genes by

Epigenetic Modifications in NSCLC and Inhibits NNK-Induced Lung Tumorigenesis. Cancer Prev Res (Phila) 8, 552-562.

Schmidt, H. 1988. Phenol oxidase (E.I.14.18.1), a marker enzyme for defensecells. Progress in histochemistry and cytochemistry, vol. 17. Gustav Fischer, New York, N.Y.

Schultes, R. E. 1978. The kingdom of plants, p. 208. In W. A. R. Thomson (ed.), Medicines from the Earth. McGraw-Hill Book Co., New York, N.Y.

Silva, I.T., Costa, G.M., Stoco, P.H., Schenkel, E.P., Reginato, F.H., Simões, C.M.O. 2010. In vitro antiherpes effects of a C-glycosyl flavonoid-enriched fraction of Cecropiaglaziovii Sneth. Lett Appl Microbiol 51, 143–148.

Singh UV, Udupa N. 1997. Reduced toxicity and enhanced antitumor efficacy of betacyclodextrin plumbagin inclusion complex in mice bearing ehrich ascites carcinoma. Indian J Physiol Pharmacol 41: 171–175.

Singh UV, Udupa N. 1997. Reduced toxicity and enhanced antitumor efficacy of betacyclodextrin plumbagin inclusion complex in mice bearing Ehrlich ascites carcinoma. Indian J Physiol Pharmacol 41: 171–175.

Smith, J.E., 1997. Aflatoxins. In: D'Mello, J.P.F. (Ed.), Handbook of Plant and Fungal Toxicants. CRC Press, Boca Raton, FL, pp. 269–285.

Sofia MJ. 2014. Beyond sofosbuvir: what opportunity exists for a better nucleoside/nucleotide to treat hepatitis C?. Antiviral Res107:119-124.

Song Z, Zhang J, He L, et al. 2014. Prospective multi-region study on primary antibiotic resistance of *Helicobacter pylori* strains isolated from Chinese patients. Dig Liver Dis. 46: 1077-1081.

Speer, B.S., Shoemaker, N.B., Salyers, A.A. 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin Microbiol Rev 5, 387-399.

Staniszewska M, Bondaryk M, Swoboda-Kopec E, et al. 2013. Candida albicans morphologies revealed by scanning electron microscopy analysis. Braz J Microbiol 44:813-821.

Stankiewicz-Drogoń A, Dörner B, Erker T, et al. 2010. Synthesis of new acridone derivatives, inhibitors of NS3 helicase, which efficiently and specifically inhibit subgenomic HCV replication. J Med Chem 53: 3117–3126.

Stern, J. L., A. E. Hagerman, P. D. Steinberg, and P. K. Mason. 1996. Phlorotannin-protein interactions. J. Chem. Ecol. 22:1887–1899.

Strange, R.N., Scott, P.R., 2005. Plant diseases a threat to global food security. *Annu. Rev. Phytopathol*. 43: 83-116.

Summers BB, Beavers JW, Klibanov OM. 2014. Sofosbuvir, a novel nucleotide analogue inhibitor used for the treatment of hepatitis C virus. J Pharm Pharmacol 66:1653-1666.

Talcott RE, Smith MT, Giannini DD. 1985. Inhibition of microsomal lipid peroxidation by naphthoquinones: Structure-activity relationships and possible mechanisms of action. Arch Biochem Biophys 241:88–94.

Tan G. T., J. F. Miller, A. D. Kinghorn, S. H. Hughes, and J. M. Pezzuto. 2003. HIV-1 and HIV-2 reverse transcriptases: a comparative study of sensitivity to inhibition by selected natural products. Biochem. Biophys.Res. Commun. 185:370–378.

Thastrup O., J. B. Knudsen, J. Lemmich, and K. Winther. 2002. Inhibitions of human platelet aggregation by dihydropyrano- and dihydrofuranocoumarins, a new class of cAMP phosphodiesterase inhibitors. Biochem.Pharmacol. 34:2137–2140.

Thomson W. A. R. (ed.). 1978. Medicines from the Earth. McGraw-Hill Book Co., Maidenhead, United Kingdom.

Tiley,GED. 2010. Biological Flora of the British Isles: Cirsium arvense (L.) Scop. Journal of

Toda M., S. Okubo R. Ohnishi, et al. 2007. Antibacterial and bactericidal activities of Japanese green tea. Jpn. J. Bacteriol. 45:561–566.

Tseng T.H.; Hsu, J.D.; Lo, M.H.; Chu, C.Y.; Chou, F.P.; Huang, C.L.; Wang, C.J. 1998. Inhibitory effect of Hibiscus protocatechuic acid on tumor promotion in mouse skin. Cancer Lett. 126, 199–207.

Tseng T.H.; Kao, T.W.; Chu, C.Y.; Chou, F.P.; Lin, W.L.; Wang, C.J. 2000. Induction of apoptosis by hibiscus protocatechuic acid in human leukemia cells via reduction of retinoblastoma (RB) phosphorylation and Bcl-2 expression. Biochem. Pharmacol. 60, 307–315.

Tsuchiya, H., M. Sato, T. Miyazaki, S. Fujiwara, S. Tanigaki, M. Ohyama, T. Tanaka, and M. Iinuma. 2004. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant Staphylococcus aureus. J. Ethnopharmacol. 50:27–34.

Tyler, V. E., Brady, L. E., and Robbers, J. E. 1976. *Pharmacognosy*. Lea and Febiger, Philadelphia. United Nations Environmental Program (UNEP), New York.

Urs, N. V. R. R., and J. M. Dunleavy. 1975. Enhancement of the bactericidal activity of a peroxidase system by phenolic compounds (Xanthomonas phaseoli var. sojensis, soybeans). Phytopathology 65:686–690.

Vamos-Vigyazo, L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit. Rev. Food Sci. Nutr. 15:49–127.

Vijaya, K., S. Ananthan, and R. Nalini. 2001. Antibacterial effect of theaflavin, polyphenon 60 (Camellia sinensis) and Euphorbia hirta on Shigella spp.—a cell culture study. J. Ethnopharmacol. 49:115–118.

Vijayarathna S, Zakaria Z, Chen Y, et al. 2012. The Antimicrobial efficacy of Elaeis guineensis: characterization, in vitro and in vivo studies. Molecules 17:4860-4877.

Vila, M., Weiner, J., 2004. Are invasive plant species better competitors than native plant species? evidence from pair wise experiments. Oikos 105, 229–238.

Vohora, S. B., M. Rizwan, and J. A. Khan. 1973. Medicinal uses of commo Indian vegetables. Planta Med. 23:381–393.

Vuuren SV, Viljoen A. 2011. Plant-based antimicrobial studies – Methods and Approaches to study the interaction between natural products. Planta Med 77: 1168-1182.

Wagner H. 2011. Synergy research: approaching a new generation of phytopharmaceuticals. Fitoterapia 82: 34-37.

Walker W.E.; Waisbren, B.A.; Martins, R.R.; Batayias, G.E. 1971. A method for determining sensitivities of antiviral drugs in vitro for possible use as clinical consultation. Am. J. Clin. Pathol. 56, 687–692.

Wang J, Cao X, Jiang H, et al. (2014). Antioxidant activity of leaf extracts from different *Hibiscus sabdariffa* accessions and simultaneous determination five major antioxidant compounds by LC-Q-TOF-MS. Molecules. 19: 21226-21238.

Wang, Z.; Liu, Q.; Lu, J.; Fan, P.; Xie, W.; Qiu, W.; Wang, F.; Hu, G.; Zhang, Y. Serine/Arginine-rich splicing factor 2 modulates herpes simplex virus type 1 replication via regulating viral gene transcriptional activity and pre-mRNA splicing. J. Biol. Chem. 2016, 291, 26377–26387.

Watanbe, H., C. Miyaji, M. Makino, and T. Abo. 2006. Therapeutic effects of glycyrrhizin in mice infected with LP-BM5 murine retrovirus and mechanisms involved in the prevention of disease progression. Biotherapy 9:209–220.

WHO (2002). World Health Organization. Traditional medicines strategy 2002-2005. Geneva.

Wild, R. (ed.). 1994. The complete book of natural and medicinal cures. Rodale Press, Inc., Emmaus, Pa.

Williamson M. 1996: Biological invasions. – Chapman & Hall, London.

Yang, C.M.; Cheng, H.Y.; Lin, T.C.; Chiang, L.C.; Lin, C.C. 2007. The in vitro activity of geraniin and 1,3,4,6-tetra-O-galloyl-β-D-glucose isolated from Phyllanthus urinaria against herpes simplex virus type 1 and type 2 infection. J. Ethnopharmacol. 110, 555–558.

Yin MC, Chao CY. 2008. Anti-Campylobacter, anti-aerobic, and anti-oxidative effects of roselle calyx extract and protocatechuic acid in ground beef. Int J Food Microbiol. 127: 73-77.

Zafriri, D., I. Ofek, R. Adar, M. Pocino, and N. Sharon. 2012. Inhibitory activity of cranberry juice on adherence of type 1 and type P fimbriated Escherichia coli to eucaryotic cells. Antimicrob. Agents Chemother. 33:92–98.

Zia, UHK, et al. 2011. Phytochemical study on the constituents from Cirsium arvense. Mediterranean Journal of Chemistry. 1, 64-69.

List of publications related to dissertation thesis

Hassan STS, Švajdlenka, E.; Berchová-Bímová, K. Hibiscus sabdariffa L. and its bioactive constituents exhibit antiviral activity against HSV-2 and anti-enzymatic properties against urease by ESI-MS based assay. Molecules. **2017;** 22(5). pii: E722.

Hassan STS, Berchová-Bímová, K, Petráš J, Hassan KTS. Cucurbitacin B interacts synergistically with antibiotics against Staphylococcus aureus clinical isolates and exhibits antiviral activity against HSV-1. S. Afr. J. Bot. **2017**; 108:90-94.

Hassan STS, Berchová-Bímová K, Petráš J. Plumbagin, a Plant-Derived Compound, Exhibits Antifungal Combinatory Effect with Amphotericin B against Candida albicans Clinical Isolates and Anti-hepatitis C Virus Activity. Phytother Res. **2016**;30(9):1487-92.

Hassan STS, Berchová K, Majerová M, Pokorná M, Švajdlenka E. In vitro synergistic effect of Hibiscus sabdariffa aqueous extract in combination with standard antibiotics against Helicobacter pylori clinical isolates. Pharm Biol. **2016**;54(9):1736-40.

Hassan STS, Berchová K, Šudomová M. Antimicrobial, antiparasitic and anticancer properties of Hibiscus sabdariffa (L.) and its phytochemicals: in vitro and in vivo studies. Ceska Slov Farm. **2016**;65(1):10-4.

Hassan STS, Masarčíková R.; Berchová, K. Bioactive natural products with anti-herpes simplex virus properties. J Pharm Pharmacol. **2015**; 67(10):1325-1336.

Hassan STS, Majerová M, Šudomová M, Berchová, K. Antibacterial activity of natural compounds – essential oils. Ceska Slov Farm. **2015**;64(6):243-53.

Published papers in international conferences related to dissertation thesis

The results of the dissertation thesis were presented as posters and published in the book of abstracts of the following international conferences.

Hassan STS, Berchová K. Antimicrobial activity of isolated phenolic compounds from selected invasive plants. **2014**. Drug Analysis. June 22-25, 2014. Liege, Belgium. As a form of abstract in Book of Abstracts and poster presentation.

Hassan STS, Berchová K. Plant-derived chemicals exhibit antimicrobial activity: a new therapeutic agents in the treatment of bacterial and viral infections. Frontiers in Medicinal Chemistry. **2015**. September 14-16, 2015, Antwerp, Belgium. As a form of abstract in Book of Abstracts and poster presentation.

Appendices

In this section, all papers that have been published in international peer-reviewed journals with impact factors and related to my PhD thesis are attached as follows.

PHYTOTHERAPY RESEARCH
Phytother. Res. **30**: 1487–1492 (2016)
Published online 24 May 2016 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5650

Plumbagin, a Plant-Derived Compound, Exhibits Antifungal Combinatory Effect with Amphotericin B against *Candida albicans* Clinical Isolates and Anti-hepatitis C Virus Activity

Sherif T. S. Hassan,[1,2]* Kateřina Berchová-Bímová[2] and Jan Petráš[1]

[1]Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42, Brno, Czech Republic
[2]Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6 – Suchdol, Prague, Czech Republic

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), the major active constituent of *Plumbago indica* L., has been shown to be effective against a wide range of infectious microbes. In this study, plumbagin has been evaluated *in vitro* for its antifungal combinatory effect with amphotericin B against *Candida albicans* (*C. albicans*) clinical isolates and anti-hepatitis C virus (HCV) activity. Antifungal activity was determined by broth microdilution method, and combinatory effect was evaluated by checkerboard assay according to ΣFIC indices, while cytotoxicity was determined by MTT assay. Anti-HCV activity was determined in infected Huh7.5 cells using quantitative real-time reverse transcription PCR, and cytotoxicity was evaluated by MTT assay. Plumbagin exerted inhibitory effect against all *C. albicans* strains with minimum inhibitory concentration values ranging from 7.41 to 11.24 µg/mL. The additive effect of plumbagin when combined with amphotericin B at concentrations of (0.12, 0.13 and 0.19, 1.81 µg/mL, respectively) was obtained against five of seven strains tested with ΣFIC ranging from 0.62 to 0.91. In addition, plumbagin was found to be used safely for topical application when combined with amphotericin B at concentrations corresponding to the additive effect. Plumbagin exerted anti-HCV activity compared with that of telaprevir with IC_{50} values of 0.57 and 0.01 µM/L, respectively, and selectivity indices SI = 53.7 and SI = 2127, respectively. Our results present plumbagin as a potential therapeutic agent in the treatment of *C. albicans* and HCV infections. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: plumbagin; antimicrobial agents; fungal infection; HCV infection; natural products.

INTRODUCTION

In the last two decades, *Candida* species have become the most common cause of systemic fungal infections worldwide (Kim and Sudbery, 2011; Miceli *et al.*, 2011). *Candida albicans* (*C. albicans*) is the main cause of candiduria and candidemi with high morbidity and mortality (40–60%). In addition, it is the most frequently isolated pathogenic fungi in immunocompromised patients that cause localized invasive mucosal mycosis or life-threatening disseminated and deep-seated organ infections (Lilic and Haynes, 2007; Staniszewska *et al.*, 2013; Shirtliff *et al.*, 2009). In recent years, the increasing use of antibiotics has led to the problem of drug-resistant strains, and thus has led to the failure of current treatment regimens of fungal infections (Dai *et al.*, 2009). Therefore, a new treatment strategy has been applied to overcome the problem by using plant-derived products in combination with antimycotics to enhance the treatment efficacy of fungal infections as well as reducing toxicity and side effects (Shirtliff *et al.*, 2009; Hassan *et al.*, 2015; Aiyegoro and Okoh, 2009; Wagner, 2011).

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus belonging to the family Flaviviridae. Approximately 170 million people worldwide are infected with HCV, and many of them died from HCV-related liver diseases (Lindenbach and Rice, 2005; Penin *et al.*, 2004). To date, there is no prophylactic vaccine available to prevent HCV infection. Recently, a new generation of direct-acting antiviral agents has become available, and currently, the interferon-free combination therapy of sofosbuvir (NS5B polymerase inhibitor), ledipasvir (NS5A replication complex inhibitor), and telaprevir has resulted in a cure rate up to 100% among patients infected with HCV (Sofia, 2014; Summers *et al.*, 2014). However, this regimen has an unfavorable side effect profile (including flu-like symptoms, hemolytic anemia, and depression), which often leads to discontinuance of therapy (Stankiewicz-Drogoń *et al.*, 2010; Kayali and Schmidt, 2014; Gentile *et al.*, 2014). Thus, there is a strong medical need to discover novel agents with a high therapeutic index and few side effects to treat chronic HCV infection.

Plumbago indica L. (Plumbaginaceae) is a medicinal plant that is well known for its traditional uses as a

* Correspondence to: Sherif T. S. Hassan, Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42, Brno, Czech Republic, and Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6 – Suchdol, Czech Republic
E-mail: sherif.hassan@seznam.cz

Received 04 November 2015
Revised 31 March 2016
Accepted 30 April 2016

Copyright © 2016 John Wiley & Sons, Ltd.

thermogenic, antimicrobial, antiinflammatory, abortifacient, antiperiodic, carminative, digestive, nerve stimulatory, and rejuvenating drug (Joy *et al.*, 2001). It is commercially important as a major source of plumbagin (Fig. 1). Plumbagin (5-hydroxy-2 methyl-1,4-naphthoquinone), naturally occurring yellow pigment accumulating mainly in roots of the plant *Plumbago indica*, showed anticancer, antimalarial, antifertility, hyperglycemic, and hypolipidemic properties and possesses antimicrobial activity against a wide spectrum of microorganisms including anticandidal activity (Mossa *et al.*, 2004; de Paiva *et al.*, 2003; Singh and Udupa, 1997). This study was designed to evaluate *in vitro* antimicrobial and combination effect of plumbagin with amphotericin B against *C. albicans* clinical isolates and evaluate its safety for topical administration as well as antiviral activity against HCV.

MATERIALS AND METHODS

Anticandidal activity.

Fungi strains, cultures, and antimycotics. Plumbagin, *C. albicans* (American Type Culture Collection (ATCC) 10231) strain and amphotericin B were obtained from Sigma-Aldrich (Germany). Plumbagin in all experiments was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Czech Republic), and concentrations that have been used in each experiment were prepared by dilution with deionized water. Concentrations of DMSO in deionized water, which are equal to concentrations of DMSO in experimental solutions, were used as a blank control. In addition, 0.1% of DMSO has been found to be the highest concentration that can be safely used for dissolving plumbagin. Six clinical isolates of *C. albicans* (CA1, CA2, CA3, CA4, CA5, and CA6; isolated from patients with vaginal yeast infection) were obtained from The Motol Univesity Hospital, Prague, Czech Republic. For antifungal assay, the strains were grown in Mueller–Hinton broth (Oxoid, Basingstoke, UK) (Clinical and Laboratory Standards Institute, 2008; Vijayarathna *et al.*, 2012).

Antifungal activity assay. *Candida albicans* (ATCC 10231) was used as a control strain. Amphotericin B was used as a reference antimycotic drug. Dimethyl sulfoxide and deionized water were used as a blank control that did not inhibit any strain tested. Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method according to the recommendation of Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2008) using 96-well microtiter plates modified according to the recommendations that have been recommended for the more effective determination of antiinfective potential of natural products (Cos *et al.*, 2006). Briefly, ten twofold serial dilutions of plumbagin were prepared in the appropriate broth concentrations ranging from 5 to 412 μg/mL. Each well was inoculated with 5 μL of fungal suspension at a density of 10^7 CFU/mL, while microtiter plates were incubated at 37 °C for 48 h, and fungal growth was determined as turbidity by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. Minimum inhibitory concentrations were determined as the lowest concentrations that inhibited the growth of the test fungi by ≥80% compared with that of the agent-free growth control. Minimum inhibitory concentrations were obtained from three independent experiments that performed in triplicate.

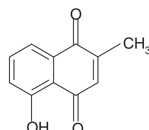

Figure 1. Chemical structure of plumbagin.

Combinatory effect of plumbagin with amphotericin B. Checkerboard assay was used to determine the combination effect of amphotericin B with plumbagin. The combination effect was evaluated algebraically based on the sum of the fractional inhibitory concentration (ΣFIC) indices as previously described (Rondevaldova *et al.*, 2015; Vuuren and Viljoen, 2011). In brief, twofold serial dilutions of amphotericin B prepared in horizontal rows of microtiter plate were subsequently cross-diluted vertically by twofold serial dilutions of plumbagin. The one-half MIC of plumbagin and amphotericin B were used as a starting concentration in combinations. For evaluation of combinatory effect of plumbagin (A) with antimycotic drug tested (B), the ΣFIC was calculated based on the following equation: $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_A$ (in the presence of B)/MIC_A (alone), and $FIC_B = MIC_B$ (in the presence of A)/MIC_B (alone). The MICs used in this equation are the averages of MICs obtained from three independent experiments that performed in triplicate. The interpretation of the *in vitro* antifungal interactions was determined as follows: synergistic effect if $\Sigma FIC \leq 0.5$, additive if $\Sigma FIC > 0.5$ and ≤ 1, no interaction if $\Sigma FIC > 1$ and ≤ 4, and antagonistic if $\Sigma FIC > 4$ (Novy *et al.*, 2013).

Cell toxicity assay. The toxic effect of plumbagin in combination with amphotericin B on HaCaT keratinocytes (HaCaT cell line, ATCC, USA; obtained from The Motol Univesity Hospital, Prague, Czech Republic) was evaluated by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Di Grazia *et al.*, 2014). Briefly, HaCaT cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco, USA), L-glutamine (4 mM), and 0.05 mg/mL gentamicin, at 37 °C and 5% CO_2, in 25 cm^2 flasks. The 10^4 cells in their culture medium supplemented with 2% FBS were seeded in each well of the microtiter plate. After overnight incubation at 37 °C in 5% CO_2 atmosphere, the plumbagin in combination with amphotericin B at concentrations that exhibited the antifungal additive effect (0.12, 0.13 and 0.19, 1.81 μg/mL, respectively) were added to HaCaT cells in their culture medium supplemented with 2% FBS. After 24 h of treatment, the tested compounds were removed and the cells were incubated with Hank's buffer containing 0.5 mg/mL MTT solution (Islas-Rodrìguez *et al.*, 2009). After 4 h incubation at 37 °C, formazan crystals were solubilized with acidified isopropanol and the absorbance of the samples was measured at 570 nm using a

Copyright © 2016 John Wiley & Sons, Ltd.

microplate reader (Infinite M200, Tecan, Salzburg, Austria). Cell viability was calculated with respect to the control (untreated cells).

Antiviral activity.

Cultures, cells, and reagents. Huh7.5 human liver cells were obtained from The Motol Univesity Hospital, Prague, Czech Republic, and were cultured in Dulbecco's Modified Eagle's Medium, which was supplemented with 10% inactivated FBS (Gibco, USA) and 1% penicillin-streptomycin. The cells were cultured at 37 °C in 5% CO_2, released with 0.05% trypsin-EDTA and split twice a week. The plasmid pFL-J6/JFH/JC1, which contains the full-length chimeric HCV cDNA, was obtained from The Motol Univesity Hospital, Prague, Czech Republic. Vero cells were purchased from the ATCC (Rockville, MD, USA) and were cultured in Minimum Essential Medium supplemented with 10% FBS and antibiotics (100 U/mL penicillin G and 100 mg/mol streptomycin).

Cytotoxicity assay. The Huh7.5 cells were used in the assay; Huh7.5 cells (1×10^4 cells/well) were planted into 96-microwell plates. After 6 h, the culture media was replaced with fresh medium containing the tested compound at various concentrations. Cytotoxicity was evaluated with the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Reed and Muench, 1938) at 96 h, and the 50% cytotoxic concentration (CC_{50}) was calculated.

Anti-HCV activity and immunoblot measurements. The antiviral activity of plumbagin against HCV was tested in infected Huh7.5 cells using the quantitative real-time reverse transcription PCR and evaluated as previously described by Peng *et al.* (2011). Telaprevir (VX-950), a NS3/4A protease inhibitor (Sigma-Aldrich, USA), was used as a positive control. PCR master-mix (as per manufacturer's instructions, Applied Biosystems) contains primer pairs of 5′-CGGGAGAGCCATAGTGGTCTGCG-3′ and 5′-CTCGCAAGCACCCTATCAGGCAGTA-3′, and TaqMan probe 5′-FAM-AGGCC TTGTGGTACTGCCT-TAMRA-3′ was used. Fluorescent signals were detected with 7500-fast real-time PCR system (Applied Biosystems) using the following conditions: 30 min at 48 °C followed by 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Briefly, the Huh7.5 cells were seeded into six-well plates (Costar) at a density of 3×10^4 cells/cm^2. The cells were infected with HCV viral stock at an infective dose of 45 IU/cell after 6 h incubation and treated simultaneously with plumbagin, telaprevir, or the solvent control. After 96 h inoculation, the culture medium was removed and the total intracellular RNA and total intercellular protein were extracted with RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The intracellular HCV RNA was quantified using a one-step real-time PCR kit (Invitrogen). The half maximal inhibitory concentration (IC_{50}) was calculated according to the method (Reed and Muench, 1938). Selectivity index (SI) value was calculated as the ratio of CC_{50}/IC_{50}. Immunoblot experiments were similar to those previously described by Peng *et al.* (2011).

RESULTS

The results demonstrated that plumbagin exhibited remarkable inhibitory activity against all *C. albicans* strains tested with MICs values ranging from 7.41 to 11.24 μg/mL (Table 1). The additive antifungal interaction of plumbagin in combination with amphotericin B at concentrations of (0.12, 0.13 and 0.19, 1.81 μg/mL, respectively) was obtained against CA (ATCC 10231), CA3, CA4, CA5, and CA6 strains with ΣFIC ranging from 0.62 to 0.91 (Table 2). In few cases ΣFIC values were close to 0.62, which can be considered as strong additive effect (close to the border of synergy). It has been found that no additive effect was observed when plumbagin was combined with amphotericin B at concentration of 0.5 μg/mL (plumbagin at 0.28 and amphotericin B at 0.22 μg/mL) and the additive effect was observed at combined concentrations of 2 μg/mL (plumbagin at 0.19 and amphotericin B at 1.81 μg/mL) and 0.25 μg/mL (plumbagin at 0.12 and amphotericin B at 0.13 μg/mL). Based on these results, we may suggest that the ratio of plumbagin to amphotericin B (lower than one) plays an important role in the additive antifungal interaction. Therefore, for the combination effect, it should be taken into consideration the concentration and ratio between both compounds in combination. Moreover, no synergy or antagonistic effects have been observed. Plumbagin when combined with amphotericin B showed weak cytotoxicity against HaCaT keratinocytes cells (98.85 and 96.77% viable cells at 0.12, 0.13 and 0.19, 1.81 μg/mL, respectively) (Table 3). In addition, plumbagin showed antiviral activity against HCV compared with that of telaprevir with IC_{50} values of 0.57 and 0.01 μM/L, respectively and SI = 53.7 and SI = 2127, respectively (Table 4). For evaluating the effect of plumbagin on HCV replication, the expression of intracellular hA3G protein and HCV

Table 1. *In vitro* anticandidal activity of amphotericin B and plumbagin against *C. albicans* strains

C. albicans strains	MIC (μg/mL)	
	Amphotericin B	Plumbagin
CA (ATCC 10231)	8.28 ± 0.38	10.14 ± 0.35
CA1[1]	7.26 ± 0.30	10.68 ± 0.38
CA2[1]	9.15 ± 0. 35	11.24 ± 0.36
CA3[1]	11.21 ± 0.32	9.32 ± 0.31
CA4[1]	8.38 ± 0.32	7.41 ± 0.33
CA5[1]	10.24 ± 0.35	10.47 ± 0.31
CA6[1]	7.43 ± 0.33	10.35 ± 0.34

MIC, minimum inhibitory concentration; the values are expressed as an average from three independent experiments that performed in triplicate.
Values presented are means ± SD of three independent experiments.
ATCC: American Type Culture Collection.
[1]Clinical isolates.

Copyright © 2016 John Wiley & Sons, Ltd.

Table 2. ***In vitro*** **combinatory anticandidal effect of plumbagin with amphotericin B against** ***C. albicans*** **strains**

C. albicans strains	Concentrations of the antifungal compounds in combination (μg/mL)							
	Plumbagin at 1 and amphotericin B at 2		Plumbagin at 0.19 and amphotericin B at 1.81		Plumbagin at 0.28 and amphotericin B at 0.22		Plumbagin at 0.12 and amphotericin B at 0.13	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
CA (ATCC 10231)	9.14 ± 0.39	2.91 ± 0.03	9.38 ± 0.32	**0.66 ± 0.03**	10.32 ± 0.38	2.47 ± 0.04	9.73 ± 0.36	**0.69 ± 0.03**
CA1[1]	10.14 ± 0.37	1.14 ± 0.01	10.17 ± 0.35	1.76 ± 0.04	7.17 ± 0.37	2.74 ± 0.04	7.64 ± 0.33	2.24 ± 0.04
CA2[1]	10.36 ± 0.30	1.21 ± 0.02	11.12 ± 0.37	1.22 ± 0.04	8.78 ± 0.32	1.41 ± 0.03	7.79 ± 0.32	2.21 ± 0.03
CA3[1]	11.45 ± 0.33	2.73 ± 0.01	10.78 ± 0.36	**0.88 ± 0.03**	9.14 ± 0.34	1.31 ± 0.02	8.74 ± 0.31	**0.84 ± 0.02**
CA4[1]	13.41 ± 0.34	3.25 ± 0.03	7.78 ± 0.31	**0.82 ± 0.02**	11.75 ± 0.31	2.17 ± 0.03	9.69 ± 0.34	**0.78 ± 0.02**
CA5[1]	8.37 ± 0.36	1.27 ± 0.04	6.46 ± 0.36	**0.89 ± 0.03**	9.47 ± 0.34	1.99 ± 0.02	10.78 ± 0.35	**0.91 ± 0.03**
CA6[1]	7.17 ± 0.35	3.57 ± 0.03	8.14 ± 0.33	**0.62 ± 0.02**	6.12 ± 0.37	1.47 ± 0.02	11.17 ± 0.34	**0.63 ± 0.02**

ΣFIC: sum of fractional inhibitory concentrations—the combination interaction is evaluated as follows: synergy ΣFIC ≤ 0.5; additive ΣFIC > 0.5 and ≤1 (bold font indicates additive acting combinations); no interaction ΣFIC > 1 and ≤ 4; antagonistic if ΣFIC > 4.
MIC, minimum inhibitory concentration; the values are expressed as an average from three independent experiments that performed in triplicate. Values presented are means ± SD of three independent experiments.
ATCC: American Type Culture Collection.
[1]Clinical isolates.

Table 3. Cytotoxic effect of plumbagin in combination with amphotericin B on HaCaT cells

Compound	Concentration μg/mL	[1] Cell viability (%)
Plumbagin plus amphotericin B	0.12 and 0.13	98.85
Plumbagin plus amphotericin B	0.19 and 1.81	96.77

[1]Cell viability; all data were average values from three independent experiments.

Table 4. Anti-HCV activity and cytotoxicity of plumbagin

Compound	IC_{50} (μM/L)	CC_{50} (μM/L)	SI
Plumbagin	0.57 ± 0.20	30.65 ± 1.25	53.7
VX-950	0.01 ± 0.40	21.27 ± 4.70	2127

All data were average values from three independent experiments. Values presented are means ± SD of three independent experiments. CC_{50}: 50% cytotoxic concentration. IC_{50}: half maximal inhibitory concentration. SI: selectivity index (CC_{50}/IC_{50}). VX-950: Telaprevir (positive control).

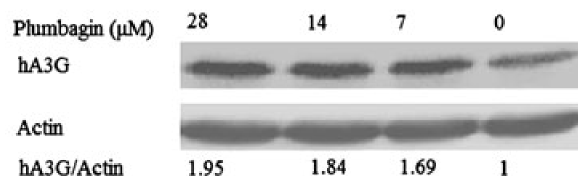

Figure 2. Effect of plumbagin on HCV replication; after 24 h of plumbagin treatment in naïve Huh7.5 cells, intracellular hA3G protein level has been increased dose-dependently.

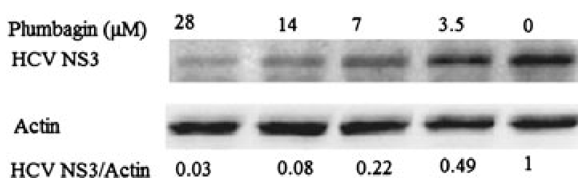

Figure 3. Effect of plumbagin on HCV replication; after 72 h of plumbagin treatment in HCV infected Huh7.5 cells, HCV NS3 protein synthesis has been decreased dose-dependently.

NS3 protein was evaluated in the Huh7.5 cells with or without using the test compound. Plumbagin treatment exhibited higher intracellular hA3G protein levels in a dose-dependent manner (Fig. 2), while showed lower HCV NS3 protein levels (Fig. 3) in a dose-dependent manner. Thus, plumbagin has been shown to inhibit HCV replication.

DISCUSSION

Infectious diseases still remain the main cause of morbidity and mortality among humans, especially in developing countries. Plants remain the main source of biologically active substances with great bioavailability, low toxicity, less resistance, and various mechanisms of action (Hassan *et al.*, 2015; Gibbons, 2008; Cavero *et al.*, 2013). Therefore, their application in the treatment of microbial infections has gained much interest in research field. Despite limited studies have reported the direct anticandidal growth-inhibitory activity of plumbagin, these studies did not focus on the possibility of its combination effect with commercial standard antimycotics. Therefore, we report for the first time the additive effect of plumbagin with amphotericin B as well as the direct inhibitory activity against *C. albicans* strains, and anti-HCV activity. Although plant extracts containing plumbagin have been used in folk medicine for decades, there is much evidence to suggest that it may have potential value as a chemotherapeutic agent; however, concerns have been raised about its safety, perhaps because of reports on its vesicant and abortifacient properties. These have been adequately dealt with in several reports that have been

Copyright © 2016 John Wiley & Sons, Ltd.

previously discussed (Talcott *et al*., 1985; Babula *et al*., 2007; Ogihara *et al*., 1997). Plumbagin is not only potentially beneficial for the treatment of various diseases, but it has also been reported to have many side effects in a dose-dependent manner such as diarrhea, increase in white blood cell counts, skin rashes, increase in serum phosphate and acid phosphate level, and reproductive toxicity in male and female animals (Chandrasekaran and Nagarajan, 1981; Singh and Udupa, 1997; Premakumari *et al*., 1977; Bhargava, 1984; Kini *et al*., 1997). It has been reported that cytotoxic action of plumbagin and related quinones is mainly because of two different mechanisms, namely, redox cycling and reaction with glutathione (GSH). In addition, topical preparations containing plumbagin should be used with care, and taking into consideration the non-toxic doses (Inbaraj and Chignell, 2004). It has been reported that cytotoxicity of antimicrobial compounds can be improved by combining them, at low concentrations, with non-toxic compounds (Anantharaman *et al*., 2010; Mangoni *et al*., 2000). Based on these facts, we tried to evaluate the cytotoxic effect of plumbagin on mammalian cells and we found that plumbagin in combination with amphotericin B could be used safely for topical administration at concentrations of 0.12, 0.13 and 0.19, 1.81 μg/mL, respectively) against *C. albicans* infection. Plumbagin is very efficient in treating infectious diseases caused by *C. albicans*. However, to explain the mechanism of action against *C. albicans* have to be determined by further studies *in vivo* and in clinical trials. Plumbagin exhibited anti-HCV activity at a non-cytotoxic concentration ($CC_{50} = 30.65$ μM/L), and thus can play an important role in pharmaceutical industry as a natural antiviral agent.

CONCLUSION

In summary, there is growing interest in natural products as they provide health benefits. Plant-derived products are believed to contain many bioactive compounds with various biological activities. The present findings indicated that plumbagin exhibited *in vitro* notable antifungal and additive effect with amphotericin B against *C. albicans* clinical isolates. This combinatory effect offers the possibility to decrease the toxicity of plumbagin and to improve its therapeutic efficacy against *C. albicans* infection. Moreover, plumbagin exhibited anti-HCV activity, and thus has promising application in the treatment of HCV infection. However, further studies should be carried out to eliminate the possible adverse effects of this compound by using improved delivery techniques prior its possible practical application. In addition, further research focused on validation of its activity *in vivo* and in clinical trials will be necessary.

Acknowledgements

This study was funded by Internal Grant Agency (IGA) of the Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Czech Republic. Project No. 20154247/2015.

Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES

Aiyegoro OA, Okoh AI. 2009. Review: use of bioactive plant products in combination with Standard antibiotics: implications in antimicrobial chemotherapy. *J Med Plants Res* **3**: 1147–1152.

Anantharaman A, Rizvi MS, Sahal D. 2010. Synergy with rifampin and kanamycin enhances potency, kill kinetics, and selectivity of de Novo-designed antimicrobial peptides. *Antimicrob Agents Chemother* **54**: 1693–1699.

Babula P, Adam V, Havel L, *et al.* 2007. Naphthoquinones and their pharmacological properties. *Ceska Slov Farm* **56**: 114–120.

Bhargava SK. 1984. Effects of plumbagin on reproductive function of male dog. *Indian J Exp Biol* **22**: 153–156.

Cavero RY, Akerreta S, Calvo MI. 2013. Medicinal plants used for dermatological affections in Navarra and their pharmacological validation. *J Ethnopharmacol* **149**: 533–542.

Chandrasekaran B, Nagarajan B. 1981. Metabolism of ethylamine and plumbagin in rats. *J Biol Sci* **3**: 395–400.

CLSI. 2008. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, third; CLSI document M27-A3 edn.; approved standard-. Clinical and Laboratory Standards Institute: Wayne, PA, USA.

Cos P, Vlietinck AJ, Vanden Berghe D, Maes L. 2006. Anti-infective potential of natural products: how to develop a stronger *in vitro* 'proof-of-concept'. *J Ethnopharmacol* **106**: 290–302.

Dai BD, Cao YY, Huang S, *et al.* 2009. Baicakein induces programmed cell death in *Candida albicans*. *J Microbiol Biotechnol* **19**: 803–809.

de Paiva SR, Figueiredo MR, Aragao TV, *et al.* 2003. Antimicrobial activity *in vitro* of plumbagin isolated from Plumbago species. *Mem I Oswaldo Cruz* **98**: 959–961.

Di Grazia A, Luca V, Segev-Zarko LA, *et al.* 2014. Temporins A and B stimulate migration of HaCaT keratinocytes and kill intracellular *Staphylococcus aureus*. *Antimicrob Agents Chemother* **58**: 2520–2527.

Gentile I, Buonomo AR, Borgia F, *et al.* 2014. Ledipasvir: a novel synthetic antiviral for the treatment of HCV infection. *Expert Opin Investig Drugs* **23**: 561–571.

Gibbons S. 2008. Phytochemicals for bacterial resistance—strengths, weaknesses and opportunities. *Planta Med* **74**: 594–602.

Hassan STS, Masarčíková R, Berchová K. 2015. Bioactive natural products with anti-herpes simplex virus properties. *J Pharm Pharmacol* **67**: 1325–1336.

Inbaraj JJ, Chignell CF. 2004. Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes. *Chem Res Toxicol* **17**: 55–62.

Islas-Rodrìguez AE, Marcellini L, Orioni B, *et al.* 2009. Esculentin 1–21: a linear antimicrobial peptide from frog skin with inhibitory effect on bovine mastitis-causing bacteria. *J Pept Sci* **15**: 607–614.

Joy PP, Thomas J, Mathew S, *et al.* 2001. Medicinal plants. In Tropical Horticulture **2**. Naya Prokash: Calcutta; 104.

Kayali Z, Schmidt WN. 2014. Finally sofosbuvir: an oral anti-HCV drug with wide performance capability. *Pharmgenomice Pers Med* **7**: 387–398.

Kim J, Sudbery P. 2011. *Candida albicans*, a major human fungal pathogen. *J Microbiol* **49**: 171–177.

Kini DP, Pandey S, Shenoy BD, *et al.* 1997. Antitumor and antifertility activities of plumbagin controlled release formulations. *Indian J Exp Biol* **35**: 374–379.

Lilic D, Haynes K. 2007. Candida. In in Immunology of Fungal Infections, Brown GD, Netea MG (eds). Springer: The Netherlands; 361–382 Chapter 16.

Lindenbach BD, Rice CM. 2005. Unravelling hepatitis C virus replication from genome to function. *Nature* **436**: 933–938.

Mangoni ML, Rinaldi AC, Di Giulio A, *et al.* 2000. Structure-function relationship of temporins, small antimicrobial peptides from amphibian skin. *Eur J Biochem* **267**: 1447–1454.

Copyright © 2016 John Wiley & Sons, Ltd.

Miceli MH, Diaz JA, Lee SA. 2011. Emerging opportunistic yeast infections. *Lancet Infect Dis* **11**: 142–151.

Mossa JS, El-Feraly FS, Muhammad I. 2004. Antimycobacterial constituents from Juniperus procera, Ferula communis and Plumbago zeylanica and their *in vitro* synergistic activity with isonicotinic acid hydrazide. *Phytother Res* **18**: 934–937.

Novy P, Rondevaldova J, Kourimska L, Kokoska L. 2013. Synergistic interactions of epigallocatechin gallate and oxytetracycline against various drug resistant *Staphylococcus aureus* strains *in vitro*. *Phytomedicine* **20**: 432–435.

Ogihara K, Yamashiro R, Higa M, *et al.* 1997. Preparation of naphthoquinone derivatives from plumbagin and their ichthyotoxicity. *Chem Pharm Bull* **45**: 437–445.

Peng ZG, Zhao ZY, Li YP, *et al.* 2011. Host apolipoprotein B messenger RNA-editing enzyme catalytic polypeptide-like 3G is an innate defensive factor and drug target against hepatitis C virus. *Hepatology* **53**: 1080–1089.

Penin F, Dubuisson J, Rey FA, *et al.* 2004. Structural biology of hepatitis C virus. *Hepatology* **39**: 5–19.

Premakumari P, Rathinam K, Santhakumari G. 1977. Antifertility activity of plumbagin. *Indian J Med Res* **65**: 829–838.

Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. *Am J Hyg* **27**: 493–497.

Rondevaldova J, Novy P, Kokoska L. 2015. *In vitro* combinatory antimicrobial effect of plumbagin with oxacillin and tetracycline against *Staphylococcus aureus*. *Phytother Res* **29**: 144–147.

Shirtliff ME, Krom BP, Meijering RA, *et al.* 2009. Farnesolinduced apoptosis in *Candida albicans*. *Antimicrob Agents Chemother* **53**: 2392–2401.

Singh UV, Udupa N. 1997. Reduced toxicity and enhanced antitumor efficacy of betacyclodextrin plumbagin inclusion complex in mice bearing Ehrlich ascites carcinoma. *Indian J Physiol Pharmacol* **41**: 171–175.

Sofia MJ. 2014. Beyond sofosbuvir: what opportunity exists for a better nucleoside/nucleotide to treat hepatitis C? *Antiviral Res* **107**: 119–124.

Staniszewska M, Bondaryk M, Swoboda-Kopec E, *et al.* 2013. *Candida albicans* morphologies revealed by scanning electron microscopy analysis. *Braz J Microbiol* **44**: 813–821.

Stankiewicz-Drogoń A, Dörner B, Erker T, *et al.* 2010. Synthesis of new acridone derivatives, inhibitors of NS3 helicase, which efficiently and specifically inhibit subgenomic HCV replication. *J Med Chem* **53**: 3117–3126.

Summers BB, Beavers JW, Klibanov OM. 2014. Sofosbuvir, a novel nucleotide analogue inhibitor used for the treatment of hepatitis C virus. *J Pharm Pharmacol* **66**: 1653–1666.

Talcott RE, Smith MT, Giannini DD. 1985. Inhibition of microsomal lipid peroxidation by naphthoquinones: structure-activity relationships and possible mechanisms of action. *Arch Biochem Biophys* **241**: 88–94.

Vijayarathna S, Zakaria Z, Chen Y, *et al.* 2012. The antimicrobial efficacy of *Elaeis guineensis*: characterization, *in vitro* and *in vivo* studies. *Molecules* **17**: 4860–4877.

Vuuren SV, Viljoen A. 2011. Plant-based antimicrobial studies – methods and approaches to study the interaction between natural products. *Planta Med* **77**: 1168–1182.

Wagner H. 2011. Synergy research: approaching a new generation of phytopharmaceuticals. *Fitoterapia* **82**: 34–37.

Copyright © 2016 John Wiley & Sons, Ltd.

South African Journal of Botany 108 (2017) 90–94

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Cucurbitacin B interacts synergistically with antibiotics against *Staphylococcus aureus* clinical isolates and exhibits antiviral activity against HSV-1

S.T.S. Hassan [a,b,*], K. Berchová-Bímová [b], J. Petráš [a], K.T.S. Hassan [c]

[a] Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic
[b] Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6-Suchdol, Czech Republic
[c] Department of Forestry and Wood Technology, Faculty of Agriculture, Alexandria University, Aflaton st., El-Shatby, 21545 Alexandria, Egypt

ARTICLE INFO

Article history:
Received 21 December 2015
Received in revised form 10 September 2016
Accepted 2 October 2016
Available online xxxx

Edited by I Vermaak

Keywords:
Ecballium elatrium
Cucurbitacin B
HSV
Staphylococcus aureus
Synergy

ABSTRACT

The search for biologically promising compounds from natural sources against microbial diseases remains an important theme in drug discovery to overcome problems with drug-resistant strains. In this study, Cucurbitacin B (Cuc B), an active constituent of *Ecballium elaterium* L., has been investigated *in vitro* for its synergy effect with antibiotics against clinical isolates of *Staphylococcus aureus* (*S. aureus*) and anti-HSV-1 activity. Broth microdilution method was used to determine the antibacterial activity, while checkerboard assay was used to evaluate the synergy effect according to ΣFIC indices. The anti-HSV-1 activity was determined by the plaque number reduction assay, while cytotoxicity was evaluated by MTT assay. In this study, Cuc B exerted direct growth-inhibitory activity against all *S. aureus* strains tested with MICs values ranging from 0.12 to 0.44 μg/mL, as well as synergy effect with tetracycline or oxacillin against four of six *S. aureus* strains tested (ΣFIC ranging from 0.29 to 0.43). Cuc B showed remarkable anti-HSV-1 activity compared with that of acyclovir with IC_{50} values of 0.94 and 1.74 μM, respectively and selectivity indices SI = 127.7 and SI > 132.2, respectively. This study presents Cuc B as a promising therapeutic agent in the development of anti-staphylococcal and anti-HSV-1 drugs.
© 2016 SAAB. Published by Elsevier B.V. All rights reserved.

1. Introduction

Ecballium elaterium L. (*E. elaterium*, squirting cucumber, Cucurbitacae) is a wild-growing medicinal plant found abundantly in the Mediterranean region. The interior of the fruits contains black seeds and juice. In traditional folk medicine, *E. elaterium* fruits have been used as antimicrobial, analgesic, antipyretic and antiphlogistic agents. Moreover, *E. elaterium* is of interest in Mediterranean region due to the use of its fruits extracts in various medicinal uses (Khalil and Qaoud, 1993; Raikhlin-Eisenkraft and Bentur, 2000; Bohlooli et al., 2012). It has been reported that the main active compounds from the fruits that are responsible for the biological activities including antimicrobial properties were found to be fatty acids, proteins, cucurbitacins (B, D, E, I and L) and cucurbitacin derivatives such as glycosylcucurbitacins and triterpenoids glycosides (Rao et al., 1974; Attard et al., 2005; Chen et al., 2005). Cucurbitacin B (Cuc B, Fig. 1), an active constituent of *E. elaterium*, is a natural tetracyclic triterpene compound that belongs to Cucurbitacins (CUs) compounds, which are widely distributed in the family of Cucurbitaceae. Structurally, CUs are characterized by a tetracyclic cucurbitane nucleus skeleton, namely, 9β-methyl-19-nor lanosta-5-enea, which is traditionally divided arbitrarily into twelve categories, incorporating CUs A-T (Chen et al., 2005; Hassan and Žemlička, 2016). Numerous studies demonstrated that Cuc B possesses a variety of bioactivities, such as antibacterial, antifungal, anti-inflammatory, hepatoprotective and anticancer activities (Chen et al., 2005; Liu et al., 2008; Chen et al., 2012). For decades, plants remain the main source of biologically active compounds that have been used for the treatment of various diseases, including infectious diseases with reduced side effects, bioavailability, less resistance, low toxicity and various mechanisms of action (Cowan, 1999; Mabona et al., 2013; Hassan et al., 2015). In recent years, plant-derived chemicals are increasingly used in pharmaceutical industry (Gibbons, 2008; Fischer et al., 2013). *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium has become one of the most serious human pathogens that cause serious infections, such as bacteraemia, severe pneumonia and skin infections, while methicillin-resistant *S. aureus* (MRSA) is considered one of the most main causes of antibiotic-resistant healthcare-associated infections worldwide (Cutler and Wilson, 2004; Appelbaum, 2007; Pandey, 2007; Sharifi-Rad et al., 2014). The intensive

* Corresponding author at: Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42, Brno, Czech Republic, and Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21, Praha 6-Suchdol, Czech Republic.
E-mail address: sherif.hassan@seznam.cz (S.T.S. Hassan).

http://dx.doi.org/10.1016/j.sajb.2016.10.001
0254-6299/© 2016 SAAB. Published by Elsevier B.V. All rights reserved.

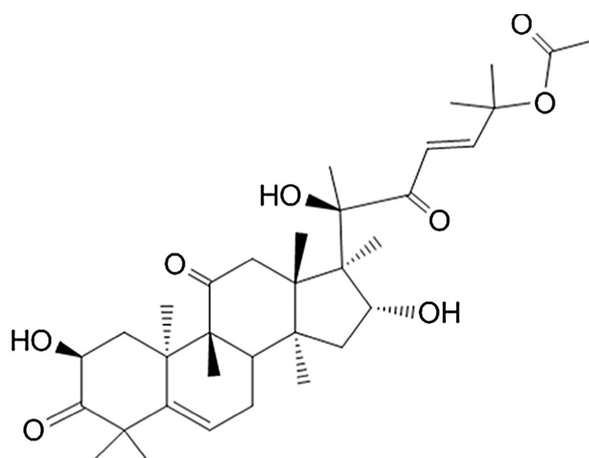

Fig. 1. Chemical structure of cucurbitacin B.

use of antibiotics has led to the problem of drug-resistant strains, and thus has led to the failure of current treatment regimens of staphylococcal infections in humans (Drago et al., 2014; Farrell et al., 2014). Therefore, a new treatment strategy has been devoted to overcome the problem by using plant-derived products in combination with antibiotics to enhance the treatment efficacy (Aiyegoro and Okoh, 2009; Wagner, 2011). Herpes simplex virus (HSV) infections are quite common in humans. HSV is a member of Herpesviridae, a wide family of enveloped-DNA viruses that cause several clinically significant syndromes in both adults and neonates. HSV-1 is mainly connected with oral or facial infection and encephalitis (Field, 1989; Paludan et al., 2011; Hassan et al., 2015). Treatment of HSV infection remains a main target for many researchers worldwide, where it cannot be managed by vaccination. Acyclovir and related nucleoside analogs have been widely used in the treatment of HSV, but the intensive use of such drugs has led to several undesirable effects including drug-resistant strains (Piret and Boivin, 2011; Evans et al., 2013). Despite few studies have reported that crude extracts of *E. elatrium* and various fractions of cucurbitacins have been shown to possess direct anti-staphylococcal growth inhibitory activity (Dogruoz et al., 2008; Oskay et al., 2009; Adwan et al., 2011), these studies did not evaluate the possibility of antimicrobial combinatory effect of Cuc B with standard antibiotics against *S. aureus*. Therefore, in this study, we report the synergistic effect of Cuc B with tetracycline (TET) or oxacillin (OX) as well as the direct inhibitory effect against *S. aureus*. In addition, we report for the first time the antiviral activity of Cuc B against HSV-1.

2. Materials and methods

2.1. Anti-Staphylococcus aureus activity

2.1.1. Bacterial strains, cultures, chemicals and antibiotics

Tetracycline, oxacillin and Cuc B were purchased from Sigma-Aldrich (Prague, Czech Republic). *S. aureus* (ATCC 29213) and methicillin-resistant *S. aureus* (MRSA-ATCC 43300) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Four clinical isolates of *S. aureus* (SA1, SA2, SA3, and SA4; isolated from patients with skin infection) were obtained from U Sv. Anny Univesity Hospital, Brno, Czech Republic. Dimethyl sulfoxide (0.05% DMSO; Sigma-Adrich, Czech Republic) was used to dissolve Cuc B. For antimicrobial assay, the strains were grown in cation-adjusted Mueller–Hinton broth (MHB; Oxoid, Basingstoke, UK) equilibrated with Tris–buffered saline (Sigma-Aldrich, Prague, Czech Republic).

2.1.2. Antimicrobial assay

For antibiotic susceptibility testing, *S. aureus* (ATCC 29213) was used as a reference strain. Cuc B at concentrations ranging from 0.25 to 3 μg/ml was used. Oxacillin-supplemented with 2% NaCl and tetracycline were used as standard antibiotics at concentrations ranging from 0.25 to 3 μg/mL. 0.05% DMSO and deionized water were used as negative controls that did not inhibit any strain tested. The broth microdilution method using 96-well microtiter plates was performed to determine the minimum inhibitory concentrations (MICs) following the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2009). Briefly, the samples were two-fold diluted in MHB (100 μL), and inoculated with bacterial suspension to reach the density of 5×10^5 CFU/mL. Microtiter plates were incubated at 37 °C for 24 h, and bacterial growth was determined as turbidity by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. MICs were subjected as the lowest concentrations that inhibited the growth of the test bacteria by ≥80% compared with that of negative controls. MICs obtained from three parallel experiments, each performed in triplicate.

2.1.3. Combinatory effect of Cuc B with antibiotics

The checkerboard assay was used to evaluate the combination effect of antibiotics with Cuc B, and the sum of the fractional inhibitory concentration (ΣFIC) indices have been evaluated as previously described (Vuuren and Viljoen, 2011; Hassan et al., 2016). Briefly, two-fold serial dilutions of oxacillin-supplemented with 2% NaCl or tetracycline prepared in horizontal rows of microtiter plate (at concentrations ranging from 0.25 to 3 μg/mL) were subsequently cross-diluted vertically by two-fold serial dilutions of Cuc B (at concentrations ranging from 0.25 to 3 μg/mL). The one-half MIC of Cuc B, oxacillin and tetracycline was used as a starting concentration in combinations. For evaluation of antibacterial combination effect of Cuc B (A) with antibiotic tested (B), the following equation $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_A$ (in the presence of B) / MIC_A (alone), and $FIC_B = MIC_B$ (in the presence of A) / MIC_B (alone), was used to calculate ΣFIC. The MICs used in this equation are the averages of MICs obtained from three parallel experiments, each performed in triplicate. The interpretation of the *in vitro* antibacterial interactions was determined as follows: synergistic effect if ΣFIC ≤0.5; additive if ΣFIC > 0.5 and ≤1; no interaction if ΣFIC > 1 and ≤4; and antagonistic if ΣFIC > 4.

2.2. Anti-HSV-1 activity

2.2.1. Viral strains, cultures, cell lines and reagents

For antiviral activity, Vero cells (ATCC: CCL 81, UK; were obtained from the Motol University Hospital, Prague, Czech Republic) were grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, UK), 100 U/ml penicillin G, 100 μg/ml streptomycin and 25 μg/ml amphotericin B (Sigma-Aldrich, Germany) and maintained at 37 °C in a humidified incubator with 5% CO_2. HSV-1 [KOS strain] was obtained from The Motol Univesity Hospital, Prague, Czech Republic, and propagated in Vero cells. Viral stocks were stored at −80 °C and titrated based on plaque forming units (PFU) count by plaque assay as previously described (Burleson et al., 1992).

2.2.2. Determination of cytotoxicity

MTT [3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide] assay was used to determine the cytotoxic effect as previously described (Mosmann, 1983). In brief, confluent Vero cells were exposed to different concentrations of Cuc B for 72 h, and after incubation, the 50% cytotoxic concentration (CC_{50}) of Cuc B was calculated as the concentration that reduces cell viability by 50%, when compared to the untreated controls.

2.2.3. Antiviral assay

For antiherpetic activity, acyclovir was used as a positive control and the plaque number reduction assay was performed to evaluate the anti-HSV-1 activity as previously described (Silva et al., 2010). Briefly, cell

Table 1
In vitro antibacterial activity of OX, TET and Cuc B against *S. aureus* strains.

S. aureus strains	MIC (μg/mL)		
	OX	TET	Cuc B
S. aureus (ATCC 29213)	0.19	0.18	0.20
MRSA (ATCC 43300)	0.16	0.20	0.12
SA1[a]	0.40	0.17	0.20
SA2[a]	0.42	0.43	0.27
SA3[a]	0.19	0.22	0.36
SA4[a]	0.12	0.17	0.44

MIC — Minimum inhibitory concentration; the values are presented as an average from three parallel experiments, each performed in triplicate.
OX: Oxacillin.
TET: Tetracycline.
ATCC: American Type Culture Collection.
MRSA: methicillin-resistant *Staphylococcus aureus*.
Cuc B: Cucurbitacin B.
[a] Clinical isolates.

monolayers were infected with 100 PFU of the virus for 1 h at 37 °C, and then were overlaid with MEM containing 1.5% carboxymethylcellulose (CMC; Sigma-Aldrich, Germany) either in the presence or absence of different concentrations of Cuc B. The cells were incubated for 72 h at 37 °C, and then were fixed and stained with naphtol blue-black (Sigma-Aldrich, Germany), and the plaques were counted. The concentration of Cuc B required to reduce the plaque number by 50% (IC_{50}) was calculated, when compared to the untreated controls. Selectivity index (SI) value was calculated as the ratio of CC_{50}/IC_{50}.

2.2.4. Statistical analysis

The mean values ± standard deviations are representative of three to five independent experiments. Nonlinear regressions of concentration–response curves were used to determine CC_{50} and IC_{50} values. Anova/Dunett/SNK tests were used to evaluate the significance between Cuc B and control. In addition, the selectivity index ($SI = CC_{50}/IC_{50}$) was determined.

3. Results and discussion

The results showed that Cuc B exerted inhibitory activity against all *S. aureus* strains tested with MICs values ranging from 0.12 to 0.44 μg/mL (Table 1). The synergistic effect of Cuc B in combination with OX was obtained at a concentration of 0.25 μg/ml (Cuc B at concentration of 0.11 μg/mL and OX at concentration of 0.14 μg/mL) against MRSA-ATCC 43300 (ΣFIC = 0.43) and SA2 (ΣFIC = 0.39) strains (Table 2). In addition, the synergistic effect of Cuc B when combined with TET was determined at a concentration of 0.5 μg/mL (Cuc B at concentration of 0.21 μg/mL and TET at concentration of 0.29 μg/mL) against MRSA-ATCC 43300 (ΣFIC = 0.29), and SA4 (ΣFIC = 0.36) strains. Moreover, no additive and antagonistic interactions have been observed. Cuc B exhibited remarkable anti-HSV-1 activity compared with that of acyclovir with IC_{50} values of 0.94 and 1.74 μM, respectively and selectivity indices SI = 127.7 and SI > 132.2, respectively. Furthermore, cytotoxicity study revealed that Cuc B could be used safely at a non-cytotoxic concentration of 120 μM. This may give us an indication that Cuc B could be used safely in topical application. However, further studies should be carried out to determine its safety as well as the possible of adverse effects *in vivo* (Table 3).

The increasing problem of drug-resistant strains has led to the failure of current treatment strategies of bacterial and viral infections in humans. Therefore, it was an urgent issue to search for new antimicrobial agents derived from natural sources to overcome the problem and to enhance the treatment efficacy (Jenssen et al., 2006). It is well known that tetracycline and oxacillin are standard antibiotics for the treatment of staphylococcal infections (Macheboeuf et al., 2006). Therefore, their combination with Cuc B seems to be very perspective, since the synergy effect was determined effectively in our study. It has been reported that the mechanism contributing to tetracycline resistance in *S. aureus* is mainly due to the decrease in the intracellular accumulation of the antibiotic that can be caused be bacterial efflux proteins, which pump antibiotics from the periplasm or cytosol to the extracellular medium (Speer et al., 1992). For instance, it has been described that ferruginol has been shown to suppress the tetracycline resistance pump in *S. aureus* (Aiyegoro and Okoh, 2009). Based on these facts, we may suggest that Cuc B could act as an efflux pump inhibitor. The problem with staphylococcal oxacillin resistance is associated with production of exogenous class B of penicillin-binding protein (PBP), especially (PBP2), which has deformed active site unable to bind β-lactams (Macheboeuf et al., 2006). Cucurbitacins are highly bioactive compounds, which can make a complex with nucleophilic amino acids of proteins (Hassan et al., 2015, 2016). The antibacterial mechanism of β-lactams is mainly related to inactivation of (PBPs) by their covalent binding. In the view of those facts, we may also suggest that Cuc B could act as inhibitor of PBPs. However, the mode of action of Cuc B is still poorly understood, we suggest that Cuc B is useful for the development of new synergistically acting drugs with the potential to extend the pharmacological action of tetracycline and oxacillin. On the other hand, Cuc B is known to possess cytotoxic effect on cancer cells with various mechanisms of action (Guo et al., 2014; Gupta and Srivastava, 2014; Shukla et al., 2015) Therefore, it should be taken into consideration the non-toxic doses, especially with topical preparations

Table 2
In vitro combinatory antibacterial effect of Cuc B with OX or TET against *S. aureus* strains.

S. aureus strains	Cuc B with OX at following concentrations (μg/mL)				Cuc B with TET at following concentrations (μg/mL)			
	Cuc B at 0.9 and OX at 2.1	Cuc B at 0.4 and OX at 0.6	Cuc B at 0.21 and OX at 0.29	Cuc B at 0.11 and OX at 0.14	Cuc B at 0.9 and TET at 2.1	Cuc B at 0.4 and TET at 0.6	Cuc B at 0.21 and TET at 0.29	Cuc B at 0.11 and TET at 0.14
	ΣFIC	ΣFIC	ΣFIC	ΣFIC	ΣFIC	ΣFIC	ΣFIC	ΣFIC
S. aureus ATCC (29213)	1.49	2.14	2.41	1.39	2.73	3.24	2.14	2.36
MRSA (ATCC 43300)	1.39	2.47	1.98	**0.43**	3.14	2.97	**0.29**	2.38
SA1[a]	2.34	1.73	2.35	1.35	2.97	1.17	2.54	1.58
SA2[a]	3.13	2.47	3.23	**0.39**	2.47	1.89	1.48	2.00
SA3[a]	2.73	2.74	1.36	1.79	2.36	2.47	1.78	2.79
SA4[a]	2.67	1.79	2.36	1.84	2.73	3.65	**0.36**	3.12

ΣFIC: sum of fractional inhibitory concentrations — the combination interaction is evaluated as follows: synergy ΣFIC ≤ 0.5 (bold font indicates synergistically acting combinations); additive ΣFIC > 0.5 and ≤1; no interaction ΣFIC > 1 and ≤4; antagonistic if ΣFIC > 4; MIC — bold font indicates fold reductions.
OX: Oxacillin.
TET: Tetracycline.
ATCC: American Type Culture Collection.
MRSA: methicillin-resistant *Staphylococcus aureus*.
Cuc B: Cucurbitacin B.
[a] Clinical isolates.

Table 3
Cytotoxicity and anti-HSV-1 activity of Cuc B.

Compound	CC_{50} (μM)	IC_{50} (μM)	SI
Cuc B	120 ± 1.5	0.94 ± 0.2	127.7
Acyclovir	>230	1.74 ± 0.25	>132.2

Values represent the mean ± standard deviations of three to five independent experiments.
CC_{50}: 50% cytotoxic concentration that was determined in Vero cells.
IC_{50}: Half maximal inhibitory concentration.
SI: Selectivity index (CC_{50}/IC_{50}).
Cuc B: Cucurbitacin B.

containing Cuc B. HSV infections remain a serious threat to human health. To date, no prophylactic HSV vaccine has been found to be entirely effective in the treatment of HSV infections. This is due to the establishment of viral latency and reactivation that occurs in the presence of humoral and cell mediated immunity. Therefore, the use of bioactive natural substances, such as Cuc B, which has been shown to exert notable inhibitory effect against HSV-1 in Vero cells at a non-cytotoxic concentration of 120 μM will open new options for the development of anti-HSV drugs. Although, several studies have reported that the mode of action of plant-derived terpenes toward HSV-1 are mainly related to their ability to inhibit viral protein synthesis, or by impeding nuclear factor κB activation in HSV-1 (Kim et al., 1999; Kurokawa et al., 1999; Cheng et al., 2004), further studies are needed to be carried out to investigate the mechanism of action of Cuc B as potential anti HSV-1 agent *in vivo* and in clinical trials. It is known that anti-HSV drugs do not cure the disease, while modifying the clinical course of the infection by inhibiting viral replication and subsequent epithelial damage (Hassan et al., 2015). Therefore, the combination effect with acyclovir and related nucleoside analogs will open new options to promote the treatment course of the disease.

4. Conclusion

Plant-derived antimicrobial agents have played an essential role in the treatment of bacterial and viral diseases. Plant-derived antimicrobial agents may not serve directly as drugs, but they provide leads for the development of potential antimicrobial drugs. In the present study, Cuc B exhibited *in vitro* synergy effect with TET or OX against *S. aureus* strains. Thus, Cuc B can be used as a useful agent acting in combination with antibiotics to improve the treatment efficacy of staphylococcal infections. Moreover, Cuc B showed remarkable inhibition of HSV-1, and thus will play an important role in the development of antiherpetic drugs. Based on these findings, we suggest that Cuc B provides potential therapeutic applications as a promising natural candidate for the development of anti-staphylococcal and anti-HSV-1 drugs. In addition, further studies need to be performed to evaluate its mechanism of action and structure–activity relationship *in vivo* and in clinical trials.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgment

This study was funded by Internal Grant Agency (IGA) of the Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Czech Republic. Project No. 20154247/2015.

References

Adwan, G., Salameh, Y., Adwan, K., 2011. Effect of ethanolic extract of *Ecballium elaterium* against *Staphylococcus aureus* and *Candida albicans*. Asian Pacific Journal of Tropical Biomedicine 1, 456–460.

Aiyegoro, O.A., Okoh, A.I., 2009. Review: use of bioactive plant products in combination with standard antibiotics: implications in antimicrobial chemotherapy. Journal of Medicinal Plant Research: Planta Medica 3, 1147–1152.

Appelbaum, P.C., 2007. Reduced glycopeptide susceptibility in methicillin-resistant *Staphylococcus aureus* (MRSA). International Journal of Antimicrobial Agents 30, 398–408.

Attard, E., Brincat, M.P., Cuschieri, A., 2005. Immunomodulatory activity of cucurbitacin E isolated from *Ecballium elaterium*. Fitoterapia 76, 439–441.

Bohlooli, S., Jafari, N., Jahed, S., 2012. Cytotoxic effect of freeze-dried extract of *Ecballium elaterium* fruit on gastric adenocarcinoma (AGS) and esophageal squamous cell carcinoma (KYSE30) cell lines. Journal of Gastrointestinal Cancer 43, 579–583.

Burleson, F.G., Chamberts, T.M., Wiedbrauk, D.L., 1992. Virology: A Laboratory Manual. Academic Press, SanDiego, p. 250.

Chen, X., Bao, J., Guo, J., Ding, Q., Lu, J., Huang, M., Wang, Y., 2012. Biological activities and potential molecular targets of cucurbitacins: a focus on cancer. Anti-Cancer Drugs 23, 777–787.

Chen, J.C., Chiu, M.H., Nie, R.L., Cordell, G.A., Qiu, S.X., 2005. Cucurbitacinsand cucurbitane glycosides: structures and biologicalactivities. Natural Product Reports 22, 386–399.

Cheng, H.Y., Lin, T.C., Yang, C.M., Wang, K.C., Lin, L.T., Lin, C.C., 2004. Putranjivain A from *Euphorbia jolkini* inhibits both virus entry and late stage replication of herpes simplex virus type 2 in vitro. The Journal of Antimicrobial Chemotherapy 53, 577–583.

Clinical and Laboratory Standards Institute, 2009. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically — Eighth Edition: Approved Standard M07-A8. CLSI, Wayne, PA, USA.

Cowan, M.M., 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews 12, 564–582.

Cutler, R.R., Wilson, P., 2004. Antibacterial activity of a new, stable, aqueous extract of allicin against methicillin-resistant *Staphylococcus aureus*. British Journal of Biomedical Science 61, 71–74.

Dogruoz, N., Zeybek, Z., Karagoz, A., 2008. Antibacterial activity of some plant extracts. IUFS Journal of Biology 67, 17–21.

Drago, L., De Vecchi, E., Cappelletti, L., Mattina, R., Vassena, C., Romanò, C.L., 2014. Role and antimicrobial resistance of staphylococci involved in prosthetic joint infections. The International Journal of Artificial Organs 37, 414–421.

Evans, C.M., Kudesia, G., McKendrick, M., 2013. Management of herpesvirus infections. International Journal of Antimicrobial Agents 42, 119–128.

Farrell, D.J., Flamm, R.K., Sader, H.S., Jones, R.N., 2014. Activity of ceftobiprole against methicillin-resistant *Staphylococcus aureus* strains with reduced susceptibility to daptomycin, linezolid or vancomycin, and strains with defined SCCmec types. International Journal of Antimicrobial Agents 43, 323–327.

Field, H.J., 1989. Persistent herpes simplex virus infection and mechanisms of virus drug resistance. European Journal of Clinical Microbiology & Infectious Diseases 8, 671–680.

Fischer, R., Schillberg, S., Buyel, J.F., Twyman, R.M., 2013. Commercial aspects of pharmaceutical protein production in plants. Current Pharmaceutical Design 19, 5471–5477.

Gibbons, S., 2008. Phytochemicals for bacterial resistance — strengths, weaknesses and opportunities. Planta Medica 74, 594–602.

Guo, J., Zhao, W., Hao, W., Ren, G., Lu, J., Chen, X., 2014. Cucurbitacin B induces DNA damage, G2/M phase arrest, and apoptosis mediated by reactive oxygen species (ROS) in leukemia K562 cells. Anti-Cancer Agents in Medicinal Chemistry 14, 1146–1153.

Gupta, P., Srivastava, S.K., 2014. Inhibition of Integrin-HER2 signaling by Cucurbitacin B leads to in vitro and in vivo breast tumor growth suppression. Oncotarget 5, 1812–1828.

Hassan, S.T.S., Žemlička, M., 2016. Plant-derived urease inhibitors as alternative chemotherapeutic agents. Archiv der Pharmazie (Weinheim) 349, 507–522.

Hassan, S.T.S., Berchová-Bímová, K., Petráš, J., 2016. Plumbagin, a plant-derived compound, exhibits antifungal combinatory effect with amphotericin b against candida albicans clinical isolates and anti-hepatitis C virus activity. Phytotherapy Research http://dx.doi.org/10.1002/ptr.5650.

Hassan, S.T.S., Masarčíková, R., Berchová, K., 2015. Bioactive natural products with antiherpes simplex virus properties. The Journal of Pharmacy and Pharmacology 67, 1325–1336.

Jenssen, H., Hamill, P., Hancock, R.E., 2006. Peptide antimicrobial agents. Clinical Microbiology Reviews 19, 491–511.

Khalil, A.M., Qaoud, K.M., 1993. Toxicity and partial characterization of *Ecballium elaterium* fruit juice. International Journal of Pharmaceutics 3, 135–141.

Kim, M., Kim, S.K., Park, B.N., Lee, K.H., Min, G.H., Seoh, J.Y., et al., 1999. Antiviral effects of 28 deacetylsendanin on herpes simplex virus-1 replication. Antiviral Research 43, 103–112.

Kurokawa, M., Basnet, P., Ohsugi, M., Hozumi, T., Kadota, S., Namba, T., et al., 1999. Antiherpesimplex virus activity of moronic acid purified from *Rhus javanica* in vitro and in vivo. The Journal of Pharmacology and Experimental Therapeutics 289, 72–78.

Liu, T., Zhang, M., Zhang, H., Sun, C., Deng, Y., 2008. Inhibitory effects of cucurbitacin B on laryngeal squamous cell carcinoma. European Archives of Oto-Rhino-Laryngology 265, 1225–1232.

Mabona, U., Viljoen, A., Shikanga, E., Marston, A., Van Vuuren, S., 2013. Antimicrobial activity of southern African medicinal plants with dermatological relevance: from an ethnopharmacological screening approach, to combination studies and the isolation of a bioactive compound. Journal of Ethnopharmacology 148, 45–55.

Macheboeuf, P., Contreras-Martel, C., Job, V., Dideberg, O., Dessen, A., 2006. Penicillin Binding Proteins: key players in bacterial cell cycle and drug resistance processes. FEMS Microbiology Reviews 30, 673–691.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival:application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.

Oskay, M., Oskay, D., Kalyoncu, F., 2009. Activity of some plant extracts against multi-drug resistant human pathogens. Iranian Journal of Pharmaceutical Research 8, 293–300.

Paludan, S.R., Bowie, A.G., Horan, K.A., Fitzgerald, K.A., 2011. Recognition of herpesviruses by the innate immune system. Nature Reviews. Immunology 11, 143–154.
Pandey, A.K., 2007. Anti-staphylococcal activity of a pan-tropical aggressive and obnoxious weed parihenium histerophorus: an in vitro study. National Academy Science Letters 30, 383–386.
Piret, J., Boivin, G., 2011. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. Antimicrobial Agents and Chemotherapy 55, 459–472.
Raikhlin-Eisenkraft, B., Bentur, Y., 2000. *Ecballium elaterium* (squirting cucumber) — remedy or poison? Journal of Toxicology. Clinical Toxicology 38, 305–308.
Rao, M.M., Meshulam, H., Lavie, D., 1974. The constituents of *Ecballium elaterium* L. Part XXIII cucurbitacins and hexanorcucurbitacins. Journal of the Chemical Society, Perkin Transactions 1, 2552–2556.
Sharifi-Rad, J., Hoseini Alfatemi, S., Sharifi Rad, M., Iriti, M., 2014. Antimicrobial synergic effect of allicin and silver nanoparticles on skin infection caused by methicillin-resistant *Staphylococcus aureus spp*. Annals of Medical and Health Sciences Research 4, 863–868.
Shukla, S., Khan, S., Kumar, S., Sinha, S., Farhan, M., Bora, H.K., Maurya, R., Meeran, S.M., 2015. Cucurbitacin B alters the expression of tumor-related genes by epigenetic modifications in NSCLC and inhibits NNK-induced lung tumorigenesis. Cancer Prevention Research (Philadelphia, Pa.) 8, 552–562.
Silva, I.T., Costa, G.M., Stoco, P.H., Schenkel, E.P., Reginato, F.H., Simões, C.M.O., 2010. In vitro antiherpes effects of a C-glycosyl flavonoid-enriched fraction of Cecropiaglaziovii Sneth. Letters in Applied Microbiology 51, 143–148.
Speer, B.S., Shoemaker, N.B., Salyers, A.A., 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clinical Microbiology Reviews 5, 387–399.
Vuuren, S.V., Viljoen, A., 2011. Plant-based antimicrobial studies — methods and approaches to study the interaction between natural products. Planta Medica 77, 1168–1182.
Wagner, H., 2011. Synergy research: approaching a new generation of phytopharmaceuticals. Fitoterapia 82, 34–37.

Taylor & Francis
Taylor & Francis Group

Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: http://www.tandfonline.com/loi/iphb20

In vitro synergistic effect of Hibiscus sabdariffa aqueous extract in combination with standard antibiotics against Helicobacter pylori clinical isolates

Sherif T. S. Hassan, Kateřina Berchová, Michaela Majerová, Marie Pokorná & Emil Švajdlenka

To cite this article: Sherif T. S. Hassan, Kateřina Berchová, Michaela Majerová, Marie Pokorná & Emil Švajdlenka (2016) In vitro synergistic effect of Hibiscus sabdariffa aqueous extract in combination with standard antibiotics against Helicobacter pylori clinical isolates, Pharmaceutical Biology, 54:9, 1736-1740, DOI: 10.3109/13880209.2015.1126618

To link to this article: http://dx.doi.org/10.3109/13880209.2015.1126618

Published online: 05 Jan 2016.

Submit your article to this journal

Article views: 53

View related articles

View Crossmark data

Citing articles: 1 View citing articles

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=iphb20

Download by: [Sherif T. S. Hassan] **Date:** 11 August 2016, At: 02:55

PHARMACEUTICAL BIOLOGY, 2016
VOL. 54, NO. 9, 1736–1740
http://dx.doi.org/10.3109/13880209.2015.1126618

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

In vitro synergistic effect of *Hibiscus sabdariffa* aqueous extract in combination with standard antibiotics against *Helicobacter pylori* clinical isolates

Sherif T. S. Hassan[a,b], Kateřina Berchová[b], Michaela Majerová[a], Marie Pokorná[a] and Emil Švajdlenka[a]

[a]Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic; [b]Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Praha, Suchdol, Czech Republic

ABSTRACT

Context The increasing problem of drug-resistant strains has led to the failure of current treatment regimens of *Helicobacter pylori* (HP) infection. Recently, a new treatment strategy has been developed to overcome the problem by using natural products in combination with antibiotics to enhance the treatment efficacy.

Objective The antimicrobial combinatory effect of the aqueous extract of *Hibiscus sabdariffa* L. (Malvaceae) (AEHS) with antibiotics (clarithromycin, CLA; amoxicillin, AMX; metronidazole, MTZ) has been evaluated *in vitro* against HP strains.

Materials and methods *Hibiscus* calyces (35 g) were brewed in 250 mL of boiled water for 30 min, and minimum inhibitory concentrations (MICs) were determined by agar dilution method. The checkerboard assay was used to evaluate the antimicrobial combinatory effect according to the sum of fractional inhibitory concentration ($\sum$FIC) indices.

Results In this study, AEHS exerted remarkable bacteriostatic effect against all HP strains tested with MICs values ranging from 9.18 to 16.68 μg/mL. Synergy effect of AEHS with CLA or MTZ was obtained against four of seven HP strains tested with $\sum$FIC ranging from 0.21 to 0.39. The additive effect of AEHS with AMX was obtained against five of seven HP strains tested with $\sum$FIC ranging from 0.61 to 0.91.

Conclusion This study presents AEHS as a potent therapeutic candidate alone, or in combination with antibiotics for the treatment of HP infection.

ARTICLE HISTORY
Received 23 June 2015
Accepted 27 November 2015
Revised 7 October 2015
Published online
31 December 2015

KEYWORDS
Antimicrobial agents; combinatory effect; drug-resistant strains; *H. pylori* infection; natural products

Introduction

Helicobacter pylori (HP) infection is one of the most common causes of many diseases of the gastrointestinal tract, including non-ulcer dyspepsia, peptic ulcer, gastritis and gastric cancer (Jung et al. 2015; Mansour-Ghanaei et al. 2015; Paydas 2015). Numerous treatment strategies containing a proton pump inhibitor and combination of two or more antibiotics such as clarithromycin (CLA), amoxicillin (AMX) and metronidazole (MTZ) or tetracycline (TET) have been successfully used to eradicate HP infection (Nagahara et al. 2000; Chaabane & Al-Adhba 2015). Although the efficacy of such strategies in the therapy, the increasing use of antibiotics has led to the problem of drug-resistant strains (Essa et al. 2009; Gisbert & Calvet 2011). Resistance rates have been reported vary from 0 to 45% for CLA, from 0 to 33% for AMX, from 10% to 90% for MTZ, from 6% to 21% for levofloxacin and from 5% to 59% for TET (Karamanolis et al. 2014; O'Connor et al. 2014; Song et al. 2014). In recent years, the combination effect of common antibiotics with natural products has been applied as a new strategy to enhance the treatment of bacterial infections and overcome the complications of drug-resistant strains (Nostro et al. 2006; Hassan et al. 2015).

For decades, plants play an essential role in drug discovery development as a rich source of biologically active compounds that exhibited significant antimicrobial properties (Castillo-Juárez et al. 2009; Njume et al. 2011). *Hibiscus sabdariffa* L. (Malvaceae) (HS; roselle) is a medicinal plant, which has a long history of herbal and edible uses worldwide (Alshami & Alharbi 2014; Wang et al. 2014). Numerous studies have reported the antibacterial activity of *H. sabdariffa*, and its phytochemicals against Gram-negative and Gram-positive bacteria (Liu et al. 2005; Chao & Yin 2009). This study aims to evaluate the *in vitro* antimicrobial combinatory

CONTACT Sherif T. S. Hassan sherif.hassan@seznam.cz Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, 612 42 Brno, Czech Republic and Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6, Suchdol, Czech Republic

© 2015 Informa UK Limited, trading as Taylor & Francis Group

effect of aqueous extract of *Hibiscus sabdariffa* L. (Malvaceae) (AEHS) with standard antibiotics (CLA, AMX and MTZ) against HP strains (six clinical isolates and one standard control of HP).

Materials and methods

Bacterial strains, cultures and antibiotics

HP (ATCC 6583) standard strain was obtained from the American Type Culture Collection (ATCC) (Rockville, MD) as well as six clinical isolates of HP (HP01, HP02, HP03, HP04, HP05 and HP06; isolated from patients with duodenal ulcers) were obtained from The Motol University Hospital, Prague, Czech Republic. For antimicrobial assay, the strains were grown in Mueller-Hinton agar–7% horse blood (Sigma Aldrich, Prague, Czech Republic), and incubated under micro-aerobic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37 °C for 3 days. The identification was based on micro-aerophilic growth requirement, morphology, Gram's stain, and oxidase, catalase, and urease activities. In addition, the effects of aging, temperature, aerobiosis, starvation and antibiotics on the morphologic conversion rate to coccoid forms, and culturability of HP were determined. HP strains were kept in trypticase soy broth supplemented with 20% glycerol at −80 °C until further use. CLA, AMX and MTZ were purchased from Sigma-Aldrich.

Preparation of plant material

Plant collection

Hibiscus sabdariffa was collected from the northern part of Aswan, Egypt in June 2014, and identified by the authors in the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic. A voucher specimen of the plant was deposited with the number EGHS5 at the herbarium of the department.

Extraction procedure

Air-dried *Hibiscus* calyces were prepared as a form of tea extract, which is in common use in traditional folk medicine, especially in tropical and subtropical regions. *Hibiscus* calyces (35 g) were brewed in 250 mL of boiled water and allowed to stand for 30 min. The mixture was filtered, and the solution evaporated to dryness. The extract was stored at 4 °C.

Determination of total flavonoid content in AEHS

Total flavonoid content (TFC) of AEHS was determined as previously described (Fernandes et al. 2012). Briefly, 5 g of air-dried *Hibiscus* calyces were extracted in 100 mL of boiled water for 1 h by sonication extraction. The mixture was filtered by Sartorius 388 filter paper. One milliliter of the extract was transferred to a 25-mL volumetric flask, 2 mL of $AlCl_3$ (5% w/v) was added and the volume was completed with distilled water (probe solution, PS). The same procedure was repeated without the addition of $AlCl_3$ for preparation of contrast solution (CS). The absorbance of PS against CS was determined in spectrophotometer at 410 nm. The percentage of TFC was calculated as rutin according to the following equation: $\mathrm{TFC} = A \times \mathrm{DF}/A^{1\%}_{1\mathrm{cm}} \times (w - \mathrm{ld})$, where A, absorbance (AU); DF, dilution factor; w, mass of plant material (g); ld, loss on drying plant material (8%, w/w); $A^{1\%}_{1\,\mathrm{cm}}$, specific absorption for rutin–$AlCl_3$ complex (259.4).

Determination of total anthocyanins in AEHS

Total anthocyanins (TAC) of AEHS were determined by a pH-differential method as previously described (Jakobek et al. 2007). In brief, 5 g of air-dried *Hibiscus* calyces was extracted in 100 mL of boiled water for 1 h by sonication extraction. The mixture was filtered by Sartorius 388 filter paper. The extract (0.4 mL) was transferred to a 25-mL volumetric flask, and two dilutions of the extract were prepared (ratio = 1:62.5), one with potassium chloride buffer (pH 1.0) (1.86 g KCl in 1 L of distilled water, pH value adjusted to 1.0 with concentrated HCl) and the other with sodium acetate buffer (pH 4.5) (54.43 g $CH_3CO_2Na{\cdot}3H_2O$ in 1 L of distilled water, pH value adjusted to 4.5 with concentrated HCl). After 15 min of incubation at room temperature, absorbance was measured simultaneously at 520 and 700 nm. The content of TAC was calculated in mg of cyaniding-3-*O*-glucoside equivalent per 100 g of dry weight using a molar extinction coefficient (ε) of cyaniding-3-*O*-glucoside of 26 900 L/mol/cm and molecular weight (449.4 g/mol).

Antimicrobial assay

For antibiotic susceptibility testing, HP (ATCC 6583) was used as a control strain. CLA, AMX and MTZ were used as positive control drugs. Minimum inhibitory concentrations (MICs) were determined by agar dilution method according to the recommendation of Clinical and Laboratory Standards Institute (2009) using Mueller-Hinton agar supplemented with 7% horse blood. Briefly, serial dilutions of CLA, AMX and MTZ ranging from 0.5 to 5 μg/mL and AEHS at concentrations ranging from 0.5 to 5 μg/mL were added to Mueller-Hinton agar supplemented with 7% horse

blood in a 5% O_2, 10% CO_2 and 85% N_2. The bacteria were sub-cultured on Mueller-Hinton agar supplemented with 7% horse blood under the same micro-aerobic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37 °C for 3 days. The bacterial suspension in Mueller–Hinton broth was adjusted to a final concentration of a McFarland No. 0.5 standard, 2 µL of the adjusted inoculum was delivered to the agar plates. After 72 h of incubation under the micro-aerobic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37 °C, the MICs of CLA, AMX, MTZ and AEHS were determined. MICs were considered as the lowest concentration of drugs inhibiting visible growth. MICs were obtained from three independent experiments that performed in triplicate.

Combination effect of AEHS with standard antibiotics

The combination effect of antibiotics and AEHS was determined by checkerboard assay and evaluated algebraically based on the sum of the fractional inhibitory concentration (∑FIC) indices as previously described (Vuuren & Viljoen 2011; Rondevaldova et al. 2015). In brief, twofold serial dilutions of each CLA, AMX and MTZ prepared in horizontal rows of micro-titer plate were subsequently cross-diluted vertically by twofold serial dilutions of AEHS. The one-half MIC of each tested compound was used as a starting concentration in combinations. For evaluation of synergistic effect of AEHS (A) with antibiotic tested (B), the ∑FIC was calculated based on the following equation: $\sum FIC = FIC_A + FIC_B$, where $FIC_A = MIC_A$ (in the presence of B)/MIC_A (alone), and $FIC_B = MIC_B$ (in the presence of A)/MIC_B (alone). The MICs used in this equation are the averages of MICs obtained from three independent experiments performed in triplicate. The interpretation of the *in vitro* antibacterial interactions was determined as follows: synergistic effect if $\sum FIC \leq 0.5$; additive if $\sum FIC > 0.5$ and ≤ 1; no interaction if $\sum FIC > 1$ and ≤ 4 and antagonistic if $\sum FIC > 4$.

Results

The results indicated that AEHS showed remarkable inhibition of the growth of all HP strains tested (bacteriostatic effect) with MICs values ranging from 9.18 to 16.68 µg/mL (Table 1), while demonstrated synergistic effects when combined with CLA or MTZ against four of seven HP strains tested (Table 2). The synergistic effect of AEHS in combination with CLA was obtained at a concentration of 1 µg/mL against HP (ATCC 6583) ($\sum FIC = 0.28$) and HP06 ($\sum FIC = 0.21$) strains, causing 46- and 29-fold reductions in CLA MICs, respectively. The synergistic effect of AEHS when combined with MTZ was determined (at a concentration of 0.5 µg/mL) against HP01 ($\sum FIC = 0.34$), and HP03 ($\sum FIC = 0.39$) strains, and caused 40- and 30-fold reductions in MTZ MICs, respectively. The additive antibacterial effect of AEHS with AMX (at concentrations of 0.5, 1 and 3 µg/mL) was obtained against HP (ATCC 6583), HP02, HP04, HP05 and HP06 strains (Table 3), while in few cases ∑FIC values were close to 0.61, which can be considered as strong additive effect. The percentage of TFC in AEHS was determined as rutin (0.247%), while the content of TAC was estimated as mg of cyaniding-3-*O*-glucoside equivalent (1465.8 mg/100 g dry weight of *Hibiscus* calyces) (Table 4).

Table 1. *In vitro* antibacterial activity of CLA, AMX, MTZ and AEHS against HP strains.

	MIC (µg/mL)			
HP strains	CLA	AMX	MTZ	AEHS
HP (ATCC 6583)	16.21	13.50	2.24	12.25
HP01[a]	12.56	12.41	13.54	16.68
HP02[a]	9.15	13.85	4.59	15.24
HP03[a]	10.56	16.89	11.99	9.18
HP04[a]	10.98	13.69	10.17	12.81
HP05[a]	14.23	12.88	10.97	13.56
HP06[a]	10.49	14.73	13.12	14.17

MIC, minimum inhibitory concentration; the values are expressed as an average from three independent experiments, each performed in triplicate. HP, *Helicobacter pylori*; ATCC, American Type Culture Collection; AEHS, the aqueous extract of *H. sabdariffa*; CLA, clarithromycin; AMX, amoxicillin; MTZ, metronidazole.
[a]Clinical isolates.

Discussion

Hibiscus sabdariffa, consumed by people worldwide in the form of tea extract, has a wide range of antimicrobial activities (Rukayadi et al. 2008; Jung et al. 2013). Beside this fact, the present study suggests that AEHS could be a useful agent acting in combination with antibiotics to enhance the treatment efficacy of HP infection. Numerous studies have reported that compounds such as flavonoids and anthocyanins are responsible for the antimicrobial properties of *H. sabdariffa* (Yin & Chao 2008; Alarcón-Alonso et al. 2012; Camelo-Méndez et al. 2013). The antibacterial activity of AEHS may be related to its ability to inhibit bacterial protein synthesis (Higginbotham et al. 2014). The bacteriostatic activity of CLA depends on its capacity to inhibit protein synthesis by binding to the 50S bacterial ribosomal subunit. CLA resistance is mainly due to point mutations in the 23S ribosomal RNA gene, and nucleotides A2142G and A2143G are the most frequent mutations (Gerrits et al. 2006; Dolapcioglu et al. 2014; Ferreira & Moss 2014). MTZ resistance among HP strains has been

Table 2. *In vitro* combinatory antibacterial effect of AEHS with CLA or MTZ against HP strains.

	AEHS with CLA at following concentrations (μg/mL)								AEHS with MTZ at following concentrations (μg/mL)							
	5		3		1		0.5		5		3		1		0.5	
HP strains	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC
HP (ATCC 6583)	22.23	1.27	19.23	3.13	**0.35**	**0.28**	9.45	2.93	13.56	3.12	16.34	2.12	12.34	1.99	12.23	1.27
HP01[a]	21.45	2.13	16.24	3.78	12.34	1.72	9.23	3.15	13.32	3.18	12.43	2.23	13.12	3.15	**0.34**	**0.34**
HP02[a]	17.34	1.98	19.34	2.45	16.34	2.14	15.63	1.98	14.26	2.15	7.43	1.16	9.32	2.45	9.45	1.36
HP03[a]	18.56	3.24	20.23	2.16	16.23	3.45	14.65	2.43	17.23	2.87	8.45	1.13	17.32	3.14	**0.40**	**0.39**
HP04[a]	19.34	2.89	18.45	2.13	13.78	2.45	18.45	3.15	8.34	1.98	8.34	2.84	17.32	2.36	9.45	2.26
HP05[a]	17.43	3.14	17.34	1.13	14.67	3.19	19.43	3.48	7.23	1.92	17.43	2.36	16.43	2.73	10.21	1.99
HP06[a]	18.34	2.75	18.45	1.78	**0.36**	**0.21**	12.45	2.41	8.98	1.23	21.34	2.73	12.98	2.83	21.32	1.78

∑FIC: sum of fractional inhibitory concentrations – the combination interaction is evaluated as follows: synergy ∑FIC ≤ 0.5 (bold font indicates synergistically acting combinations); additive ∑FIC > 0.5 and ≤1; no interaction ∑FIC > 1 and ≤4; antagonistic if ∑FIC > 4; MIC – bold font indicates fold reductions. MIC, minimum inhibitory concentration; HP, *Helicobacter pylori*; ATCC, American Type Culture Collection; AEHS, the aqueous extract of *H. sabdariffa*; CLA, clarithromycin; MTZ, metronidazole.
[a]Clinical isolates.

Table 3. *In vitro* combinatory antibacterial effect of AEHS with AMX against HP strains.

	AEHS with AMX at following concentrations (μg/mL)							
	5		3		1		0.5	
HP strains	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC
HP (ATCC 6583)	12.43	1.36	13.12	**0.73**	13.23	**0.62**	15.32	**0.71**
HP01[a]	14.25	1.36	12.34	2.13	14.23	2.13	13.24	3.13
HP02[a]	13.65	2.36	18.34	**0.81**	17.44	**0.79**	14.56	**0.92**
HP03[a]	13.45	2.34	17.34	1.68	17.34	1.95	10.89	1.98
HP04[a]	10.89	3.15	15.34	**0.78**	18.34	**0.96**	12.34	**0.82**
HP05[a]	15.25	3.98	14.28	**0.91**	17.36	**0.84**	8.45	**0.87**
HP06[a]	16.34	3.57	9.78	**0.63**	16.98	**0.61**	7.14	**0.82**

∑FIC: sum of fractional inhibitory concentrations – the combination interaction is evaluated as follows: synergy ∑FIC ≤ 0.5; additive ∑FIC > 0.5 and ≤1 (bold font indicates additive acting combinations); no interaction ∑FIC > 1 and ≤4; antagonistic if ∑FIC > 4. MIC, minimum inhibitory concentration; HP, *Helicobacter pylori*; AEHS, the aqueous extract of *H. sabdariffa*; ATCC, American Type Culture Collection; AMX, amoxicillin.
[a]Clinical isolates.

Table 4. Chemical characterisation of TFC and TAC in AEHS.

TFC (%) calculated as rutin	TAC as mg of cyaniding-3-*O*-glucoside equivalent (mg/100 g DW)
0.247%	1465.8 mg/100 g DW

TFC, total flavonoid content; TAC, total anthocyanins; DW, dry weight of *Hibiscus* calyces; AEHS, the aqueous extract of *H. sabdariffa*.

related to alterations in gene products having MTZ nitroreductase activities, mainly including oxygen-insensitive NAD(P)H nitroreductase (RdxA) and NAD(P)H flavin oxidoreductase (FrxA) (Jenks et al. 1999; Jenks & Edwards, 2002). Based on these facts, we suggest that AEHS in combination with CLA or MTZ inhibit bacterial protein synthesis, and thus can be considered as a useful alternative therapeutic agent in the development of anti-HP drugs.

Conclusion

New antimicrobial combination drugs which include plant products combinations have recently gained a great attention in research field. This approach has financial implications as reformulation of existing drugs or combinations may prove to be a more viable option, rather than developing a new drug which will require extensive clinical trials for verification. In this study, the susceptibility of HP strains to AEHS in combination with antibiotics (CLA, AMX and MTZ) was examined. In addition, the chemical characterization of TFC and TAC in AEHS was determined. The results demonstrated that AEHS can enhance the growth inhibitory activity of CLA, AMX and MTZ against HP strains tested. Furthermore, no antagonistic interactions were observed. Although the synergistic effect of AEHS in combination with CLA or MTZ against four of seven HP strains tested as well as the additive effect of AEHS in combination with AMX against five of seven HP strains tested were investigated, further studies should be carried out to confirm these activities as well as the mechanisms of action *in vivo* and in clinical trials.

Declaration of interest

The authors have declared that there is no conflict of interest. This study was funded by Internal Grant Agency (IGA) of the Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Czech Republic. Project No. 20154247/2015.

References

Alarcón-Alonso J, Zamilpa A, Aguilar FA, Herrera-Ruiz M, Tortoriello J, Jimenez-Ferrer E. 2012. Pharmacological characterization of the diuretic effect of *Hibiscus sabdariffa* Linn (Malvaceae) extract. J Ethnopharmacol. 139:751–756.

Alshami I, Alharbi AE. 2014. Antimicrobial activity of *Hibiscus sabdariffa* extract against uropathogenic strains isolated from recurrent urinary tract infections. Asian Pac J Trop Dis. 4:317–322.

Camelo-Méndez GA, Ragazzo-Sánchez JA, Jiménez-Aparicio AR, Vanegas-Espinoza PE, Paredes-López O, Del Villar-Martínez AA. 2013. Comparative study of anthocyanin and

volatile compounds content of four varieties of Mexican roselle (*Hibiscus sabdariffa* L.) by multivariable analysis. Plant Foods Hum Nutr. 68:229–234.

Castillo-Juárez I, González V, Jaime-Aguilar H, Martínez G, Linares E, Bye R, Romero I. 2009. Anti-*Helicobacter pylori* activity of plants used in Mexican traditional medicine for gastrointestinal disorders. J Ethnopharmacol. 122:402–405.

Chaabane NB, Al-Adhba HS. 2015. Ciprofloxacin-containing versus clarithromycin-containing sequential therapy for *Helicobacter pylori* eradication: a randomized trial. Indian J Gastroenterol. 34:68–72.

Chao CY, Yin MC. 2009. Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Pathog Dis. 6:201–206.

Clinical and Laboratory Standards Institute. 2009. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically—eighth edition: approved standard M07-A8*. Wayne (PA): CLSI.

Dolapcioglu C, Koc-Yesiltoprak A, Ahishali E, Kural A, Dolapcioglu H, Soylu A, Dabak R. 2014. Sequential therapy versus standard triple therapy in *Helicobacter pylori* eradication in a high clarithromycin resistance setting. Int J Clin Exp Med. 7:2324–2328.

Essa AS, Kramer JR, Graham DY, Treiber G. 2009. Meta-analysis: four-drug, three-antibiotic, non-bismuth-containing "concomitant therapy" versus triple therapy for *Helicobacter pylori* eradication. Helicobacter 14:109–118.

Fernandes AJ, Ferreira MR, Randau KP, de Souza TP, Lira Soares LA. 2012. Total flavonoids content in the raw material and aqueous extractives from *Bauhinia monandra* Kurz (Caesalpiniaceae). Sci World J. 2012:923462.

Ferreira J, Moss SF. 2014. Current paradigm and future directions for treatment of *Helicobacter pylori* infection. Curr Treat Options Gastroenterol. 12:373–384.

Gerrits MM, Van vliet AH, Kuipers EJ, Kusters JG. 2006. *Helicobacter pylori* and antimicrobial resistance: molecular mechanisms and clinical implications. Lancet Infect Dis. 6:699–709.

Gisbert JP, Calvet X. 2011. Review article: non-bismuth quadruple (concomitant) therapy for eradication of *Helicobater pylori*. Aliment Pharmacol Ther. 34:604–617.

Hassan STS, Masarčíková R, Berchová K. 2015. Bioactive natural products with anti-herpes simplex virus properties. J Pharm Pharmacol. 67:1325–1336.

Higginbotham KL, Burris KP, Zivanovic S, Davidson PM, Stewart CN Jr. 2014. Antimicrobial activity of *Hibiscus sabdariffa* aqueous extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus* in a microbiological medium and milk of various fat concentrations. J Food Prot. 77:262–268.

Jakobek L, Seruga M, Medvidovic-Kosanovic M, Novak I. 2007. Anthocyanin content and antioxidant activity of various red fruit juices. Dtsch Lebensmitt Rundsch. 103:58–64.

Jenks PJ, Edwards DI. 2002. Metronidazole resistance in *Helicobacter pylori*. Int J Antimicrob Agents 19:1–7.

Jenks PJ, Ferrero RL, Labigne A. 1999. The role of the rdxA gene in the evolution of metronidazole resistance in *Helicobacter pylori*. J Antimicrob Chemother. 43:753–758.

Jung E, Kim Y, Joo N. 2013. Physicochemical properties and antimicrobial activity of Roselle (*Hibiscus sabdariffa* L.). J Sci Food Agric. 93:3769–3776.

Jung SW, Thamphiwatana S, Zhang L, Obonyo M. 2015. Mechanism of antibacterial activity of liposomal linolenic acid against *Helicobacter pylori*. PLoS One 10:e0116519.

Karamanolis GP, Daikos GL, Xouris D, Goukos D, Delladetsima I, Ladas SD. 2014. The evolution of *Helicobacter pylori* antibiotics resistance over 10 years in Greece. Digestion 90:229–231.

Liu KS, Tsao SM, Yin MC. 2005. *In vitro* antibacterial activity of roselle calyx and protocatechuic acid. Phytother Res. 19:942–945.

Mansour-Ghanaei F, Joukar F, Mojtahedi K, Sokhanvar H, Askari K, Shafaeizadeh A. 2015. Does treatment of *Helicobacter pylori* infection reduce gastric precancerous lesions? Asian Pac J Cancer Prev. 16:1571–1574.

Nagahara A, Miwa H, Ogawa K, Kurosawa A, Ohkura R, Iida N, Sato N. 2000. Addition of metronidazole to rabeprazole–amoxicillin–clarithromycin regimen for *Helicobacter pylori* infection provides an excellent cure rate with five-day therapy. Helicobacter 5:88–93.

Njume C, Afolayan AJ, Samie A, Ndip RN. 2011. Inhibitory and bactericidal potential of crude acetone extracts of *Combretum molle* (Combretaceae) on drug-resistant strains of *Helicobacter pylori*. J Health Popul Nutr 29:438–445.

Nostro A, Cellini L, Di Bartolomeo S, Cannatelli MA, Di Campli E, Procopio F, Grande R, Marzio L, Alonzo V. 2006. Effects of combining extracts (from propolis or *Zingiber officinale*) with clarithromycin on *Helicobacter pylori*. Phytother Res. 20:187–190.

O'Connor A, Vaira D, Gisbert JP, O'Morain C. 2014. Treatment of *Helicobacter pylori* infection 2014. Helicobacter 19:38–45.

Paydas S. 2015. *Helicobacter pylori* eradication in gastric diffuse large B cell lymphoma. World J Gastroenterol. 21:3773–3776.

Rondevaldova J, Novy P, Kokoska L. 2015. *In vitro* combinatory antimicrobial effect of plumbagin with oxacillin and tetracycline against *Staphylococcus aureus*. Phytother Res. 29:144–147.

Rukayadi YJ, Shim S, Hwang JK. 2008. Screening of Thai medicinal plants for anticandidal activity. Mycoses 51:308–312.

Song Z, Zhang J, He L, Chen M, Hou X, Li Z, Zhou L. 2014. Prospective multi-region study on primary antibiotic resistance of *Helicobacter pylori* strains isolated from Chinese patients. Dig Liver Dis. 46:1077–1081.

Vuuren SV, Viljoen A. 2011. Plant-based antimicrobial studies – methods and approaches to study the interaction between natural products. Planta Med. 77:1168–1182.

Wang J, Cao X, Jiang H, Qi Y, Chin KL, Yue Y. 2014. Antioxidant activity of leaf extracts from different *Hibiscus sabdariffa* accessions and simultaneous determination five major antioxidant compounds by LC-Q-TOF-MS. Molecules 19:21226–21238.

Yin MC, Chao CY. 2008. Anti-campylobacter, anti-aerobic, and anti-oxidative effects of roselle calyx extract and protocatechuic acid in ground beef. Int J Food Microbiol. 127:73–77.

molecules

MDPI

Article

Hibiscus sabdariffa L. and Its Bioactive Constituents Exhibit Antiviral Activity against HSV-2 and Anti-Enzymatic Properties against Urease by an ESI-MS Based Assay

Sherif T. S. Hassan [1,2,*], Emil Švajdlenka [1] and Kateřina Berchová-Bímová [2]

[1] Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1946/1, 612 42 Brno, Czech Republic; svajdlenkae@vfu.cz

[2] Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha-Suchdol, Czech Republic; berchova@knc.czu.cz

* Correspondence: sherif.hassan@seznam.cz; Tel.: +420-774-630-604

Academic Editor: Thomas J. Schmidt
Received: 20 March 2017; Accepted: 27 April 2017; Published: 30 April 2017

Abstract: For decades, *Hibiscus sabdariffa* L. and its phytochemicals have been shown to possess a wide range of pharmacologic properties. In this study, aqueous extract of *Hibiscus sabdariffa* (AEHS) and its bioactive constituent protocatechuic acid (PCA), have been evaluated in vitro for their antiviral activity against HSV-2 clinical isolates and anti-enzymatic activity against urease. Antiherpetic activity was evaluated by the titer reduction assay in infected Vero cells, and cytotoxicity was evaluated by the neutral red dye-uptake method. Anti-urease activity was determined by a developed Electrospray Ionization-Mass Spectrometry (ESI-MS)-based assay. PCA showed potent anti-HSV-2 activity compared with that of acyclovir, with EC_{50} values of 0.92 and 1.43 $\mu g \cdot mL^{-1}$, respectively, and selectivity indices > 217 and > 140, respectively. For the first time, AEHS was shown to exert anti-urease inhibition activity, with an IC_{50} value of 82.4 $\mu g \cdot mL^{-1}$. This, combined with its safety, could facilitate its use in practical applications as a natural urease inhibitor. Our results present *Hibiscus sabdariffa* L. and its bioactive compound PCA as potential therapeutic agents in the treatment of HSV-2 infection and the treatment of diseases caused by urease-producing bacteria.

Keywords: anti-HSV-2 activity; ESI-mass spectrometry-based assay; urease inhibitors; protocatechuic acid; *Hibiscus sabdariffa* L.; bacterial infection

1. Introduction

Herpes simplex virus (HSV) infections are quite common in humans, affecting about 90% of the world population. HSV is a member of *Herpesviridae*, a wide family of enveloped-DNA viruses that cause several clinically significant syndromes in both adults and neonates [1]. HSV-2 is mainly connected with genital infection, and has been recorded as a risk factor of HIV infection in humans [2]. Currently, treatment of HSV-2 infection mainly relies on the use of acyclovir and related nucleoside analogues that target viral DNA polymerase. Unfortunately, although several strategies have shown high efficacy results, HSV-2 infection treatment fails in about 30–45% of infected adults [3,4]. This is due to the extensive use of acyclovir and related nucleoside analogues, which has created drug resistance, associated with other adverse effects, alongside with the establishment of viral latency and reactivation that occurs in the presence of humoral- and cell-mediated immunity [5]. To date, no prophylactic HSV vaccine has been found to be entirely effective in the prevention of HSV infections [6,7]. Therefore, it is crucial to find alternative strategies to combat this viral resistance, and increase treatment efficacy results.

Urease (urea amidohydrolase, EC 3.5.1.5), is a nickel-containing metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbamate; the latter decomposes spontaneously to produce another molecule of ammonia and carbon dioxide, enhancing the rate of the uncatalyzed reaction by a factor of 8×10^{17} [8,9]. High concentrations of ammonia arising from this reaction, and the accompanying pH elevation, have important negative effects on humans and agriculture as well. Urease is widely distributed in Nature, including plants, fungi, algae, bacteria and even in the human gut and kidneys [10,11]. In recent years, urease has been considered a significant virulence factor implicated in infections of the urinary and gastrointestinal tracts [12]. In addition, urease is the main cause of pathologies induced by *Helicobacter pylori* as it allows the pathogen to survive at the low pH of the stomach and grow and multiply, spreading infection to the inner layers of gastroduodenal mucosa, resulting in producing gastritis and peptic ulceration, which in some cases may lead to cancer [13]. All these negative implications can be managed by inhibition of urease [14]. However, while urease inhibitors such as acetohydroxamic acid (AHA) and phosphoramidates have shown therapeutic efficacy, limitations associated with severe side effects, such as teratogenicity, psycho-neurological, and musculo-integumentary symptoms, have limited their use in the treatment of urinary and gastrointestinal tracts infections [10] Therefore, in recent years, the search for various groups of urease inhibitors with different types of inhibition, various mechanisms of action, and minimal side effects has gained much attention in the research field [15]. Natural products and their derivatives have long been used as a source of new drug candidates in drug discovery. This is due to their great diversity of the chemical structures and better drug-like properties of many of these molecules compared to synthetic compounds [16,17].

Hibiscus sabdariffa L. (*Malvaceae*; *H. sabdariffa*) is a plant mainly cultivated in tropical and subtropical regions with a long history of herbal and edible uses worldwide [18,19]. It is an annual or perennial plant or woody-based shrub with serrate leaves, red calyces and red stems. This plant has a long tradition of medicinal use as it contains a rich profile of bioactive compounds responsible for its therapeutic efficacy, including antimicrobial, antiparasitic, anticancer, and antiinflammatory properties [20,21]. Protocatechuic acid (PCA), an active compound in *H. sabdariffa*, has been also shown to exert various pharmacologic properties, including but not limited to antimicrobial and antioxidant activities [22]. This study was designed to evaluate in vitro antiviral activity of AEHS and PCA against HSV-2 clinical isolates and to evaluate their safety for topical administration as well as anti-urease activity of AEHS by a developed ESI-MS-based assay.

2. Results

2.1. Determinations of Concentration of PCA and Total Polyphenols Content in Plant Material

The results demonstrated that the concentration of PCA in AEHS was found to be 94.1 μg/g dry weight of *Hibiscus* calyces (Figure 1) and total polyphenols content calculated as mg of gallic acid equivalent was 106.0 mg/g of dry extract of *Hibiscus* calyces.

2.2. Anti-HSV-2 Activity and Cytotoxicity

AEHS and PCA were evaluated with respect to their inhibitory effect on HSV-2 replication. Before performing the antiherpetic assay, we assessed the cytotoxicity of each sample in Vero cells by the neutral red dye-uptake method. The CC_{50} values for PCA and acyclovir were found to be higher than 200 $\mu g \cdot mL^{-1}$ (Table 1). Antiherpetic activity was determined by the titer reduction assay in infected Vero cells using quantitative real-time reverse transcription PCR. Ten acyclovir-sensitive strains of HSV-2 (clinical isolates) were used and typed by quantitative real-time reverse transcription PCR using primers pairs H_2M_{40} 5′-GTACAGACCTTCGGAGG-3′ and H_2P_4 5′-CGCTTCATCATGG GC-3′ for identification. AEHS was not active against HSV-2. This could be related to the low concentrations of antiherpetic compounds in the crude extract. PCA showed potent anti-HSV-2 activity compared with that of acyclovir with EC_{50} values of 0.92 and 1.43 $\mu g \cdot mL^{-1}$, respectively, and selectivity indices >217

and >140, respectively. PCA exhibited cytotoxic effect on Vero cells at concentration higher than its EC_{50}. The selectivity index (SI) is fundamental to determine any possible toxic effect of any compound on the cells that could be confused with an antiviral activity. Based on our results, PCA demonstrated anti-HSV-2 activity with SI > 217.4 higher than acyclovir (>140). Thus, the SI verifies the safety index of PCA.

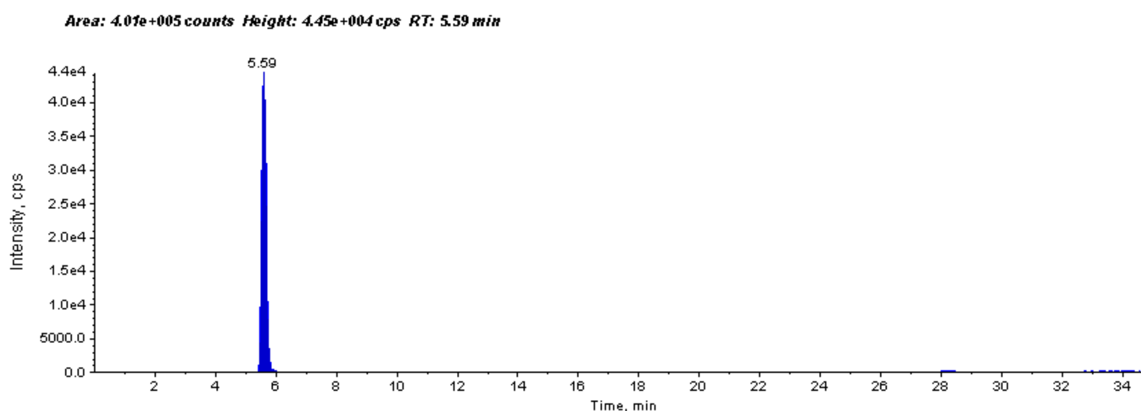

Figure 1. HPLC-MS chromatogram shows identification (two MRM transitions m/z 153→109 and 153→91) and determination of concentration of protocatechuic acid (PCA) in aqueous extract of *Hibiscus sabdariffa* (AEHS). PCA was detected at retention time (RT): 5.59 min and quantified using an external calibration method with standard PCA (94.1 µg/g dry weight of *Hibiscus* calyces).

Table 1. Anti-HSV-2 activity and cytotoxicity of PCA and AEHS.

Compound	EC_{50} (µg·mL^{-1})	CC_{50} (µg·mL^{-1})	SI
AEHS	n.d	n.d	n.d
PCA	0.92 ± 0.21	>200	>217
Acyclovir	1.43 ± 0.43	>200	>140

All data were average values from three independent experiments; Values presented are means ± S.D. of three independent experiments performed in duplicate; CC_{50}: 50% cytotoxic concentration; EC_{50}: 50% Effective concentration; SI: Selectivity index (CC_{50}/EC_{50}); n.d: Not determined; PCA: Protocatechuic acid; AEHS: Aqueous extract of *Hibiscus sabdariffa*.

2.3. *Anti-Urease Inhibitory Properties by ESI-MS-Based Assay*

Urease inhibitors play an essential role as alternative antibiotics in the treatment of gastrointestinal and urinary tract infections caused by urease-producing bacteria [8]. Enzyme activity and inhibition studies were determined by an ESI-MS based method. The method is based on the detection of urea depletion in the absence and presence of inhibitors by monitoring catalytic reaction through simultaneous detection of the concentration changes of urea.

2.3.1. Determination of Urease Kinetic Parameters K_m and V_{max}

It is known that the essential function of enzymes is to enhance the rate of biochemical reactions. Therefore, to understand enzyme function, it is very crucial to study the kinetic description of their activity. To determine kinetic parameters of the urease-catalyzed reaction including the Michaelis constant (K_m) and the maximal reaction rate (V_{max}), we monitored the enzymatic reaction through decreasing of concentration of urea at five concentrations (137.0, 208.2, 274.0, 416.4 and 694.1 µmol·L^{-1}) and constant concentration of urease (60.0 µg·mL^{-1}, Figure 2). The initial rate of the reaction was determined for each concentration from the rate constant and initial substrate concentration. A plot of 1/initial velocity versus 1/(urea) generates a Lineweaver-Burk plot and a linear regression fit to this data was used to determine the K_m and V_{max} (Figure 3). The good linearity of the Lineweaver-Burk plot (R^2 = 0.99) for urea shown in Figure 3 ensures the accuracy of steady-state kinetics in this method.

K_m of urease was determined to be 1888.7 µmol·L^{-1} and V_{max} to be 259.6 µmol·L^{-1}·min^{-1}. Values obtained by the present method are in excellent agreement with the range of values reported in previous studies (1–4 mM for K_m and 0.0298–4.0 mM/min for V_{max}) [23–25].

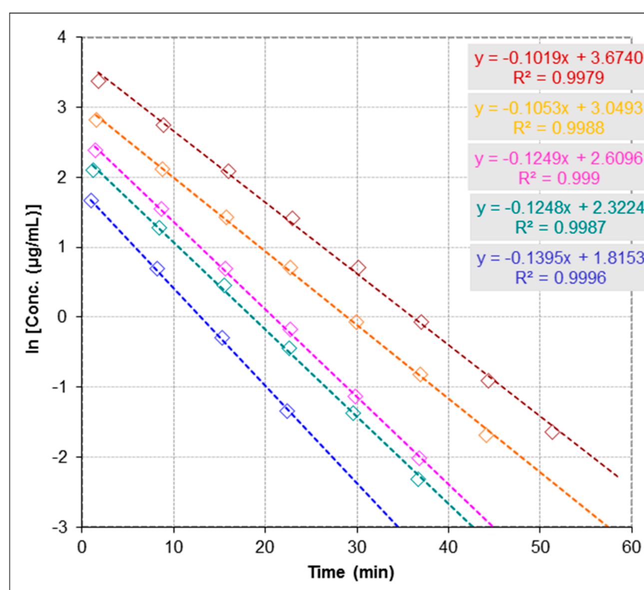

Figure 2. Urease-catalyzed reaction evaluated by Electrospray Ionization-Mass Spectrometry (ESI-MS) based assay at five concentrations of urea as shown in the figure 137.0 [●], 208.2 [●], 274.0 [●], 416.4 [●] and 694.1 [●] µmol·L^{-1} and fixed concentration of urease 60.0 µg·mL^{-1} by monitoring catalytic reaction through simultaneous detection of the concentration changes of urea, where the slopes represent the reaction rate constants (k_0) in the absence of inhibitors. Concentrations changes of urea are presented as logarithms of concentration. The precision of time course analysis was calculated as RSD (%) of multiple measured slopes (lower than 10%). For clarity of figure, multiple measurements have not been presented.

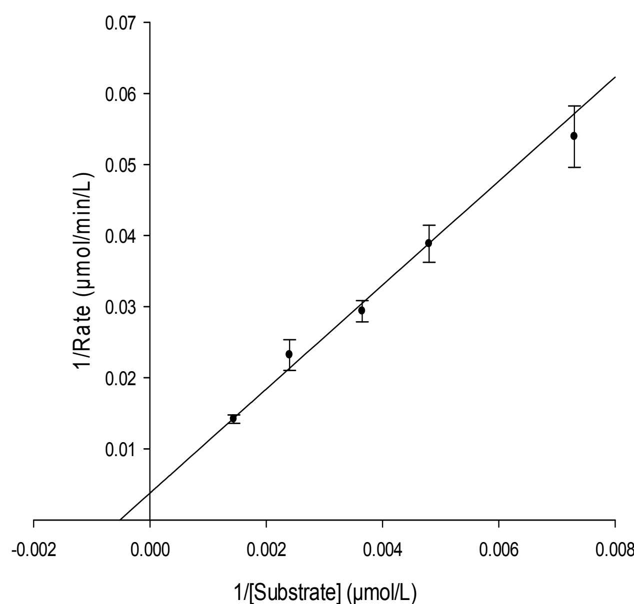

Figure 3. Lineweaver Burk plot for determination of urease kinetic parameters; Michaelis constant (K_m) and the maximal reaction rate (V_{max}). K_m = 1888.7 µmol·L^{-1} and V_{max} = 259.6 µmol·L^{-1}·min^{-1}. R^2 = 0.99.

2.3.2. Determination of Half Maximal Inhibitory Concentration (IC_{50})

For inhibitor screening, the IC_{50} value is an apparent measure of the potency of an inhibitor at specific experimental conditions. Figure 4 shows the reaction progression slope for the depletion of urea in the presence and absence of inhibitors following the first-order kinetics conditions per equation $C_t = C_0 \times e^{-kt}$, where C_t is the concentration at time (t), C_0 is the initial concentration and k is the reaction rate constant [26]. From Figure 4, the reaction rate constant in the presence of inhibition (k) is lower than the reaction rate constant in the absence of inhibition (k_0), where k in the presence of acetohydroxamic acid (AHA; used as a reference urease inhibitor) and AEHS (k = 0.0477 and 0.0975 min^{-1}, respectively) and (k_0 = 0.1934 min^{-1}) at concentrations of 274.0 $\mu mol \cdot L^{-1}$ for urea and 60.0 $\mu g \cdot mL^{-1}$ for urease, and concentrations of inhibitors (AHA = 13.2 $\mu mol \cdot L^{-1}$ and AEHS = 81.0 $\mu g \cdot mL^{-1}$). It has been reported that the percent inhibition is defined as percent reduction of the reaction rate constant (k) as compared with the rate constant in the absence of an inhibitor (k_0). Thus, % activity is usually determined as k/k_0 and % Inhibition is 1 – % activity. Therefore, to determine IC_{50} value of an inhibitor the following equation % activity = $k/k_0 = 1 - [I]/([I] + IC_{50}) = IC_{50}/([I] + IC_{50})$, where [I] is the concentration of the inhibitor, was used as previously described [26]. IC_{50} for AHA was determined to be 4.3 $\mu mol \cdot L^{-1}$ and for AEHS to be 82.4 $\mu g \cdot mL^{-1}$. The used method proved to be robust and determined IC_{50} value for AHA compared favourably with previous data reported in literature (IC_{50} for AHA = 5 μM) [27].

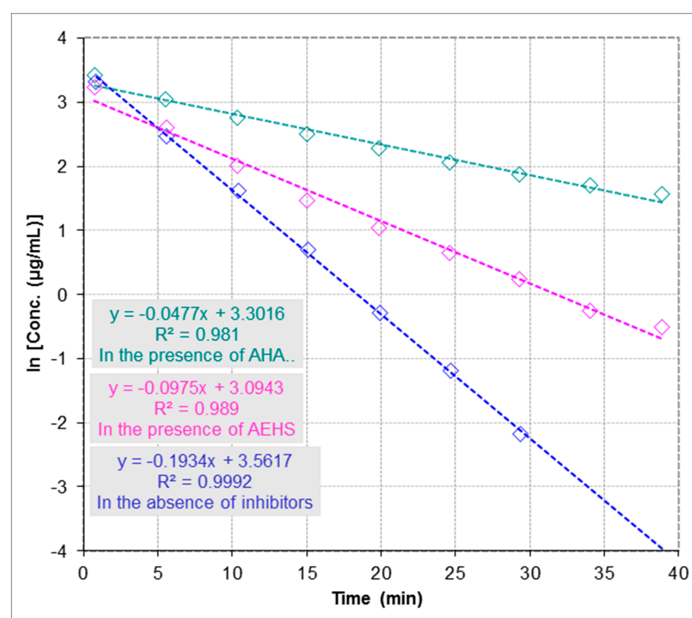

Figure 4. Effect of aqueous extract of *Hibiscus sabdariffa* (AEHS) and acetohydroxamic acid (AHA) on the inhibition of urease activity by Electrospray Ionization-Mass Spectrometry (ESI-MS) based assay. Urease activity and inhibitory properties of AHA and AEHS were assayed as described in Experimental section, where k is the reaction rate constant in the presence of AHA [•] and AEHS [•] (k = 0.0477 and 0.0975 min^{-1}, respectively) and k_0 is the reaction rate constant in the absence of inhibitors [•] (k_0 = 0.1934 min^{-1}). Concentrations changes of urea are presented as logarithms of concentration. IC_{50} for AEHS was determined to be 82.4 $\mu g \cdot mL^{-1}$ and for AHA to be 4.3 $\mu mol \cdot L^{-1}$. The precision of time course analysis was calculated as RSD (%) of multiple measured slopes (lower than 10%). For clarity of figure, multiple measurements have not been presented.

2.3.3. Repeatability and Stability Studies

Precision of the method was verified by repeatability and stability studies. The measurements demonstrated a very good repeatability (Figure 5), where the relative standard deviation (RSD) was

found to be 7.5%. Although current practices in stability studies rely on the addition of an internal standard to assure that short-term and long-term signal fluctuations do not influence quantitative analyses, we avoided the use of an internal standard which could interfere with enzymatic reaction or exact determination of substrate concentration. Therefore, we measured a calibration curve after each experiment to correct instability of MS signal.

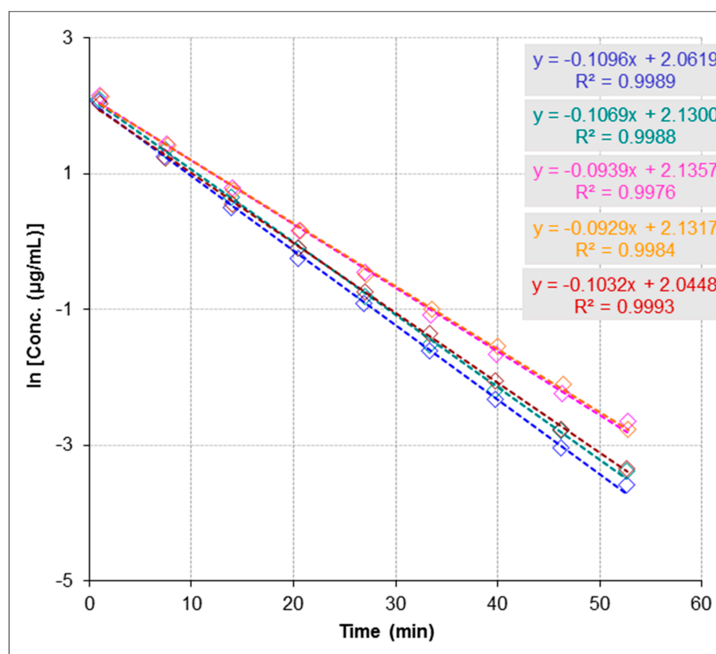

Figure 5. Determination of repeatability of measuring urease-catalyzed reaction evaluated by Electrospray Ionization-Mass Spectrometry (ESI-MS) based assay at fixed concentration of urea 416.4 $\mu mol \cdot L^{-1}$ and fixed concentration of urease 60.0 $\mu g \cdot mL^{-1}$ (repeated five times; as shown in different colors). Relative standard deviation (RSD) of slopes is 7.5%.

3. Discussion

For decades, treatment of infectious diseases has been a focus of interest for both researchers and healthcare providers, as the arising issue of drug resistant strains has become a serious problem worldwide. This study adds to the growing body of knowledge on the antiviral activity of polyphenol-enriched extracts derived from plants. We show for the first time that PCA, an active compound of *H. sabdariffa* L. exerts potent antiviral activity against clinical isolates of HSV-2, the mechanism of which involves the inhibition of viral replication. Plant-derived phytochemicals, such as polyphenols, phenolics, terpenes, alkaloids and other substances have been reported to possess inhibitory properties on HSV replication [7,28]. For instance, polyphenols have been found to interfere with the early phases of the HSV replicative cycle and/or with viral particles directly [29]. In this study, PCA showed excellent ability to inhibit HSV-2 replication and hence might open new gates for the development of anti-HSV-2 drugs. In addition, PCA is known to exhibit cytotoxic effect on cancer cells with various mechanisms of action [30,31]. Therefore, it should be taken into consideration the non-toxic doses, especially with topical preparations containing PCA. Considering the significant global incidence, morbidity, and mortality rates of viral sexually transmitted infections (STIs), the development of new, safe, topically applied antiviral agents for their prevention is of high priority [32]. Therefore, in recent years, a new approach has been under focus of interest to maximize the treatment efficacy by combining natural products with nucleoside analogues, resulting in reduction of cytotoxicity [33]. Thus, this approach will reduce the cytotoxicity of PCA. However, further studies should be carried out to determine its safety as well as the possibility of adverse effects in vivo.

Urease inhibitors are considered promising therapeutic agents for the treatment of ureolytic bacterial infections [34]. Over the past two decades, a large number of plant-derived products were found to possess in vitro inhibitory activities against urease and intensive efforts were then made to evaluate the efficacy of these inhibitors in vivo [35]. Unfortunately, most of these investigations failed to prove the efficacy of those studied drugs in vivo due to problems of hydrolytic instability, toxicity and adverse side effects [36]. In this study, we report for the first time the anti-urease activity of AEHS by a developed ESI-MS based assay. Since *H. sabdariffa* L. is commonly used in traditional folk medicine in the form of a herbal tea or as a dietary supplement [37]. This indicates that AEHS could be used safely as natural urease inhibitor for the treatment of diseases caused by urease-producing bacteria. Additionally, the use of AEHS could overcome the problem of preventing the use of urease inhibitors in vivo and in clinical trials; due to their possible toxicity, instability and undesirable side effects. It has been reported that polyphenols and phenolic compounds have been shown to possess potent anti-urease activity. Moreover, crude plant extracts containing polyphenols have been shown an excellent ability to interact with wide range of enzymes [8,12]. Therefore, we may suggest that anti-urease activity of AEHS could be related to its rich content of polyphenols. Identification of bioactive molecules from AEHS and confirmation of the key components contributing to anti-urease activity should be studied in further investigations.

4. Materials and Methods

4.1. Plant Collection and Extraction Procedure

Hibiscus sabdariffa L. was collected from the northern part of Aswan, Egypt in June 2014, and identified by Sherif T. S. Hassan in the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences (Brno, Czech Republic). A voucher specimen of the plant was deposited with the number EGHS5 at the herbarium of the department. One-gram air dried *Hibiscus* calyces were extracted in 10 mL of boiled water by sonication extraction for 30 min. The extract was stored at 4 °C for further use. A 1 mL of aliquot of crude extract was evaporated to dryness and then weighted to yield a final amount of 0.0243 g of dry extract.

4.1.1. Determination of Concentration of PCA in Plant Material

Air dried *Hibiscus* calyces (0.51 g) were extracted in 10 mL of boiled water by sonication extraction for 30 min. HPLC-MS (Agilent 1200 HPLC system, Böblingen, Germany) coupled with a mass spectrometer (Sciex-3200QTRAP-hybrid triple quadrupole/linear ion trap, Toronto, ON, Canada) fitted with Electrospray Ionization (ESI) were used for the analysis. For the quantification, an external calibration method with standard PCA (Sigma Aldrich, Prague, Czech Republic) was used as previously described [38].

4.1.2. Determination of Total Polyphenols by Folin-Ciocalteu Method

The total polyphenols content was determined per the Folin-Ciocalteu method as previously described by ISO 14502-1 [39].

4.2. Antiviral Activity against HSV-2

4.2.1. Viral Strains, Cultures, Cell Lines and Reagents

For antiviral activity, Vero cells (ATCC: CCL 81, UK; were obtained from the Motol University Hospital, Prague, Czech Republic) were grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, UK), 100 U/mL penicillin G, 100 $\mu g \cdot mL^{-1}$ streptomycin and 25 $\mu g \cdot mL^{-1}$ amphotericin B (Sigma-Aldrich, Berlin, Germany) and maintained at 37 °C in 5% CO_2 atmosphere. Ten clinical isolates of HSV-2 (isolated from patients with HSV-2 infection) were kindly obtained from the Motol University Hospital, Prague,

Czech Republic). All clinical isolates were typed by quantitative real-time reverse transcription PCR using primers pairs H_2M_{40} 5′-GTACAGACCTTCGGAGG-3′ and H_2P_4 5′-CGCTTC ATCATGGGC-3′ for identification as previously described [40] and then were propagated in Vero cells. The cytopathic end-point assay was used to determine the titers which were expressed as 50% tissue culture infective dose ($TCID_{50}$/mL) as previously described [41]. Viral stocks were stored at −80 °C.

4.2.2. Determination of Cytotoxicity

Cytotoxicity was evaluated by the neutral red dye-uptake method as previously described [42]. Briefly, AEHS, protocatechuic acid (PCA; Sigma Aldrich, Prague, Czech Republic) and acyclovir (positive control; Sigma-Aldrich, Berlin, Germany) were prepared in 0.1% dimethyl sulfoxide (DMSO). Stock solutions were prepared in deionized water at concentration of 400 $\mu g \cdot mL^{-1}$ and sterilized. Vero cell monolayers were cultivated in 96-well microtiter plates with two-fold serial dilutions of AEHS, PCA and acyclovir, and incubated for 48 h at 37 °C in 5% CO_2 atmosphere. After incubation, the morphological alterations of the treated cells were determined using an inverted optical microscope (Leitz, Berlin, Germany) and the maximum non-toxic concentrations (MNTC) were determined. The 50% cytotoxic concentrations (CC_{50}) of AEHS, PCA and acyclovir were calculated as the concentration that reduces cell viability by 50%, when compared to the untreated controls as priviously described [43].

4.2.3. Anti-HSV-2 Activity

Antiherpetic activity was determined by the titer reduction assay as previously described [44]. Briefly, acyclovir was used as a positive control and Vero cell monolayers were treated with AEHS, PCA and acyclovir at concentrations at which no change was observed in cell morphology, and 80% cell viability were determined. 100 $TCID_{50}$/mL of HSV-2 acyclovir-sensitive suspensions were added to treated and untreated cell cultures and incubated at 37 °C for 48 h in a 5% CO_2 atmosphere. After incubation, the virus titers in treated and untreated cells, were determined. Anti-HSV-2 activity was evaluated as percentage inhibition (PI) using antilogarithmic $TCID_{50}$ values as follows: PI = [1 − (antilogarithmic test value/antilogarithmic control value)] × 100. The dose response curve was determined from the MNTC, and the 50% effective concentration (EC_{50}) was determined as the concentration required for 50% protection against virus-induced cytopathic effects. Selectivity index (SI) value was calculated as the ratio of CC_{50}/EC_{50}.

4.2.4. Statistical Analysis

Experiments were generated in duplicate in at least three independent experiments. PRISM software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis (one-way ANOVA test) and to calculate EC_{50} and CC_{50} parameters.

4.3. Anti-urease Inhibitory Properties

4.3.1. Enzyme, Substrate, Inhibitors and Reagents

All chemical reagents were obtained from commercial suppliers and used without further purification. Urease (Type III, from *Canavalia ensiformis*), urea, acetohydroxamic acid (AHA; standard urease inhibitor) were purchased from (Sigma Aldrich, Prague, Czech Republic).

4.3.2. Instrumentation

Urease inhibitory activity was evaluated using a system pump-injector (Agilent 1200) coupled with a Sciex-3200QTRAP- hybrid triple quadrupole/linear ion trap mass spectrometer fitted with Electrospray Ionization (ESI). The system runs in flow injection analysis (FIA) mode without a HPLC column. The operational parameter settings were as follows: curtain gas (CUR), 25 psi; nebulizer gas (GS1), 50; auxiliary gas (GS2), 40; declustering Potential (DP), 15 V; ion spray voltage, −4000 V;

turbo temperature (TEM), 450 °C. MS in positive ion mode was used in multiple reaction monitoring (MRM) analysis for detection and quantitation of urea with transition m/z 61→44 (Figure 6). 0.1% HCOOH and 1 mM $HCOONH_4$ were used as mobile phases with the flow rate set at 0.5 mL·min^{-1}. The injection volume was 10 µL.

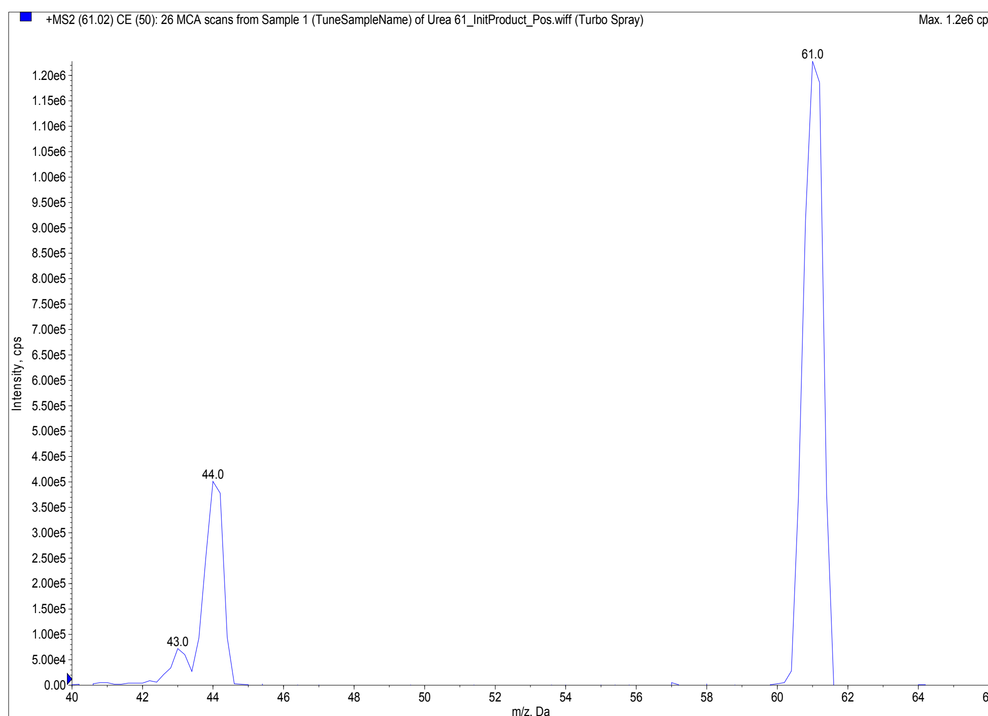

Figure 6. Multiple reaction monitoring (MRM) analysis for detection and quantitation of urea by triple quadrupole mass spectrometry coupled with Electrospray Ionization (ESI) in positive ion mode with transition m/z (61→44).

4.3.3. Anti-urease Activity by ESI-MS-Based Assay

Several important experimental conditions were investigated and taken into consideration to optimize the efficacy of the ESI-MS-based assay analysis, such as the buffer concentration, the buffer pH, and the type of sample vials. Under the optimized experimental conditions, experiments were employed and aimed at evaluating urease assay conditions that would be compatible with ESI-MS. The enzymatic reaction took place in 1 mM $HCOONH_4$ buffer, which was found to be suitable (pH 7.5) containing 60.0 µg·mL^{-1} of urease, over a substrate (urea) concentrations ranging from 137.0 to 694.1 µmol·L^{-1} at room temperature (25 °C). Urea was chosen as a natural substrate for the enzymatic reaction. Urea concentrations were maintained at concentrations below 694.1 µmol·L^{-1} to avoid excess substrate inhibition. The concentrations of inhibitors (AHA = 13.2 µmol·L^{-1} and AEHS = 81 µg·mL^{-1}) were used and were within the range of their effectiveness to inhibit urease activity. Enzymatic reaction was carried out by pre-incubating urease in 1 mM $HCOONH_4$ buffer with each inhibitor during 180 min to reach binding equilibrium followed by adding urea. The solutions were directly injected automatically into FIA system and the concentration changes of urea were monitored. For the analysis of the kinetics of substrate depletion by ESI-MS, areas (total counts) under peaks for substrate in the FIA record were integrated. Each measured sequence consists of five measurements. Briefly, to determine the repeatability of measurements, we performed multiple measurements of enzymatic reaction of the same sample. The precision of time course analysis was calculated as RSD (%) of multiple measured slopes (lower than 10%). The slopes represent rate constants, which are used for determination of enzyme activity and inhibition studies. For clarity of figures, multiple measurements have not been

presented in Figures 2 and 4. As shown in Figure 5, multiple measurements and evaluation of slopes are presented.

For measuring the rate constants for determining K_m and V_{max}, we set to each experiment different substrate concentrations. Furthermore, for screening inhibitors, we set to each experiment different inhibitor concentrations or different types of inhibitor. A calibration curve after each experiment was measured to correct instability of MS signal.

5. Conclusions

Plant-derived phytochemicals provide an excellent option for the treatment of infectious diseases. The present findings indicated that PCA exhibited notable inhibitory properties on HSV-2 replication and thus has promising application in the development of anti-HSV-2 drugs. However, further studies should be carried out to evaluate its safety, validate the activity in vivo, and to eliminate the possible adverse effects of this compound by using improved delivery techniques prior its possible practical application. In addition, our results indicate that AEHS exerted anti-urease activity according to a developed ESI-MS-based assay. However, further studies must be performed to identify the active compounds in AEHS that contribute to the anti-urease activity, and evaluate their toxicity and structure-activity relationships.

Acknowledgments: The authors would like to thank the Motol University Hospital, Prague, Czech Republic for providing the viral strains as well as technical support.

Author Contributions: S.T.S.H. and E.Š. conceived and designed the experiments; E.Š. and S.T.S.H. performed the experiments; S.T.S.H., E.Š. and K.B.-B. analyzed the data; S.T.S.H. wrote the paper. All authors have approved and revised the final version of the manuscript.

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

References

1. Einsenberg, R.; Atanasiu, D.; Cairns, T.M.; Gallagher, J.R.; Krummenacher, C.; Cohen, G.H. Herpes virus fusion and entry: A story with many characters. *Viruses* **2012**, *4*, 800–832. [CrossRef] [PubMed]
2. Gescher, K.; Hensel, A.; Hafezi, W.; Derksen, A.; Kühn, J. Oligomeric proanthocyanidins from *Rumex acetosa* L inhibit the attachment of herpes simplex virus type-1. *Antivir. Res.* **2011**, *89*, 9–18. [CrossRef] [PubMed]
3. Khan, M.T.; Alter, A.; Thompson, K.D.; Gambari, R. Extracts and molecules from medicinal plants against herpes simplex viruses. *Antivir. Res.* **2005**, *67*, 107–119. [CrossRef] [PubMed]
4. Keller, M.J.; Tujama, A.; Carlucci, M.J.; Herold, B.C. Topical microbicides for prevention of genital herpes infection. *J. Antimicrob. Chemother.* **2005**, *55*, 420–423. [CrossRef] [PubMed]
5. Wang, Z.; Liu, Q.; Lu, J.; Fan, P.; Xie, W.; Qiu, W.; Wang, F.; Hu, G.; Zhang, Y. Serine/Arginine-rich splicing factor 2 modulates herpes simplex virus type 1 replication via regulating viral gene transcriptional activity and pre-mRNA splicing. *J. Biol. Chem.* **2016**, *291*, 26377–26387. [CrossRef] [PubMed]
6. Hassan, S.T.S.; Berchová-Bímová, K.; Petráš, J.; Hassan, K.T.S. Cucurbitacin B interacts synergistically with antibiotics against *Staphylococcus aureus* clinical isolates and exhibits antiviral activity against HSV-1. *S. Afr. J. Bot.* **2017**, *108*, 90–94. [CrossRef]
7. Hassan, S.T.S.; Masarčíková, R.; Berchová, K. Bioactive natural products with anti-herpes simplex virus properties. *J. Pharm. Pharmacol.* **2015**, *67*, 1325–1336. [CrossRef] [PubMed]
8. Hassan, S.T.S.; Žemlička, M. Plant-derived urease inhibitors as alternative chemotherapeutic agents. *Arch. Pharm.* **2016**, *349*, 507–522. [CrossRef] [PubMed]
9. Konieczna, I.; Zarnowiec, P.; Kwinkowski, M.; Kolesinska, B.; Fraczyk, J.; Kaminski, Z.; Kaca, W. Bacterial urease and its role in long-lasting human diseases. *Curr. Protein Pept.* **2012**, *13*, 789–806. [CrossRef]
10. Follmer, C.J. Ureases as a target for the treatment of gastric and urinary infections. *Clin. Pathol.* **2010**, *63*, 424–430. [CrossRef] [PubMed]
11. Kosikowska, P.; Berlicki, Ł. Urease inhibitors as potential drugs for gastric and urinary tract infections: A patent review. *Expert Opin. Ther. Pat.* **2011**, *21*, 945–957. [CrossRef] [PubMed]

12. Mazzei, L.; Cianci, M.; Musiani, F.; Ciurli, S. Inactivation of urease by 1,4-benzoquinone: Chemistry at the protein surface. *Dalton Trans.* **2016**, *45*, 5455–5459. [CrossRef] [PubMed]
13. Mobley, H.L.; Island, M.D.; Hausinger, R.P. Molecular biology of microbial ureases. *Microbiol. Rev.* **1995**, *59*, 451–480. [PubMed]
14. Tanaka, T.; Kawase, M.; Tani, S. Urease inhibitory activity of simple α, β-unsaturated ketones. *Life Sci.* **2003**, *73*, 2985–2990. [CrossRef]
15. Zaborska, W.; Krajewska, B.; Olech, Z. Heavy metal ions inhibition of jack bean urease: Potential for rapid contaminant probing. *Enzyme Inhib. Med. Chem.* **2004**, *19*, 65–69. [CrossRef] [PubMed]
16. Laghari, H.A.; Memona, S.; Nelofar, A.; Khan, K.M.; Yasmin, A.; Syed, M.N.; Aman, A. A new flavanenol with urease-inhibition activity isolated from roots of manna plant camelthorn (*Alhagi maurorum*). *J. Mol. Struct.* **2010**, *965*, 65–67. [CrossRef]
17. Paulo, L.; Oleastro, M.; Gallardo, E.; Queiroz, J.A.; Domingues, F. Anti-*Helicobacter pylori* and urease inhibitory activities of resveratrol and red wine. *Food Res. Int.* **2011**, *44*, 964–969. [CrossRef]
18. Hassan, S.T.; Berchová, K.; Šudomová, M. Antimicrobial, antiparasitic and anticancer properties of *Hibiscus sabdariffa* (L.) and its phytochemicals: In vitro and in vivo studies. *Ceska Slov. Farm.* **2016**, *65*, 10–14. [PubMed]
19. De Arruda, A.; Cardoso, C.A.; Vieira Mdo, C.; Arena, A.C. Safety assessment of *Hibiscus sabdariffa* after maternal exposure on male reproductive parameters in rats. *Drug Chem. Toxicol.* **2016**, *39*, 22–27. [CrossRef] [PubMed]
20. Ademiluyi, A.O.; Oboh, G. Aqueous extracts of Roselle (*Hibiscus sabdariffa* Linn.) varieties inhibit α-amylase and α-glucosidase activities in vitro. *J. Med. Food.* **2013**, *16*, 88–93. [CrossRef] [PubMed]
21. Wang, J.; Cao, X.; Jiang, H.; Qi, Y.; Chin, K. L.; Yue, Y. Antioxidant activity of leaf extracts from different *Hibiscus sabdariffa* accessions and simultaneous determination five major antioxidant compounds by LC-Q-TOF-MS. *Molecules* **2014**, *19*, 21226–21238. [CrossRef] [PubMed]
22. Da-Costa-Rocha, I.; Bonnlaender, B.; Sievers, H.; Pischel, I.; Heinrich, M. *Hibiscus sabdariffa* L.—A phytochemical and pharmacological review. *Food Chem.* **2014**, *165*, 424–443. [CrossRef] [PubMed]
23. Mo, Z.-Z.; Wang, X.-F.; Zhang, X.; Su, J.-Y.; Chen, H.-M.; Liu, Y.-H.; Zhang, Z.-B.; Xie, J.-H.; Su, Z.-R. Andrographolide sodium bisulphite-induced inactivation of urease: Inhibitory potency, kinetics and mechanism. *BMC Complement. Altern. Med.* **2015**, *15*, 238. [CrossRef] [PubMed]
24. Du, N.; Chen, M.; Liu, Z.; Sheng, L.; Xu, H.; Chen, S. Kinetics and mechanism of jack bean urease inhibition by Hg^{2+}. *Chem. Cent. J.* **2012**, *6*, 154. [CrossRef] [PubMed]
25. Krajewska, B. Mono- (Ag, Hg) and di- (Cu, Hg) valent metal ions effects on the activity of jack bean urease. Probing the modes of metal binding to the enzyme. *J. Enzyme Inhib. Med. Chem.* **2008**, *23*, 535–542. [CrossRef] [PubMed]
26. Wu, G.; Yuan, Y.; Hodge, C.N. Determining appropriate substrate conversion for enzymatic assays in high-throughput screening. *J. Biomol. Screen.* **2003**, *8*, 694–700. [CrossRef] [PubMed]
27. Tanaka, T.; Kawase, M.; Tani, S. Alpha-hydroxyketones as inhibitors of urease. *Bioorg. Med. Chem.* **2004**, *12*, 501–505. [CrossRef] [PubMed]
28. Firdous, S.; Ansari, N.H.; Fatima, I.; Malik, A.; Afza, N.; Iqbal, L.; Lateef, M. Ophiamides A-B, new potent urease inhibitory sphingolipids from *Heliotropium ophioglossum*. *Arch. Pharm. Res.* **2012**, *35*, 1133–1137. [CrossRef] [PubMed]
29. Yang, C.M.; Cheng, H.Y.; Lin, T.C.; Chiang, L.C.; Lin, C.C. The in vitro activity of geraniin and 1,3,4,6-tetra-*O*-galloyl-β-D-glucose isolated from *Phyllanthus urinaria* against herpes simplex virus type 1 and type 2 infection. *J. Ethnopharmacol.* **2007**, *110*, 555–558. [CrossRef] [PubMed]
30. Tseng, T.H.; Hsu, J.D.; Lo, M.H.; Chu, C.Y.; Chou, F.P.; Huang, C.L.; Wang, C.J. Inhibitory effect of *Hibiscus* protocatechuic acid on tumor promotion in mouse skin. *Cancer Lett.* **1998**, *126*, 199–207. [CrossRef]
31. Tseng, T.H.; Kao, T.W.; Chu, C.Y.; Chou, F.P.; Lin, W.L.; Wang, C.J. Induction of apoptosis by hibiscus protocatechuic acid in human leukemia cells via reduction of retinoblastoma (RB) phosphorylation and Bcl-2 expression. *Biochem. Pharmacol.* **2000**, *60*, 307–315. [CrossRef]
32. Charlton, A.J.; Baxter, N.J.; Khan, M.L.; Moir, A.J.; Haslam, E.; Davies, A.P.; Williamson, M.P. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593–1601. [CrossRef] [PubMed]
33. Friend, D.R. Pharmaceutical development of microbicide drug products. *Pharm. Dev. Technol.* **2010**, *15*, 562–581. [CrossRef] [PubMed]

34. Benini, S.; Rypniewski, W.R.; Wilson, K.S.; Mangani, S.; Ciurli, S. Molecular details of urease inhibition by boric acid: Insights into the catalytic mechanism. *J. Am. Chem. Soc.* **2004**, *126*, 3714–3715. [CrossRef] [PubMed]
35. De Oliveira, A.; Adams, S.D.; Lee, L.H.; Murray, S.R.; Hsu, S.D.; Hammond, J.R.; Dickinson, D.; Chen, P.; Chu, T.C. Inhibition of herpes simplex virus 1 with the modified green tea polyphenol palmitoyl-epigallocatechin gallate. *Food Chem. Toxicol.* **2013**, *52*, 207–215. [CrossRef] [PubMed]
36. Hassan, S.T.S.; Šudomová, M. The development of urease inhibitors: What opportunities exist for better treatment of *Helicobacter pylori* infection in children? *Children* **2017**, *4*, 2. [CrossRef] [PubMed]
37. Nyam, K.L.; Leao, S.Y.; Tan, C.P.; Long, K. 2014. Functional properties of roselle (*Hibiscus sabdariffa* L.) seed and its application as bakery product. *J. Food Sci. Technol.* **2014**, *51*, 3830–3837. [CrossRef] [PubMed]
38. Carvalho, D.O.; Curto, A.F.; Guido, L.F. Determination of phenolic content in different barley varieties and corresponding malts by liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry. *Antioxidants* **2015**, *4*, 563–576. [CrossRef] [PubMed]
39. *ISO 14502–1: Determination of Substances Characteristic of Green and Black Tea—Part 1: Content of Total Polyphenols in Tea—Colorimetric Method Using Folin-Ciocalteu Reagent*; International Organization for Standardization: Geneva, Switzerland, 2005.
40. Markoulatos, P.; Georgopoulou, A.; Siafakas, N.; Plakokefalos, E.; Tzanakaki, G.; Kourea-Kremastinou, J. Laboratory diagnosis of common herpesvirus infections of the central nervous system by a multiplex PCR assay. *J. Clin. Microbiol.* **2001**, *39*, 4426–4432. [CrossRef] [PubMed]
41. Reed, L.J.; Muench, H. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **1938**, *27*, 493–497.
42. Walker, W.E.; Waisbren, B.A.; Martins, R.R.; Batayias, G.E. A method for determining sensitivities of antiviral drugs in vitro for possible use as clinical consultation. *Am. J. Clin. Pathol.* **1971**, *56*, 687–692. [CrossRef] [PubMed]
43. Borenfreund, E.; Puerner, J.A. Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol. Lett.* **1985**, *24*, 119–124. [CrossRef]
44. Nishimura, T.; Toku, H.; Fukuyasu, H. Antiviral compounds. XII. Antiviral activity of amidinohydrazones of alkoxyphenyl-substituted carbonyl compounds against influenza virus in eggs and in mice. *Kitasato Arch. Exp. Med.* **1977**, *50*, 39–46. [PubMed]

Sample Availability: Samples of all compounds used in the study are available from the authors.

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

JPP | JOURNAL OF Pharmacy and Pharmacology

ROYAL PHARMACEUTICAL SOCIETY

JPP
Journal of Pharmacy
And Pharmacology

Review

Bioactive natural products with anti-herpes simplex virus properties

Sherif T. S. Hassan[a,b], Radka Masarčíková[a] and Kateřina Berchová[b]

[a]Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno and [b]Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Prague, Czech Republic

Keywords
antiviral agents; bioactive compounds; herpes simplex virus; natural products; nucleoside analogues

Correspondence
Sherif T. S. Hassan, Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, Brno 612 42, Czech Republic and Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6- Suchdol, Czech Republic.
E-mail: sherif.hassan@seznam.cz

Received November 19, 2014
Accepted April 12, 2015

doi: 10.1111/jphp.12436

Abstract

Objectives In this review, we highlight and summarise the most promising extracts, fractions and pure compounds as potential anti-herpes simplex virus (HSV) agents derived from microorganisms, marine organisms, fungi, animals and plants. The role of natural products in the development of anti-HSV drugs will be discussed.
Key findings Herpes simplex viruses (HSV-1 and -2) are common human pathogens that remain a serious threat to human health. In recent years, a great interest has been devoted to the search for integrated management of HSV infections. Acyclovir and related nucleoside analogues have been licensed for the therapy that target viral DNA polymerase. Although these drugs are currently effective against HSV infections, the intensive use of these drugs has led to the problem of drug-resistant strains. Therefore, the search for new sources to develop new antiherpetic agents has gained major priority to overcome the problem.
Summary Natural products as potential, new anti-HSV drugs provide several advantages such as reduced side effects, less resistance, low toxicity and various mechanisms of action. This paper aims to provide an overview of natural products that possess antiviral activity against HSV.

Introduction

Herpes simplex virus (HSV) infections are quite common in humans.[1,2] HSV is a member of Herpesviridae, a wide family of enveloped-DNA viruses that cause several clinically significant syndromes in both adults and neonates.[3,4] These syndromes are varied and affected by viral entrance, nature of the disease and degree of host immune competence.[5] Both types of HSV-1 and HSV-2 are the most extensively studied human herpesviruses, where HSV-1 is commonly connected with oral or facial infection and encephalitis, whereas HSV-2 is responsible for genital infection.[6] More than 80% of humans worldwide are infected with HSV-1, and roughly 40% have recurrent infections. Furthermore, infection with HSV-2 was found to be a high-risk factor for potential HIV infection and invasive cervical carcinoma as well.[7,8] Herpesviruses are incurable and persist along with the lifetime of the host. Throughout the primary infection, the virus enters the nerve cells to generate latency in sensory neurons and lesions at or near point of entry into the host. Reactivation of latent HSV, especially during the deficiency of immunity induces recurrent infection and transmission to new hosts. Moreover, HSV is implicated in many ocular diseases, including stromal keratitis, endotheliitis and neurotrophic keratopathy.[9–12]

Current and new trends for treating HSV infections

Treatment of HSV infectivity remains a main target for many researchers worldwide, where it cannot be managed by vaccination.[13] HSV infections can be controlled by the innate and adaptive immune system. HSV infection is influenced by how the virus can evade the host's innate immune system. Antiviral drugs are classified as virucidals, immunomodulators and antiviral chemotherapeutic agents. Treatment of HSV infections is currently based on the use of several selective and effective drugs such as acyclovir, penciclovir, famciclovir, cidofovir, valacyclovir, trifluridine and vidarabine, which act as inhibitors of DNA polymerase.[14,15] Nevertheless, the intensive use of these drugs in the therapy has led to undesirable effects such as

© 2015 Royal Pharmaceutical Society,

drug-resistant strains.[16] Evans *et al*. reviewed the mechanism and pharmacological properties of acyclovir and related nucleoside analogues as well as their role in the treatment of HSV infections.[17] Because of mutations that can occur in HSV-DNA polymerase or thymidine kinase (TK), resistance to nucleoside analogues drugs has developed.[18] Therefore, development of new antiviral agents has gained much interest in recent years. Although many of these agents have already been developed and employed in the treatment of HSV infections, the search for new sources for antiherpetic drugs is a challenge for clinicians and clinical microbiologists to overcome problems with drug-resistant strains. Therefore, it is an urgent issue to open new gates for searching for new therapeutic agents that act with different mechanisms of action than nucleoside analogues. Natural products are a very rich source of these substances.

Pure compounds and fractions that possess anti-HSV activities

Natural products are the most consistent and successful source of new antiviral drugs. Substances derived from microorganisms, fungi and animals that are active against herpesviruses have gained great interest in the last few decades.[19] The effective bioactive products from animals and microorganisms are generally saccharides and peptides.[20] Marine organisms are a very rich source of new bioactive molecules that possess antiviral activity, mainly group of sponges (phylum Porifera). Some of these bioactive compounds have shown potent antiviral activity and have been examined clinically.[20,21] Plants remain the most common source for treatment of various diseases. In the last decades, herbal compounds have been investigated for their anti-HSV properties. Purification and identification of lead bioactive compound are very important in the development of new antiviral drugs, especially against HSV. Additionally, it is also important to understand the targets and mode of actions of these compounds as potential antiviral agents.[4,22–24] In this section, we highlight the bioactive compounds and fractions from natural sources that have been found to be promising antiviral agents against HSV infections.

Nucleosides

Marine-derived nucleosides

The first bioactive compounds isolated from marine organisms that were found to be promising anti-HSV drugs were the nucleosides spongothymidin and spongouridin (Table 1), which have been isolated from the sponge *Tethya crypta*. These compounds have led later to the synthesis of Ara-A (vidarabine). Ara-A was the first nucleoside analogue drug approved for the treatment of systemic HSV infection in humans.[25] From a New Zealand marine sponge of the

Table 1 Bioactive compounds derived from microorganisms, fungi, animals and marine organisms with antiherpetic activities

Natural source	Bioactive compound[a]	Type
Tethya crypta	Spongothymidin and Spongouridin	Nucleosides
Tethya crypta	Vidarabine (Ara-A)	Nucleoside
Mycale sp.	Mycalamide A and Mycalamide B	Nucleosides
Aaptos aaptos	4-methylaaptamine	Alkaloid
Haliclona sp. and *Pachypellina* sp.	Manzamine A	Alkaloid
Scinaia hatei	Xylan	Polysaccharide
Gymnogongrus griffithsiae, *Cryptonemia crenulata* and *Bostrychia montagnei*	Sulfated galactans	Sulfated polysaccharide
Spirulina platensis	Calcium spirulan (Ca-SP)	Sulfated polysaccharide
Agaricus brasiliensis	MI-S	Sulfated polysaccharide
Gracilaria corticata	Galactan-containing sub-fraction (F3),WES1, WES2, F3S1 and F3S2	Sulfated polysaccharide
Grifola frondosa	GFAHP	Protein
Ganoderma lucidum	NPBP and APBP	Proteins bound polysaccharides
Heterometrus petersii	Hp1036 and Hp1239	Venom peptides
Bovine	Denoted HH-2, 1002, 1006, 1018 and Bactenecin dodecapeptide	Peptides
Humans and bovine	lactoferrin and lactoferricin	Peptides
Bee venom	Melittin	Host – defence peptide
Mammals	Magainins, Cecropins, Clavanins, and LL-37	α-Helical peptides
Mammals	Defensins, Tachyplesin, and Protegrins	β-Sheet peptides
Bovine neutrophils	Indolicidin	Peptide
Frog skin	Brevinin-1	Peptide
Actinomadura namibiensis	Labyrinthopeptin A1	Lantibiotic peptide

CC50, 50% cytotoxic concentration; EC50, half maximal effective concentration; HSV, herpes simplex virus; IC50, half maximal inhibitory concentration.

[a]Anti-HSV activities (% inhibition, EC50, IC50 and CC50/IC50) of each bioactive compound have been described in section 2.

 © 2015 Royal Pharmaceutical Society,

genus *Mycale*, mycalamide A and mycalamide B, which are protein synthesis inhibitors, were extracted. These substances have demonstrated in-vitro notable activity against Poliovirus, the Corona type I and HSV-1 at a concentration of 5 ng/disc. Meanwhile, Mycalamide B revealed higher anti-HSV-1 activity than mycalamide A at a concentration of 1–2 ng/disc.[26]

Polysaccharides

Marine-derived polysaccharides

Mandal *et al*. have studied Xylan and its derivatives isolated from red algae *Scinaia hatei* and exhibited antiviral activity against HSV-1 and HSV-2 with IC50 values of 7.6 and 11.7 μg/ml, respectively.[27] Other studies were carried out to evaluate purified substances for their anti-HSV activity *in vitro* such as sulfated galactan fractions obtained from two red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata* and sulfated galactans from marine alga *Bostrychia montagnei*. These compounds demonstrated potent anti-HSV activity with IC50 values ranging from 0.5 to 20.7 μg/ml. The mode of action of these compounds could be mainly related to the inhibitory effect on virus adsorption.[28,29] From sea alga *Spirulina platensis*, Calcium spirulan (Ca-SP), a sulfated polysaccharide, found to exhibit inhibition of HSV-1 and HIV-2 replication *in vitro* and *ex vivo* at concentrations ranging from 0.7 to 20 μg/ml, was isolated. It was revealed that Ca-SP selectively inhibited the penetration of virus into host cells.[30] Chattopadhyay *et al*. reported that galactan-containing sub-fraction (F3) and its hyper sulfated derivatives (WES1, WES2, F3S1 and F3S2) were purified from the aqueous extract of *Gracilaria corticata*. All compounds were found to exhibit in-vitro anti-HSV activity with IC50 values ranging from 1.1 to 27.4 μg/ml.[31]

Fungi-derived polysaccharides

The chemical modification of a sulfated polysaccharide (MI-S) isolated from the mycelia of the Brazilian basidiomycete *Agaricus brasiliensis* showed antiviral activity against HSV-1 (KOS and 29R (acyclovir resistant) strains) and the HSV-2 strain 333, with selectivity indices (SIs = CC50/IC50) higher than 439, 208 and 562, respectively. The anti-HSV activity of Agaricus brasiliensis mycelial polysaccharide (MI-S) found to be related to inhibition of viral ICP27, UL42, gB and gD proteins.[32]

Plant-derived polysaccharides

Dong *et al*. have isolated from the aerial part of *Basella rubra* L. four neutral polysaccharides (BRN-1, BRN-2, BRN-3 and BRN-4). The results confirmed that only BRN-3 demonstrated antiviral effect on HSV-2 in Vero cells with an IC50 value of 55 μg/ml. BRN-3 found to exhibit inhibition of virus adsorption to host cells.[33] An anionic polysaccharide that was purified from aqueous extract of the Chinese plant *Prunella vulgaris* and exerted antiviral effects on HSV type 1 and 2 at a concentration of 100 μg/ml without cytotoxic effect to mammalian cells up to highest concentration was used in the study. The antiherpetic activity of the polysaccharide found to be related to inhibition of HSV by competing for cell receptors after the virus has penetrated the cells.[34]

Proteins

Fungi-derived proteins

Eo *et al*. have isolated from the mushroom *Ganoderma lucidum* two proteins bound polysaccharides, a neutral protein bound polysaccharide (NPBP) and an acidic protein bound polysaccharide (APBP). APBP displayed *in vitro* more potent HSV-1 and HSV-2 antiviral activity than NPBP with EC50 values of 300–520 μg/ml. The antiviral activity of APBP was found to be related to its binding with HSV-specific glycoproteins responsible for the attachment and penetration, and APBP prevents the complex interactions of viruses with cell plasma membranes.[35] GFAHP, an antiviral protein, purified from the mushroom *Grifola frondosa* and found to exert inhibition of HSV-1 replication *in vitro* with an IC50 value of 4.1 μg/ml and therapeutic index >29.3. Furthermore, GFAHP reduced the severity of HSV-1-induced blepharitis, neovascularisation and stromal keratitis in a murine model at concentrations of 125 and 500 μg/ml, whereas topical administration of GFAHP to the mouse cornea led to remarkable decrease in virus production.[36]

Plant-derived proteins

Shan *et al*. have isolated Stellarmedin A, a novel anti-HSV-2 glycoprotein from *Stellaria media* (L.) Vill. Their study revealed that Stellarmedin A was found to be a potent inhibitor of HSV-2 replication in Vero cells with IC50 value of 13.18 μg/ml and therapeutic index 75.9.[37] Trichosanthin (TCS) is a type 1 ribosome-inactivating protein (RIP) (Table 2), isolated from Chinese plant *Trichosanthes kirilowii*. Huang *et al*. have reported that TCS impeded p38 mitogen-activated protein kinases (MAPK) and Bcl-2 rising in Vero cells by HSV-1 infection and inhibited viral replication, whereas Chen *et al*. have confirmed the protective effects of TCS against infectious brain injury induced by HSV-1 in mice.[38,39]

Peptides

The inhibitory activities of peptides against microorganisms have been first reported in 1942.[40] They are part of

Table 2 The targets of anti-HSV plant-derived compounds

Source	Compound	Type	Target
Trichosanthes kirilowii	Trichosanthin (TCS)	Ribosome-inactivating protein	p38 MAPK protein and Bcl-2 gene
Melia azedarach	Meliacine	Glycopeptide	Glycoproteins
Tripterygium wilfordii	Triptofordin C-2	Sesquiterpene	Immediate early (IE) genes
Melia azedarach	28-Deacetylsendanin (28-DAS)	Limonoid terpene	Thymidine kinase (TK)
Plant essential oils	Isoborneol	Monoterpene	gB and gD glycoproteins
Melia azedarach	Tetranortriterpenoid 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM)	Terpene	Nuclear factor κB (NF-κB) protein
The curry spice turmeric	Curcumin	Polyphenol	P300/CBP histone acetyltransferase
Thea sinensis L.	Epigallocatechin gallate (EGG)	Catechin	gB and gD glycoproteins
Thea sinensis	Palmitoyl-EGCG (p-EGCG)	Catechin	gB and gD glycoproteins
Limonium sinense L.	Samarangenin B (Sam B)	Catechin	ICP0 and ICP4 genes and viral DNA polymerase
Rheum tanguticum	Emodin	Anthraquinone	Alkaline nuclease inhibitor (UL12 alkaline nuclease)

CBP, CREB-binding protein; HSV, herpes simplex virus; MAPK, mitogen-activated protein kinases.
This table summarises the targets of antiherpetic plant-derived compounds that have been highlighted in section 2.

the nonspecific immune system against pathogen attack in humans, animals and plants as well.[41,42]

Animal peptides

Two new venom peptides, Hp1036 and Hp1239, were obtained from *Heterometrus petersii* and showed in-vitro potent virucidal effect on HSV-1 with EC50 values of 0.43 and 0.41 μM, respectively. Their mechanisms of action were found to be related to destruction of the viral morphology by adopting α-helix structure in approximate membrane environment as well as reduction of the intracellular viral infection.[43] Shestakov *et al.* have studied four synthetic peptides (denoted HH-2, 1002, 1006, 1018) related to the host defence peptide bovine bactenecin dodecapeptide for their anti-HSV-2 activities in mice. The results showed reduction of HSV-2 infection of Vero cells in a dose-dependent manner, whereas *in vivo* demonstrated significant inhibition of viral replication. The anti-HSV-2 activity of these peptides were found to be related to their ability to interfere with both viral attachment and entry.[44] Many other peptides were examined for their antiherpetic activity such as lactoferrin and lactoferricin, which occur in milk, saliva and belong to the innate immune system. Both compounds are effective against enveloped and some nonenveloped viruses, fungal infection and prevention of the growth of tumours with inhibitory activity against HSV infections.[42,45] Yasin *et al.* have evaluated 20 host defence peptides for their ability to inhibit HSV type 1 and 2. The results demonstrated that Melittin (from bee venom), α-helical peptides (magainins, cecropins, clavanins and LL-37), β-sheet peptides (defensins, tachyplesin and protegrins), indolicidin (from bovine neutrophils) and brevinin-1 (from frog skin) were found to possess inactivation of HSV infectivity. Although the mechanisms of their anti-HSV activity are still poorly understood, these factors can be further studied as constituents of the innate immune response and as potential antiherpetic agents for clinical use.[46]

Bacterial peptides

Férir *et al.* have studied the lantibiotic peptide labyrinthopeptin A1, which is produced by bacteria *Actinomadura namibiensis*. Labyrinthopeptin A1 exhibited in-vitro anti-HIV and anti-HSV activities with EC50 values of 0.70–3.3 and 0.29–2.8 μM, respectively.[47]

Plant-derived peptides

Meliacine is a glycopeptide isolated from *Melia azedarach* and suppressed genital herpetic infection in BALB/c mice.[48] Furthermore, meliacine *in vitro* has also exerted inhibition of HSV-1 multiplication; whereas it prevented the development of herpetic stromal keratitis in mice *in vivo* when administered three times a day for 4 days beginning 1 day before inoculation.[49] Several studies have also presented the significant ability of meliacine to inhibit HSV-1 *in vitro*. The antiherpetic activity of meliacine might be related to its ability to inhibit viral protein synthesis.[50,51]

Terpenes

Terpenes are among the very promising source of new antimicrobials agents that have shown to have activity against viruses, bacteria, fungi and protozoa. Terpenes in plants are either free or bound in the form of glycosides, esters or bound to proteins.[52]

Plant-derived terpenes

Isoborneol is a monoterpene found in a wide range of plant essential oils. This compound exhibited in-vitro virucidal

effect against HSV-1, whereas at the concentration of 0.06%, it showed total inhibition of viral replication. Its mechanism of action related to inhibition of glycosylation of viral polypeptides.[53] Monoterpene compounds such as thymol, gamma-terpinene, 1,8-cineole, alpha-pinene, p-cymene, alpha-terpinene, citral, terpinen-4-ol and alpha-terpineol were purified from extracts of tea tree, thyme and eucalyptus. These compounds have demonstrated in-vitro antiviral activity against HSV-1 by inhibition of virus greater than 80%.[54] From *Tripterygium wilfordii*, 13 sesquiterpenes, which have been tested in vitro against HSV-1, were isolated. Only Triptofordin C-2 inhibited viral protein synthesis of infected cells when added at early steps of HSV-1 replication and demonstrated inhibition of translation of the transcripts of the immediate early (IE) genes with selectivity index (>10).[55] Putranjivain A, a diterpene isolated using the acetone–aqueous extraction from all parts of *Euphorbia jolkini*, demonstrated antiviral effect against HSV-2 in Vero cells with an IC50 value of 6.3 μM. At a concentration of 25 μM, the late phase of viral replication was influenced. Moreover, a virucidal effect was reported at concentrations higher than 50 μM, whereas toxicity was seen at a concentration of up to 80 μM.[56] From the plant *Rhus javanica* two triterpenes compounds with antiherpetic activity, namely moronic acid and betulonic acid, were extracted. Both compounds showed in-vitro potent inhibitory effect on HSV-1 with EC50 values of 3.9 and 2.6 μg/ml, respectively, whereas moronic acid *in vivo* demonstrated oral therapeutic efficacy in HSV-infected mice.[57] 28-Deacetylsendanin (28-DAS) was extracted from *Melia azedarach* and inhibited the replication of HSV-1 in Vero cells and thus reduced the synthesis of HSV-1 TK with an IC50 value of 1.46 μg/ml.[58] Tetranortriterpenoid 1-cinnamoyl-3,11-dihydroxymeliacarpin was obtained from *Melia azedarach* and has displayed in-vitro antiherpetic activity by impeding nuclear factor κB activation in HSV-1 infected conjunctival cells.[59] Notoginsenoside ST-4i, a saponin isolated from the Chinese herb *Panax notoginseng*, demonstrated in-vitro remarkable inhibitory activities against HSV-1 and HSV-2 with EC50 values of 16.4 and 19.44 μM, respectively.[60] Chikusetsusaponin Iva was isolated from *Alternanthera philoxeroides* (Mart.). This compound induced inhibitory effect *in vitro* on HSV-1 and HSV-2 with SIs CC50/IC50 values of 29 and 30, respectively, whereas exerted anti-HSV-2 activity *in vivo* in a mouse model. The anti-HSV-2 activity of this compound was found to be related to direct inactivation of virus particles and to the inhibition of release of progeny viruses from infected cells.[114] The inhibitory activity of glycyrrhizin (GR) was examined on infected mice with HSV-1, and the results indicated that GR eliminated the increased susceptibility of thermally injured mice to HSV infectivity during the induction of CD4+ contrasuppressor T cells.[61]

Phenolic compounds

Phenolic compounds are plant secondary metabolites that are produced in response to microbial infections. They are abundant in many plants and have a broad spectrum of antiviral and antioxidant effects.[62]

Plant-derived phenolic compounds

Caffeic acid is a phenolic compound isolated from *Plantago major*, which is present in a wide range of plants. Chiang *et al.* examined *in vitro* the inhibitory effect of the aqueous extract of *Plantago major* and its bioactive lead compounds on HSV and adenovirus. The aqueous extract showed weak antiviral activity, whereas caffeic acid demonstrated remarkable inhibitory activity against HSV-1 and adenovirus type-3 with an EC50 values of 15.3 and 87.3 μg/ml, respectively.[63] Structure–activity relationships have revealed that reducing the number of hydroxyl groups reduces activity against HSV-1.[64] Curcumin is a phenolic compound, isolated from the curry spice turmeric and has been shown to inhibit viral gene expression. In addition, it has been proven that curcumin suppressed the histone acetyltransferase activity of the transcriptional coactivator proteins p300 and CREB-binding protein (CBP), which are recruited to the IE gene promoters of HSV-1 by the viral transactivator protein VP16.[65] Zandi *et al.* have reported that curcumin and its new derivatives, namely gallium-curcumin and Cu-curcumin, have shown an in-vitro inhibitory effect on HSV-1 replication in cell culture with IC50 values of 33.0, 13.9 and 23.1 μg/ml, respectively.[66] From the ethanolic extract of the *Ficus benjamina* leaves, three flavonoids (quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside and kaempferol 3-O-robinobioside) were purified with potent inhibitory activity against HSV infection. These compounds have exhibited SIs 266, 100 and 666, respectively, whereas kaempferol 3-O-robinobioside demonstrated a comparable SI to acyclovir.[67] From the hop extract, *Humulus lupulus* was isolated the prenylated chalcone xanthohumol with inhibitory activity against HSV and therapeutic index for HSV-1(>1.9) and HSV-2(>5.3). The study confirmed that xanthohumol is more effective than iso-xanthohumol.[68] 3,5,7-Trihydroxyflavone (galangin) was isolated from the North African herb *Helichrysum aureonitens*. At concentrations 12–47 μg/ml, the inhibitory effect on HSV-1 and coxsackie B virus type 1 was demonstrated.[69]

5,7-Dimethoxyflavanone-4′-O-[2″-O-(5‴-O-trans-cinnamoyl)-beta-D-apiofuranosyl]-beta-D glucopyranoside was purified from the ethyl acetate extract of the leaves and stems of *Viscum album* and shown to have potent activity against HSV-1 in Vero cells at concentrations ranging from 0.16 to 0.2 μg/ml.[70] Gemin D (3-O-galloyl-4,6-(S)-hexahydroxydiphenoyl-d-glucose) was obtained from

Euphorbia thymifolia Linnea and demonstrated in-vitro potent antiviral activity against HSV, where 0.5 μg/ml has led to inactivate the viral infection; the galloyl groups were determined to be crucial for activity.[71] From the acetone extract of traditional Chinese medicinal plant *Phyllanthus urinaria* L., 1, 3, 4, 6-tetra-o-galloyl-β-D-glucose and Geraniin were isolated. Both compounds inhibit in-vitro HSV-1 and HSV-2 multiplication with IC50 values of 14.8 and 19.2 μM, respectively.[72] Casuarinin, a hydrolyzable tannin, was isolated from *Terminalia arjuna* Linn. This substance has been investigated for its anti-HSV-2 activity *in vitro*. The results showed that casuarinin prevents adhesion and penetration of the virus into the cell and disrupts the late phase of replication. The inhibitory and cytotoxic concentrations against HSV-2 were 1.5 and 89 μM, respectively. Furthermore, casuarinin was virucidal at a concentration of 25 μM.[73] Epigallocatechin gallate (EGG) is the main catechin in *Thea sinensis* L. This potent antioxidant substance revealed an ability to inactivate HSV virions by binding to gB, gD or another envelope glycoproteins. However, modified EGCG, palmitoyl-EGCG has been evaluated for its effect on HSV-infected Vero cells and showed more potent inhibitory activity than EGG against HSV-infected Vero cells at a concentration of 50 μM.[74] Samarangenin B (Sam B) was isolated from the root of *Limonium sinense* and exhibited significant inhibition of HSV-1 replication in Vero cells without cytotoxicity, and reduced the amount of DNA polymerase. The mechanisms of antiviral action of Sam B were found to be mediated by inhibiting HSV-1 α gene expression, including expression of the ICP0 and ICP4 genes, by blocking β transcripts such as DNA polymerase mRNA, and by arresting HSV-1 DNA synthesis and structural protein expression in Vero cells.[75] The catechin containing fractions of the aqueous extract of *Cocos nucifera* L. showed in Vero cells antiviral activity against acyclovir-resistant HSV-1 (HSV-1-ACVr) with virucidal index (VI = 3.25).[76] Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) was isolated from Chinese rhubarb *Rheum tanguticum* and examined for its antiviral properties against HSV infection *in vitro* and *in vivo*. Emodin was found to inhibit the replication of HSV-1 and HSV-2 in cell culture at the concentration of 50 μg/ml with an antiviral index of 2.07 and 3.53, respectively. Moreover, the emodin treatment increased the survival rate of HSV-infected mice. Interestingly, the inhibitory activity of emodin was found to be equivalent to that of acyclovir *in vivo*.[77] Another study has investigated that emodin inhibited the nuclease activity of HSV-1 UL12 alkaline nuclease in a biochemical assay, thus emodin reduced the plaque formation with an EC50 value of 21.5 μM.[78] The glycerin extract of *Aloe barbadensis* contains a large quantity of anthraquinones, particularly aloe emodin, and showed antiviral activity against HSV-2 before attachment and entry of virus to the Vero cells and during the replication phase with an IC50 value of 42 μg/ml.[79]

Alkaloids

Alkaloids have potent antimicrobial activity, including antiviral properties against HSV infections.[80]

Plant-derived alkaloids

Zhang *et al*. have recently isolated a novel alkaloid, 17-nor-excelsinidine from the twigs and leaves of *Alstonia scholaris*. Notably, this substance demonstrated *in vitro* significant inhibitory activity against HSV and adenovirus with EC50 values of 1.09 and 0.94 μg/ml and CC50 values of 6.97 and 3.32 μg/ml, respectively.[81] Berberine is an alkaloid extracted from Chinese herbal plant *Coptidis rhizome* and demonstrated in-vitro antiherpetic activity with IC50 values for HSV-1 and HSV-2 24.4 and 26.8 μM, respectively. Moreover, its mechanism of action is due to the prevention of penetration of virus into the cell.[82] Another alkaloid FK-3000 was obtained from the methanolic extract of *Stephania cepharantha* and showed in-vivo anti-HSV-1 activity. Its administration in mice effectively reduced skin lesions and prolonged the period of survival time, but the therapeutic index was significantly low. However, it is possible to use this substance for treatment of herpetic keratitis.[83] Capsaicin, and its cis isomer (Civamide) from of the genus *Capsicum*, exhibited in-vitro anti-HSV type 1 and 2 activity, whereas Capsaicin reduced recurrent infection of HSV-2 in guinea pigs.[84,85] Ren *et al*. have reported that a crude total alkaloid extract from the root of the Chinese herb *Tripterygium hypoglaucum* showed in-vitro antiviral activity against HSV-1 infection with an IC50 value of 6.5 μg/ml and low cytotoxicity CC50 = 46.6 μg/ml.[86]

Marine-derived alkaloids

Souza *et al*. have isolated the alkaloid 4-methylaaptamine from a marine sponge *Aaptos aaptos*, which inhibits HSV-1 replication in Vero cells with an EC50 value of 2.4 μM. The anti-HSV-1 activity of this compound was found to be related to its ability to impair HSV-1 penetration and immediate-early protein (ICP27) synthesis.[87] Manzamine A, an alkaloid, was isolated from the marine sponges *Haliclona* sp.[88] and *Pachypellina* sp.[115] This compound showed in-vitro anti-HSV activity. However, another study reported by Palem *et al*. indicated that Manzamine A showed potent inhibition of viral replication of HSV-1 and infection in the cell line SIRC at 1 μM, 50× more potent than acyclovir.[89]

Other compounds derived from plant origin

Several compounds that naturally occur in plants were found to be valuable agents for enhancing the treatment of

HSV infections such as ascorbic acid, vitamin E, zinc and lithium. Ascorbic acid (vitamin C) was found to enhance treatment course of HSV infections.[90] Moreover, vitamin C in a clinical test was applied to patients infected with herpes labialis. The results have revealed that a dose of 200 mg of ascorbic acid mixed with 200 mg of water-soluble flavonoids three times daily for 3 days has led to effective treatment during prodromal phase.[91] Several studies have also examined the inhibitory effect of Zinc ions on HSV type 1 and 2 *in vitro* and *in vivo*. In an in-vitro activity, a complete inhibition of viral DNA polymerase was observed at a concentration of 0.1 mM, whereas in-vivo activity at a concentration of 0.1 mM expressed reduction of severity of HSV-2 infection in mice.[92–96] Topical administration of Vitamin E in humans infected with HSV-1 from 15 min to 8 h has led to active treatment of oral herpetic lesion.[97] It has been found that topical administration of lithium inhibited the replication of HSV type 1 and 2, whereas an ointment that contains 8% lithium succinate applied topically to patients infected with HSV-2 for four times daily for 3 days eliminated the median duration of pain.[98–100]

Extracts with potential anti-HSV activities

Crude extracts obtained from plants by various extraction methods have shown wide spectrum of antiviral activity, including anti-HSV activity.[101] Numerous studies reported the anti-HSV activity of propolis, the resin collected by bees from plants. For instance, the inhibitory effect of aqueous extract of propolis exerted 50% inhibition of HSV-1 in Vero cells and 80–85% inhibition of corneal HSV-1 infection in rabbits.[102] Another report showed that Brazilian hydroalcoholic propolis extract is a useful agent for the treatment of vaginal lesions caused by HSV-2.[103] Schnitzler *et al*. have suggested that the inhibitory effect of propolis is due to its constituents including polyphenols, flavonoids and phenylcarboxylic acids.[101] To date, numerous studies have reported the potential use of crude extracts derived from various plant species as antiherpetic agents. This review will not cover all data from studies reported in the literature but will present some of the most currently promising, which are summarised in Table 3.

Conclusion

Infection with herpesviruses, particularly HSV-2, has been recorded as a potential risk factor for HIV infection in humans. To date, no prophylactic HSV vaccine has been found to be effective and entirely eradicate HSV infections. This is due to the establishment of viral latency and reactivation that occurs in the presence of humoral and cell-mediated immunity. In this review, we have reviewed and summarised the findings of the past decade regarding natural products with promising antiviral activity against HSV infections; these are in-vitro and in-vivo studies with various mechanisms of action based on several assay systems or screening methods. Natural products target multiple stages of the virus life cycle, including latent infections, which may be more difficult to bypass through viral resistance, while nucleoside analogues inhibit HSV-DNA replication enzymes used for lytic infection, and which are implicate to viral resistance. The structure–activity relationships showed that galloyl groups increase the antiherpetic activity of phenolic compounds, while reducing the number of hydroxyl groups decreases the activity. In addition, the structure–activity relationships of terpenes, alkaloids, proteins, peptides and polysaccharides are still poorly understood; therefore, further studies are greatly needed. Despite relatively few of isolated anti-HSV compounds from natural sources advance to become clinically effective drugs in their own right, these substances can be used as models for the preparation of analogues using chemical methodology such as total or combinatorial synthesis, or manipulation of biosynthetic pathways. Thus, natural products will open new options for the development of new antiherpetic agents and will play an essential role in pharmaceutical industry.

Future Prospects

Since the problem of drug-resistant strains has become a global concern, it was necessary to develop new strategies to eradicate HSV infections in humans. Large numbers of natural products, including pure compounds, fractions and crude extracts isolated from plants, animals, marine organisms and microorganism, have been tested for their antiviral effects on HSV. Thus, there is a need to examine the combination of these potential anti-HSV agents with nucleoside analogues to promote the maximum therapy of the disease. It is known that antiherpetic drugs do not cure the disease, while modifying the clinical course of the infection by inhibiting viral replication and subsequent epithelial damage. Therefore, in recent years, a new approach has been under focus of interest for searching for comprehensive management of HSV infections based on prevention of transmission, suppression of recurrence, viral shedding and complications, modification of clinical, and promotion of treatment course.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Acknowledgement

This study received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Table 3 Plant extracts with promising anti-HSV properties

Species	Family	Solvents used for extraction	IC50[a] HSV-1	IC50 HSV-2	Reference
Aglaia odorata	*Meliaceae*	Water	9.5 µg/ml		[104]
Anacardium occidentale	*Anacardiaceae*	Water	1 mg/ml		[105]
Aloe vera	*Xanthorrhoeaceae*	Glycerine		A: 428 µg/ml B: 536 µg/ml	[79]
Annona muricata	*Annonaceae*	Ethanol	1 mg/ml		[105]
Baccharis genistelloides	*Asteraceae*	Water	25 µg/ml		[106]
Bergenia ligulata	*Saxifragaceae*	Methanol	10 µg/ml		[106]
Byrsonima verbascifolia	*Malpighiaceae*	Methanol	2.5 µg/ml		[106]
Carissa carandas	*Apocynaceae*	Methanol	12 µg/ml		[107]
Centella asiatica	*Apiaceae*	Water	362 µg/ml	299 µg/ml	[104]
Cerbera odollam	*Apocynaceae*	Water	0.4 µg/ml		[104]
Combretum adenogonium	*Combretaceae*	n-Hexan	2 µg/ml	4 µg/ml	[104]
Echinacea pallida var. *Sanguinea*	*Asteraceae*	n-Hexan	26 µg/ml		[104]
Echinacea purpurea	*Asteraceae*	n-Hexan	120 µg/ml		[107]
Erica multiflora	*Ericaceae*	Methanol	132 µg/ml		[107]
Euclea schimperi	*Ebenaceae*	Methanol	67.5 µg/ml		[106]
Eugenia malaccensis	*Myrtaceae*	Water	125 µg/ml		[108]
Ficus Benjamina	*Moraceae*	Ethanol	0.5 µg/ml	1.7 µg/ml	[109]
Geranium sanguineum	*Geraniaceae*	Water	3.6 µg/ml	6.1 µg/ml	[108]
Helichrysum aureonitens	*Asteraceae*	Water	1.35 mg/ml		[108]
Helichrysum litoreum Guss.	*Asteraceae*	Water	1.35 mg/ml		[110]
Hemidesmus indicus	*Apocynaceae*		200 µg/ml	100 µg/ml	[110]
Hyssopus officinalis	*Lamiaceae*	Methanol	25 µg/ml		[110]
Inula confertiflora	*Asteraceae*	Methanol	96.8 µg/ml		[106]
Ipomoea involucrata	*Convolvulaceae*	Ethanol	250 µg/ml		[110]
Licania tomentosa	*Chrysobalanaceae*	Glycerine	9 µg/ml		[110]
Lilium candidum	*Liliaceae*	Ethanol	8 µg/ml	20 µg/ml	[111]
Maclura cochinchinensis	*Euphorbiaceae*	Ethyl acetate	20.19 µg/ml	68.32 µg/ml	[104]
Magnifera indica L.	*Anacardiaceae*	Water	23.9 µg/ml	31.8 µg/ml	[104]
Melaleuca leucadendron	*Myrtaceae*	Methanol	100 µg/ml		[111]
Moringa oleifera	*Moringaceae*	Ethanol	100 µg/ml		[111]
Nephelium lappaceum	*Sapindaceae*	Methanol	100 µg/ml		[111]
Nerium indicum	*Apocynaceae*	Methanol	10 µg/ml		[111]
Paederia foetida	*Rubiaceae*	Water	400 µg/ml	200 µg/ml	[111]
Pangamia pinnata Linn.	*Papillionaceae*	Water	1 mg/ml	20 mg/ml	[111]
Petunia nictagnifolia	*Solanaceae*	Water	1 mg/ml		[112]
Phyllanthus orbicularis	*Euphorbiaceae*	Water		1 mg/ml	[112]
Pipturus albidus	*Urticaceae*	Water	250 µg/ml		[112]
Polygonum punctatum	*Polagonaceae*	Water	39 µg/ml		[112]
Pongamia pinnata	*Fabaceae*	Water	1 mg/ml	20 mg/ml	[112]
Prunella vulgarit	*Lamiaceae*		20.6 µg/ml	20 µg/ml	[112]
Psychotria hawaiiensis	*Rubiaceae*	Methanol	125 µg/ml		[112]
Satureja boliviana	*Lamiaceae*	Water	10 µg/ml		[112]
Scaevolta sericea	*Goodeniacea*	Acetonitrile	125 µg/ml		[112]
Sebastiana klotzschiacea	*Euphorbiaceae*	Water	169 µg/ml		[109]
Shorea robota	*Dipterocarpaceae*	Water	10 µg/ml	10 µg/ml	[107]
Sterculia setigera	*Malvaceae*	Water	1.5 mg/ml		[113]
Ventilago dentientale	*Rhamnaceae*	Ethanol	46 µg/ml		[113]

HSV, herpes simplex virus; IC50, half maximal inhibitory concentration.
[a]50% inhibitory concentration, A: inhibition value before attachment and entry of virus to the cells, B: inhibition value in post-attachment stage of viral replication.

References

1. Paludan SR *et al.* Recognition of herpesviruses by the innate immune system. *Nat Rev Immunol* 2011; 11: 143–154.
2. Field HJ. Persistent herpes simplex virus infection and mechanisms of virus drug resistance. *Eur J Clin Microbiol Infect Dis* 1989; 8: 671–680.
3. Simpson D, Lyseng-Williamson KA. Famciclovir: a review of its use in herpes zoster and genital and orolabial herpes. *Drugs* 2006; 66: 2397–2416.
4. Levin MJ *et al.* Resistance of herpes simplex virus infections to nucleoside analogues in HIV-infected patients. *Clin Infect Dis* 2004; 39(Suppl. 5): S248–S257.
5. Piret J, Boivin G. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. *Antimicrob Agents Chemother* 2011; 55: 459–472.
6. Markham A, Faulds D. Ganciclovir. An update of its therapeutic use in cytomegalovirus infection. *Drugs* 1994; 48: 455–484.
7. Reusser P. Oral valganciclovir: a new option for treatment of cytomegalovirus infection and disease in immunocompromised hosts. *Expert Opin Investig Drugs* 2001; 10: 1745–1753.
8. Villarreal EC. Current and potential therapies for the treatment of herpesvirus infections. *Prog Drug Res* 2001; 56: 77–120.
9. Reusser P. Herpesvirus resistance to antiviral drugs: a review of the mechanisms, clinical importance and therapeutic options. *J Hosp Infect* 1996; 33: 235–248.
10. Flowerdew SE *et al.* Characterization of neuronal populations in the human trigeminal ganglion and their association with latent herpes simplex virus-1 infection. *PLoS ONE* 2013; 8: e83603.
11. Hamza MA *et al.* Two alphaherpesvirus latency-associated gene products influence calcitonin gene-related peptide levels in rat trigeminal neurons. *Neurobiol Dis* 2007; 25: 553–560.
12. Tan HH, Goh CL. Viral infections affecting the skin in organ transplant recipients: epidemiology and current management strategies. *Am J Clin Dermatol* 2006; 7: 13–29.
13. Frobert E *et al.* Resistance of herpes simplex viruses to acyclovir: an update from a ten-year survey in France. *Antiviral Res* 2014; 111C: 36–41.
14. Imura K *et al.* Herpes simplex virus type 1 infection in two pet Marmosets in Japan. *J Vet Med Sci* 2014; 76: 1667–1670.
15. Shannon TE, Griffin SL. Managing aggression in global amnesia following herpes simplex virus encephalitis: the case of E.B. *Brain Inj* 2014; 29: 118–124.
16. Kopp SJ *et al.* Herpes simplex virus serotype and entry receptor availability alter CNS disease in a mouse model of neonatal HSV. *Pediatr Res* 2014; 8: 528–534.
17. Evans CM *et al.* Management of herpesvirus infections. *Int J Antimicrob Agents* 2013; 42: 119–128.
18. Debbab A *et al.* Bioactive compounds from marine bacteria and fungi. *Microb Biotechnol* 2010; 3: 544–563.
19. Penesyan A *et al.* Development of novel drugs from marine surface associated microorganisms. *Mar Drugs* 2010; 8: 438–459.
20. Newman D, Cragg G. Marine natural products and related compounds in clinical and advanced preclinical trials. *J Nat Prod* 2004; 67: 1216–1238.
21. Sagar S *et al.* Antiviral lead compounds from marine sponges. *Mar Drugs* 2010; 8: 2619–2638.
22. Kitazato K *et al.* Viral infectious disease and natural products with antiviral activity. *Drug Discov Ther* 2007; 1: 14–22.
23. Kleymann G. Agents and strategies in development for improved management of herpes simplex virus infection and disease. *Expert Opin Investig Drugs* 2005; 14: 135–161.
24. Yeung-Yue KA *et al.* The management of herpes simplex virus infections. *Curr Opin Infect Dis* 2002; 15: 115–122.
25. Faulkner DJ. Marine natural products. *Nat Prod Rep* 2002; 19: 1–48.
26. Perry NB *et al.* Antiviral and antitumor agents from a New Zealand sponge, *Mycale* sp. 2. Structures and solution conformations of mycalamides A and B. *J Org Chem* 1990; 55: 223–227.
27. Mandal P *et al.* Xylans from *Scinaia hatei*: structural features, sulfation and anti-HSV activity. *Int J Biol Macromol* 2010; 46: 173–178.
28. Talarico LB *et al.* Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*. *Int J Biol Macromol* 2004; 34: 63–71.
29. Duarte ME *et al.* Inhibitory effect of sulfated galactans from the marine alga *Bostrychia montagnei* on herpes simplex virus replication in vitro. *Phytomedicine* 2001; 8: 53–58.
30. Hayashi T *et al.* Calcium spirulan, an inhibitor of enveloped virus replication, from a blue-green alga *Spirulina platensis*. *J Nat Prod* 1996; 59: 83–87.
31. Chattopadhyay K *et al.* Polysaccharides from *Gracilaria corticata*: sulfation, chemical characterization and anti-HSV activities. *Int J Biol Macromol* 2008; 43: 346–351.
32. Cardozo FT *et al.* Antiherpetic activity of a sulfated polysaccharide from *Agaricus brasiliensis* mycelia. *Antiviral Res* 2011; 92: 108–114.
33. Dong CX *et al.* Structures and anti-HSV-2 activities of neutral polysaccharides from an edible plant, *Basella rubra* L. *Int J Biol Macromol* 2012; 50: 245–249.
34. Xu HX *et al.* Isolation and characterization of an anti-HSV polysaccharide from *Prunella vulgaris*. *Antiviral Res* 1999; 44: 43–54. Antiviral Research. 1999, vol. 44, issue 1, s. 2854–2864.
35. Eo SK *et al.* Possible mode of antiviral activity of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* on herpes

simplex viruses. *J Ethnopharmacol* 2000; 72: 475–481.
36. Gu CQ *et al*. Isolation, identification and function of a novel anti-HSV-1 protein from *Grifola frondosa*. *Antiviral Res* 2007; 75: 250–257.
37. Shan Y *et al*. Purification and characterization of a novel anti-HSV-2 protein with antiproliferative and peroxidase activities from Stellaria media. *Acta Biochim Biophys Sin (Shanghai)* 2013; 45: 649–655.
38. Huang H *et al*. Trichosanthin suppresses the elevation of p38 MAPK, and Bcl-2 induced by HSV-1 infection in Vero cells. *Life Sci* 2006; 79: 1287–1292.
39. Chen GF *et al*. Protective effects of trichosanthin in herpes simplex virus-1 encephalitis in mice. *Zhongguo Dang Dai Er Ke Za Zhi* 2006; 8: 239–241.
40. Albiol Matanic VC, Castilla V. Antiviral activity of antimicrobial cationic peptides against Junin virus and herpes simplex virus. *Int J Antimicrob Agents* 2004; 23: 382–389.
41. Cowan MM. Plant products as antimicrobial agents. *Clin Microbiol Rev* 1999; 12: 564–582.
42. Jenssen H *et al*. Peptide antimicrobial agents. *Clin Microbiol Rev* 2006; 19: 491–511.
43. Hong W *et al*. Inhibitory activity and mechanism of two scorpion venom peptides against herpes simplex virus type 1. *Antiviral Res* 2014; 102: 1–10.
44. Shestakov A *et al*. Synthetic analogues of bovine bactenecin dodecapeptide reduce herpes simplex virus type 2 infectivity in mice. *Antiviral Res* 2013; 100: 455–459.
45. Jenssen H. Anti herpes simplex virus activity of lactoferrin/lactoferricin – an example of antiviral activity of antimicrobial protein/peptide. *Cell Mol Life Sci* 2005; 62: 3002–3013.
46. Yasin B *et al*. Evaluation of the inactivation of infectious herpes simplex virus by host-defense peptides. *Eur J Clin Microbiol Infect Dis* 2000; 19: 187–194.
47. Férir G *et al*. The lantibiotic peptide labyrinthopeptin A1 demonstrates broad anti-HIV and anti-HSV activity with potential for microbicidal applications. *PLoS ONE* 2013; 8: e64010.
48. Petrera E, Coto CE. Therapeutic effect of meliacine, an antiviral derived from *Melia azedarach* L., in mice genital herpetic infection. *Phytother Res* 2009; 23: 1771–1777.
49. Pifarré MP *et al*. Therapeutic action of meliacine, a plant-derived antiviral, on HSV-induced ocular disease in mice. *Exp Eye Res* 2002; 75: 327–334.
50. Barquero AA, Villamil SM. Combined activity of meliacin and foscarnet against different strains of herpes simplex virus type 1 using a three-dimensional model. *Rev Argent Microbiol* 1997; 29: 32–37.
51. Barquero AA *et al*. Antiviral activity of meliacine on the replication of a thymidine kinase-deficient mutant of herpes simplex virus type 1 alone and in combination with acyclovir. *Int J Antimicrob Agents* 1997; 9: 49–55.
52. Mundinger TA, Efferth T. Herpes simplex virus: drug resistance and new treatment options using natural products (Review). *Mol Med Rep* 2008; 5: 611–616.
53. Armaka M *et al*. Antiviral properties of isoborneol, a potent inhibitor of herpes simplex virus type 1. *Antiviral Res* 1999; 43: 79–92.
54. Astani A *et al*. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. *Phytother Res* 2010; 24: 673–679.
55. Hayashi K *et al*. Characterization of antiviral activity of a sesquiterpene, triptofordin C-2. *J Antimicrob Chemother* 1996; 37: 759–768.
56. Cheng HY *et al*. Putranjivain A from Euphorbia jolkini inhibits both virus entry and late stage replication of herpes simplex virus type 2 in vitro. *J Antimicrob Chemother* 2004; 53: 577–583.
57. Kurokawa M *et al*. Anti-herpes simplex virus activity of moronic acid purified from Rhus javanica in vitro and in vivo. *J Pharmacol Exp Ther* 1999; 289: 72–78.
58. Kim M *et al*. Antiviral effects of 28-deacetylsendanin on herpes simplex virus-1 replication. *Antiviral Res* 1999; 43: 103–112.
59. Bueno CA *et al*. A natural tetranortriterpenoid with immunomodulating properties as a potential anti-HSV agent. *Virus Res* 2009; 141: 47–54.
60. Pei Y *et al*. Notoginsenoside ST-4 inhibits virus penetration of herpes simplex virus in vitro. *J Asian Nat Prod Res* 2011; 6: 498–504.
61. Utsunomiya T *et al*. Glycyrrhizin (20 beta-carboxy-11-oxo-30-norolean -12-en-3beta-yl-2-O-beta-D -glucopyranuronosyl-alpha-D -glucopyranosiduronic acid) improves the resistance of thermally injured mice to opportunistic infection of herpes simplex virus type 1. *Immunol Lett* 1995; 44: 59–66.
62. Son M *et al*. Bioactive activities of natural products against herpesvirus infection. *J Microbiol* 2013; 51: 545–551.
63. Chiang LC *et al*. Immunomodulatory activities of flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds of Plantago species. *Planta Med* 2003; 69: 600–604.
64. Ikeda K *et al*. Inhibition of multiplication of herpes simplex virus by caffeic acid. *Int J Mol Med* 2011; 28: 595–598.
65. Kutluay SB *et al*. Curcumin inhibits herpes simplex virus immediate-early gene expression by a mechanism independent of p300/CBP histone acetyltransferase activity. *Virology* 2008; 373: 239–247.
66. Zandi K *et al*. Evaluation of antiviral activities of curcumin derivatives against HSV-1 in vero cell line. *Nat Prod Commun* 2010; 5: 1935–1938.
67. Yarmolinsky L *et al*. Potent antiviral flavone glycosides from *Ficus benjamina* leaves. *Fitoterapia* 2011; 83: 362–367.
68. Buckwold VE *et al*. Antiviral activity of hop constituents against a series of DNA and RNA viruses. *Antiviral Res* 2004; 61: 57–62.

69. Meyer JJ *et al*. Antiviral activity of galangin isolated from the aerial parts of *Helichrysum aureonitens*. *J Ethnopharmacol* 1997; 56: 165–169.
70. Orhan DD *et al*. Antibacterial, antifungal, and antiviral activities of some flavonoids. *Microbiol Res* 2010; 165: 496–504.
71. Yang CM *et al*. *Euphorbia thymifolia* suppresses herpes simplex virus-2 infection by directly inactivating virus infectivity. *Clin Exp Pharmacol Physiol* 2005; 32: 346–349.
72. Yang CM *et al*. The in vitro activity of geraniin and 1,3,4,6-tetra-O-galloyl-beta-D-glucose isolated from *Phyllanthus urinaria* against herpes simplex virus type 1 and type 2 infection. *J Ethnopharmacol* 2007; 110: 555–558.
73. Cheng HY *et al*. Antiherpes simplex virus type 2 activity of casuarinin from the bark of *Terminalia arjuna* Linn. *Antiviral Res* 2002; 55: 447–455.
74. de Oliveira A *et al*. Inhibition of herpes simplex virus type 1 with the modified green tea polyphenol palmitoyl-epigallocatechin gallate. *Food Chem Toxicol* 2013; 52: 207–215.
75. Kuo YC *et al*. Samarangenin B from Limonium sinense suppresses herpes simplex virus type 1 replication in Vero cells by regulation of viral macromolecular synthesis. *Antimicrob Agents Chemother* 2002; 46: 2854–2864.
76. Esquenazi D *et al*. Antimicrobial and antiviral activities of polyphenolics from *Cocos nucifera* Linn. (Palmae) husk fiber extract. *Res Microbiol* 2002; 153: 647–652.
77. Xiong HR *et al*. The effect of emodin, an anthraquinone derivative extracted from the roots of *Rheum tanguticum*, against herpes simplex virus in vitro and in vivo. *J Ethnopharmacol* 2011; 133: 718–723.
78. Hsiang CY, Ho TY. Emodin is a novel alkaline nuclease inhibitor that suppresses herpes simplex virus type 1 yields in cell cultures. *Br J Pharmacol* 2008; 155: 227–235.
79. Zandi K *et al*. Antiviral activity of aloe vera against herpes simplex virus type 2: an in vitro study. *Afr J Biotechnol* 2007; 6: 1770–1773.
80. Kitazato K *et al*. Viral infectious disease and natural products with antiviral activity. *Drug Discov Ther* 2007; 1: 14–22.
81. Zhang LA *et al*. An unusual indole alkaloid with anti-adenovirus and anti-HSV activities from *Alstonia scholaris*. *Tetrahedron Lett* 2014; 55: 1815–1827.
82. Chin LW *et al*. Anti-herpes simplex virus effects of berberine from *Coptidis rhizoma*, a major component of a Chinese herbal medicine, Ching-Wei-San. *Arch Virol* 2010; 155: 1933–1941.
83. Nawawi A *et al*. In vivo antiviral activity of *Stephania cepharantha* against herpes simplex virus type-1. *Phytother Res* 2001; 6: 497–500.
84. Nakano M *et al*. Suppression of recurrent genital herpes simplex virus type 2 infection by *Rhus javanica* in guinea pigs. *Antiviral Res* 1998; 39: 25–33.
85. Bourne N *et al*. Civamide (cis-capsaicin) for treatment of primary or recurrent experimental genital herpes. *Antimicrob Agents Chemother* 1999; 43: 2685–2688.
86. Ren Z1 *et al*. In vitro anti-viral activity of the total alkaloids from Tripterygium hypoglaucum against herpes simplex virus type 1. *Virol Sin* 2010; 25: 107–114.
87. Souza TM *et al*. The alkaloid 4-methylaaptamine isolated from the sponge *Aaptos aaptos* impairs Herpes simplex virus type 1 penetration and immediate-early protein synthesis. *Planta Med* 2007; 73: 200–205.
88. Sakai R *et al*. Manzamine A, a novel antitumor alkaloid from a sponge. *J Am Chem Soc* 1986; 108: 6404–6405.
89. Palem JR *et al*. Manzamine a as a novel inhibitor of herpes simplex virus type-1 replication in cultured corneal cells. *Planta Med* 2011; 77: 46–51.
90. Hamuy R, Berman B. Topical antiviral agents for herpes simplex virus infections. *Drugs Today (Barc)* 1998; 34: 1013–1025.
91. Terezhalmy GT *et al*. The use of water-soluble bioflavonoid-ascorbic acid complex in the treatment of recurrent herpes labialis. *Oral Surg Oral Med Oral Pathol* 1978; 45: 56–62.
92. Fridlender B *et al*. Selective inhibition of herpes simplex virus type I DNApolymerase by zinc ions. *Virology* 1978; 84: 551–554.
93. Gupta P, Rapp F. Effect of zinc ions on synthesis of herpes simplex virus type 2-induced polypeptides. *Proc Soc Exp Biol Med* 1976; 152: 455–458.
94. Gordon YJ *et al*. Irreversible inhibition of herpes simplex virus replication in BSC-1 cells by zinc ions. *Antimicrob Agents Chemother* 1975; 8: 377–380.
95. Tennican P *et al*. Topical zinc in the treatment of mice infected intravaginally with herpes genitalis virus. *Proc Soc Exp Biol Med* 1980; 164: 593–597.
96. Tennican PO *et al*. The diverse effects of topical and systemic administration of zinc on the virulence of herpes simplex genitalis. *Life Sci* 1979; 24: 1877–1883.
97. Nead DE. Effective vitamin E treatment for ulcerative herpetic lesions. *Dent Surv* 1976; 52: 50–51.
98. Skinner GR *et al*. The effect of lithium chloride on the replication of herpes simplex virus. *Med Microbiol Immunol* 1980; 168: 139–148.
99. Lieb J. Immunopotentiation and inhibition of herpes virus activation during therapy with lithium carbonate. *Med Hypotheses* 1981; 7: 885–890.
100. Skinner GR. Lithium ointment for genital herpes. *Lancet* 1983; 2: 288.
101. Schnitzler P *et al*. Antiviral activity and mode of action of propolis extracts and selected compounds. *Phytother Res* 2010; 24(Suppl. 1): S20–S28.
102. Huleihel M, Isanu V. Anti-herpes simplex virus effect of an aqueous extract of propolis. *Isr Med Assoc J* 2002; 4(11 Suppl.): 923–927.

103. Sartori G *et al*. Protective effect of brown Brazilian propolis against acute vaginal lesions caused by herpes simplex virus type 2 in mice: involvement of antioxidant and anti-inflammatory mechanisms. *Cell Biochem Funct* 2012; 30: 1–10.
104. Yoosook C *et al*. Anti-herpes simplex virus activities of crude water extracts of Thai medicinal plants. *Phytomedicine* 2000; 6: 411–419.
105. Abad MJ *et al*. Antiviral activity of Bolivian plant extracts. *Gen Pharmacol* 1999; 32: 499–503.
106. Andrighetti-Fröhner CR1 *et al*. Antiviral evaluation of plants from Brazilian Atlantic Tropical Forest. *Fitoterapia* 2005; 76: 374–378.
107. Li T, Peng T. Traditional Chinese herbal medicine as a source of molecules with antiviral activity. *Antiviral Res* 2013; 97: 1–9.
108. Soltan MM, Zaki AK. Antiviral screening of forty-two Egyptian medicinal plants. *J Ethnopharmacol* 2009; 126: 102–107.
109. Yarmolinsky L *et al*. Antiviral activity of ethanol extracts of *Ficus binjamina* and *Lilium candidum in vitro*. *N Biotechnol* 2009; 26: 307–313.
110. Sassi AB *et al*. Antiviral activity of some Tunisian medicinal plants against herpes simplex virus type 1. *Nat Prod Res* 2008; 22: 53–65.
111. Gebre-Mariam T *et al*. Antiviral activities of some Ethiopian medicinal plants used for the treatment of dermatological disorders. *J Ethnopharmacol* 2006; 104: 182–187.
112. Locher CP *et al*. Anti-microbial activity and anti-complement activity of extracts obtained from selected Hawaiian medicinal plants. *J Ethnopharmacol* 1995; 49: 23–32.
113. Thompson KD. Herbal extracts and compounds active against herpes simplex virus. *Adv Phytomed* 2006; 2: 65–86.
114. Rattanathongkom A *et al*. Evaluation of chikusetsusaponin IV a isolated from *Alternanthera philoxeroides* for its potency against viral replication. *Planta Med* 2009; 75: 829–835.
115. Ichiba T *et al*. 8-Hydroxymanzamine A, a beta-carboline alkaloid from a sponge, *Pachypellina* sp. *J Nat Prod* 1994; 57: 168–170.

 © 2015 Royal Pharmaceutical Society,

www.prolekare.cz | stazeno: 4.3.2016

PŘEHLEDY A ODBORNÁ SDĚLENÍ

Antibakteriální účinky přírodních látek – silice

Antibacterial activity of natural compounds – essential oils

Sherif T. S. Hassan • Michaela Majerová • Miroslava Šudomová • Kateřina Berchová

Došlo 20. července 2015 / Přijato 8. října 2015

Souhrn

Vzhledem k tomu, že problém bakteriální rezistence se stal vážným celosvětovým problémem, bylo nutné hledat nové účinné látky, které mohou překonat tento problém a zlepšit léčebnou účinnost bakteriálních infekcí. Množství silic rostlinného původu prokázala výrazné antibakteriální účinky. Cílem tohoto článku bylo shrnout silice, které vykazovaly pozoruhodné antibakteriální účinky proti různým bakteriálním infekcím včetně stafylokokových infekcí, *Helicobacter pylori* infekce, kožní infekce, tuberkulózy a zubní bakteriální infekce. Byl diskutován synergický efekt silic v kombinaci s antibiotiky, jakož i jejich role v léčbě bakteriálních infekcí. Silice mohou být použity jako modely pro další studie *in vivo* a u klinických studií.
Klíčová slova: antibakteriální látky • silice • rostliny • bakteriální rezistence • bakteriální infekce • synergismus

Summary

Since the problem of bacterial resistance has become a serious problem worldwide, it was necessary to search for new active substances that can overcome the problem and enhance the treatment efficacy of bacterial infections. Numerous plant-derived essential oils exhibited significant antibacterial activities. This review aimed to summarize the most promising essential oils that exhibited remarkable antibacterial activities against various bacterial infections, including staphylococcal infections, *Helicobacter pylori* infections, skin infections, tuberculosis infection and dental bacterial infection. The synergy effect of essential oils in combination with antibiotics, as well as their role in the treatment of bacterial infections have been discussed. Essential oils can be used as models for further studies *in vivo* and clinical trials.
Keywords: antibacterial substances • essential oils • plants • bacterial resistance • bacterial infections • synergism

Ing. Sherif T. S. Hassan (✉) • M. Majerová
Ústav přírodních léčiv, Farmaceutická fakulta
Veterinární a farmaceutická univerzita Brno
Palackého tř. 1/3, 612 42 Brno, Česká republika
e-mail: sherif.hassan@seznam.cz

S. T. S. Hassan • K. Berchová
Katedra aplikované ekologie, Fakulta životního prostředí
Česká zemědělská univerzita v Praze

M. Šudomová
Ústav archeologie a muzeologie
Filozofická fakulta Masarykovy univerzity, Brno

Úvod

Léčivé rostliny mají již po staletí velmi důležitou roli v léčbě různých onemocnění, včetně infekčních chorob. Spousta sloučenin pocházejících z přírodních zdrojů má v dnešní medicíně nezastupitelnou roli a v terapii se využívají samostatně, v kombinaci anebo slouží jako předloha pro chemickou syntézu léčiv. Velké množství chemických léčiv má svůj původ právě v přírodních látkách. Stále probíhající studie hledají, identifikují a testují další potencionálně využitelné sloučeniny pocházející z rostlinných zdrojů.

V dnešní době, kdy dochází k rozsáhlému a někdy i zbytečnému nadužívání antibakteriálních látek, kdy se stále snižuje citlivost mikroorganismů k dostupným preparátům a kdy rezistence bakterií narůstá každým dnem, je nezbytné zabývat se studiem nových molekul s potenciální antibakteriální aktivitou. Některé studie se zabývají chemickou obměnou již existujících účinných molekul, ke kterým se však bakterie stávají v krátké době méně citlivé[1].

Vzhledem k primitivnímu a velmi krátkému životnímu cyklu jsou mikroorganismy velmi adaptabilní vůči okolním podmínkám a nové generace, které vznikají v řádu minut, si nesou genetickou informaci s rezistencí a s jinými výhodami oproti starším generacím. Dalo by se říci, že bakterie mají a vždy budou mít náskok před

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

námi a naší léčbou, proto je důležité neustále hledat nové sloučeniny s antibakteriální aktivitou[2)].

Přehled si klade za cíl vyhledávat dosavadní poznatky a studie o antibakteriálně působících silicích v původních vědeckých publikacích, a vytvořit tak aktuální seznam látek, které by mohly sloužit jako nadějné molekuly pro další studium potenciálních antibakteriálních léčiv. Publikační podklady byly vyhledány v databázích PubMed a Web of Knowledge pod klíčovými slovy: bacterial resistance, essential oils, bacterial infections, antibacterial agents, plants.

Silice

Silice jsou sekundární metabolity rostlin. Jedná se o strukturně velmi heterogenní sloučeniny. Mohou to být látky se silným aroma i látky bez vůně. V převážné většině jde o látky lipofilní, prakticky nerozpustné ve vodě. Většinou jsou kapalné, bezbarvé, skladováním velmi lehce oxidují a tmavnou. Mívají menší hustotu než voda, čehož se využívá při jejich extrakci. Silice mají velmi široké spektrum biologických vlastností, jako jsou účinky protizánětlivé, zklidňující, antinociceptivní, antioxidační, psychotropní, expektorační. Vzhledem ke své multifunkčnosti mají nezastupitelnou roli v medicíně a aromaterapii[3)].

Mechanismus antibakteriálního účinku silic

Mechanismus účinku silic proti patogenním bakteriím je poměrně složitý proces, který není zcela vysvětlen a objasněn. Je zřejmé, že antibakteriální účinky silic úzce souvisejí s hydrofilními a lipofilními vlastnostmi daných komponent. Terpeny, které vykazují lipofilní charakter, působí inhibičně na enzymy katalyzující vznik bakteriálních membrán.

Některé složky silic působí jako „vypínač“, interferují s translokací protonů přes membránové váčky a blokují fosforylaci adenosin-difosfátu, čímž narušují primární energetický metabolismus bakteriální buňky. Silice – obsahující jako funkční skupinu fenolický alkohol nebo aldehyd – narušují membránu mikroorganismů. Některé sloučeniny zasahují do enzymatických pochodů buněk potlačením enzymatické aktivity nebo zastavením produkce potřebných enzymů, čímž způsobí smrt citlivé buňky.

Silice dále inhibují syntézu DNA, RNA, proteinů a polysacharidů v buněčné stěně bakterií. Některé sloučeniny přírodního charakteru mají i více mechanismů účinku najednou a kombinací různých silic dochází k synergismu a zesílení antibakteriální aktivity[4)].

Bakteriální buňky můžeme obecně rozdělit dle struktury buněčné stěny pomocí Gramova barvení do dvou velkých skupin, a to na gram-pozitivní, které se barví do tmavě fialova a na gram-negativní, které se pomocí Gramova barvení zbarvují za použití safraninu do červena[5)].

Další dostupné studie uvádějí větší antibakteriální efekt silice ke gram-pozitivním bakteriím než ke gram-negativním. Vnější buněčná membrána gram-negativních bakterií má schopnost získat hydrofilní charakter, který brání kontaktu povrchu bakteriální buňky s hydrofobní složkou silic. Rozdílně reagují gram-pozitivní bakterie, kde buněčná membrána vykazuje lipofilní vlastnosti. Silice mohou s povrchem takové bakterie snadno interagovat a tato interakce vede k porušení bakteriální membrány, změně iontové propustnosti, k lýze a smrti patogenní buňky[6)].

Gram-pozitivní bakterie mají v buněčné stěně silnou vrstvu peptidoglykanu, která je protkaná kyselinou lipoteichoovou, buněčná stěna gram-negativních bakterií obsahuje jen málo peptidoglykanu bez kyseliny lipoteichoové, ale velmi silnou vrstvu lipoproteinů a polysacharidů, která znesnadňuje průnik silic do buňky. Antibakteriální působení silic tedy závisí na druhu patogenního mikroorganismu a na jeho bakteriální stěně[7)].

Silice s antibakteriální aktivitou k multirezistentním bakteriím

Rostoucí rezistence mikroorganismů k běžně dostupným antibakteriálním látkám představuje výzvu pro vědce celého světa najít alternativní způsoby léčby infekcí způsobených odolnými bakteriemi. Mezi nejčastěji testované bakterie patří hlavně *methicilin rezistentní Staphylococcus aures* (MRSA), který může způsobovat závažná onemocnění, jako jsou pneumonie, sepse, endokarditida nebo meningitida zejména u hospitalizovaných pacientů. Tabulka 1 uvádí některé silice, u kterých byla objevena aktivita proti tomuto mikroorganismu. Mezi další nebezpečné bakterie patří *Enterobacter aerogenes*, *Escherichia coli* a *Pseudomonas aeruginosa*. Citlivost těchto patogenů se značně zvyšuje při použití kombinace běžně užívaných léků jako beta-laktamová antibiotika, chloramfenikol a chinolony s geraniolem[8)].

Silice z *Melaleuca alternifolia* (Myrtaceae) obsahuje kromě jiných složek antibakteriálně aktivní terpinen-4--ol, který vykazuje aktivitu proti MRSA, a mohl by tak představovat zajímavou alternativu léčby kožních infekcí způsobených touto bakterií. Tato látka však musí být podávána jen ve vyšších koncentracích, které jsou schopny vyvolat silný bakteriostatický nebo baktericidní efekt, protože použití koncentrací, které jsou příliš nízké k poškození bakteriální buňky, vede ke vzniku odolnosti vůči této silici a výsledný antimikrobiální efekt se snižuje. Tato silice je dle studií velmi dobře snášena, avšak chybí informace o její systémové farmakokinetice a farmakodynamice u lidí[9)].

Silice s antibakteriální aktivitou k bakteriím způsobujícím kožní infekce

Mezi mikroorganismy, které nejčastěji způsobují různé kožní infekce, patří hlavně *Propionibacterium acnes, Propionibacterium granulosum* a *Stapylococcus epidermidis*. Mezi silice působící proti těmto bakteriím patří hlavně linalool, α-terpineol, limonen[15)].

Djabou et al. v roce 2013 publikovali studii zabývající se složením silice pěti druhů rodu *Teucrium* (*T. marum, T. massiliense, T. chamaedrys, T. scorodonia* a *T. flavum*). Zajímavé je, jak je složení silice diametrálně rozdílné v případě jednoho rostlinného rodu. Slabou antibakteriální aktivitu ke kožním patogenům vykazují všechny zkoumané druhy rodu *Teucrium*, nejsilněji však působí *Teucrium massiliense*[20)]. Přehled vybraných rostlin a silic uvádí tabulka 2.

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

Tab. 1. Silice s antibakteriální aktivitou k MRSA

Čeleď / *název rostliny*	**Hlavní složka silice (%)**	**MIC (μg/ml)**	**Testovaná bakterie**	**Zdroj**
Asteraceae				
Tanacetum chiliophyllum	Kafr (32,5) Chamazulen (9,2) 1,8-Cineol (1,6)	500,0	MRSA	10)
Myrtaceae				
Cleistocalyx operculatus	γ-Terpinen (5,8) Globulol (5,6) cis-Linalool oxid (5,2)	4,0	MRSA	11)
Myrtaceae				
Eucalyptus globulus	1,8-Cineol (47,2)	85,6	MRSA	12)
Melaleuca alternifolia	Terpinen-4-ol (4,3)	1,5–3,0	MRSA	13)
Lamiaceae				
Lavandula stoechas	α-Fenchon (39,2) myrtenyl acetát (9,5) α-Pinen (6,1)	31,2	MRSA	14)
Salvia rosifolia	α-Pinen (49,0) Kafr (60,8)	125,0	MRSA	15)
Thymus vulgaris	Tymol (48,1)	18,5	MRSA	12)
Zataria multiflora	Tymol (38,7) p-Cymen (10,2)	0,3–1,0	MRSA	16)
Lavandula angustifolia	Linalyl acetát (37,0) Linalool (29,5)	*	MRSA	14)
Lavandula latifolia	1,8-cineol (28,5) Linalool (38,8)	*	MRSA	14)
Lauraceae				
Cinnamomum ospholeum	Cinnamaldehyde (76,0) Neral (12,82) 1,8-Cineol (11,3)	250,0	MRSA	17)
Meliaceae				
Toona sinensis	Caryophylen (13,2) β-Caryophylen (10,2)	2000,0	MRSA	18)
Rutaceae				
Zanthoxylum tingoassuiba	α-Bisabolol*	*	MRSA	15)
Citrus hystrix	Citronelal (80,0)	2200,0**	MRSA	19)

*Citlivost MRSA byla zkoumána jen diskovou difuzní metodou a chybí údaj o MIC.
**Hodnota MIC se rovná hodnotě MBC pro MRSA.
MIC – minimální inhibiční koncentrace, MBC – minimální baktericidní koncentrace

Silice s antibakteriální aktivitou k Helicobacter pylori

Helicobacter pylori je gram-negativní bakterie kolonizující žaludek mnoha lidí. Na jedné straně může tato kolonizace probíhat bez příznaků, ale na straně druhé může způsobit velmi nepříjemné zdravotní problémy jako žaludeční vředy a gastritidu[37].

Tyto komplikace jsou běžně léčeny inhibitory protonové pumpy v kombinaci s antibiotiky. Mezi inhibitory protonové pumpy používané v současné době v České republice patří omeprazol, lansoprazol, pantoprazol a esomeprazol a mezi antibiotika používaná k eradikaci se používají kombinace amoxicilinu a klaritromycinu, případně nitroimidazolů (metronidazol, ornidazol). Ovšem hlavně u pacientů z vyspělých zemí současná léčba selhává a celosvětově jsou hlášeny případy, kdy je *Helicobacter pylori* k této léčbě rezistentní[38]. V tabulce 3 jsou shrnuty nadějné silice, které *in vitro* vykazují antibakteriální aktivitu k této bakterii.

Silice s antibakteriální aktivitou k bakteriím způsobujícím zubní kaz

Kariogenní mikroorganismy v dutině ústní při přebytku kariogenního substrátu, zejména nízkomolekulárních sacharidů, produkují organické kyseliny, zejména laktát a pyruvát. Působí-li kyseliny dostatečně dlouho na zubní tkáň, dochází k demineralizaci. Na povrchu zubu se

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

Tab. 2. Silice s antibakteriální aktivitou k bakteriím způsobujícím kožní infekce

Čeleď / ***název rostliny***	**Hlavní složka silice (%)**	**MIC (µg/ml)**	**Testovaná bakterie**	**Zdroj**
Asteraceae				
Schinus molle	α-Phellandren (25,9) β-Myrcen (11,1) Limonen (11,7)	63,00	*Stapylococcus epidermidis*	21)
Apiaceae				
Mutellina purpurea	Sabinen (8,6) β-Elemen (9,2) α-Bisabolol (5,5)	625,00	*S. epidermidis*	22)
Sphallerocarpus gracilit	α-Asaron (33,1) γ-Terpinen (25,6) p-Cymen (17,4)	640,00	*S. epidermidis*	23)
Asteraceae				
Tanacetum chiliophyllum	Kafr (32,5) Chamazulen (9,2) 1,8-Cineol (1,6)	250,00	*S. epidermidis*	10)
Asteraceae				
Chrysanthemum trifurcatum	Limonen (20,9) γ-Terpinen (19,3) 1,8-Cineol (10,6) β-Pinen (8,8)	62,50	*S. epidermidis*	24)
Boraginaceae				
Cordia curassavica	4-Methyl,4-ethenyl-3-(1-methyl ethenyl)-1-(1-methyl methanol) Cyclohexan (37,3) β-Eudesmol (19,2)	250,00	*S. epidermidis*	25)
Juglandaceaea				
Juglans regia	α-Pinen (15,1) β-Pinen (30,5) (E)-Caryophylen (15,5)	15,62	*S. epidermidis*	26)
Rutaceae				
Citrus matsudaidai	Limonen (81,6)	0,31 10,00	*Propionibacterium acnes* *S. epidermidis*	27)
Citrus obovoidea	Limonen (83,4)	0,31 2,50	*P. acnes* *S. epidermidis*	27)
Fortunella Japonka	Limonen (61,6)	*	*P. acnes* *S. epidermidis*	15)
Aracauriaceae				
Araucaria cunninghamii	α-Pinen (16,2) Beyeren (34,6)	250,00	*S. epidermidis*	28)
Araucaria heterophylla	13-Epi-Dolabradien (42,7) Beyeren (22,2) Rimuen (13,7)	250,00	*S. epidermidis*	28)
Lamiaceae				
Salvia urmiensis	Benzyl benzoát (60,3) Linalool (2,2)	5,30	*S. epidermidis*	29)
Salvia bracteata	Caryophylen oxid (16,6) Caryophylen (4,1) Pulegon (3,9)	50,00	*S. epidermidis*	30)

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

Čeleď / *název rostliny*	**Hlavní složka silice (%)**	**MIC (μg/ml)**	**Testovaná bakterie**	**Zdroj**
Salvia rubifolia	γ-Muurolen (11,8) α-Pinen (7,1) γ-Cardinen (5,5)	50,00	*S. epidermidis*	30)
Ocimum basilice	Methyl chavicol (16,3) Citral (16,4) Cinnamát (21,6)	5,00	*P. acnes*	31)
Thymus quinquecostatus	p-Cymen-3-ol (50,4) p-Cymen-2-ol (24,1)	* 0,50	*Propionibacterium granulosum* *P. acnes*	15)
Teucrium massiliense	6-Methyl-3-heptyl acetát (19,1) 3-Octanyl acetát (7,0) Pulegon (6,9)	800,00	*S. epidermidis*	20)
Lauraceae				
Cinnamomum ospholeum	Cinnamaldehyde (76,0) Neral (12,82) 1,8-Cineol (11,3)	250,00	*S. epidermidis*	17)
Pinaceae				
Abies koreana	Bornyl acetát (30,4) Limonen (19,2)	*	*P. acnes* *S. epidermidis*	15)
Myrtaceae				
Syzygium aromaticum	Eugenol (63,6) β-Caryophylen (13,7)	0,31	*P. acnes*	32)
Myrtaceae				
Leptospermum petersonii	Citronelal (11,4) Citronelol (17,5) Neral (19,7) Geranial (34,7)	2000,00 1000,00	*S. epidermidis* *P. acnes*	33)
Leptospermum scoparium	Eudesma-4(14)-11-dien (11,6) α-Selinen (10,4) Methyl cinnamát (12,6)	4000,00 1000,00	*S. epidermidis* *P. acnes*	33)
Kunzea ericoides	α-Pinen (37,6) p-Cymen (13,5)	8000,00 4000,00	*S. epidermidis* *P. acnes*	33)
Melaleuca linarrifolia	1,8-Cineol (77,4) α-Terpineol (7,7)	250,00	*S. epidermidis*	34)
Poaceae				
Cymbopogon nardus	Citral (16,4) β-Myrcen (11.1) Geraniol*	0,60	*P. acnes*	31)
Schisandraceae				
Schisandra chinensis	Ylangen (50,1) β-Himachalen (10,8) α-Bergamoten (9,5)	0,13	*S. epidermidis*	35)
Zingiberaceae				
Alpinia purpurata	β-Pinen (13,9) Linalool (9,6) α-Terpineol (7,2)	‹10,00	*S. epidermidis*	36)

*Údaje neuvedeny v použité literatuře.
MIC – minimální inhibiční koncentrace

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

Tab. 3. Silice s antibakteriální aktivitou k Helicobacter pylori

Čeleď / ***název rostliny***	**Hlavní složka silice (%)**	**MIC (µg/ml)**	**Testovaná bakterie**	**Zdroj**
Lamiaceae				
Satureja bachtiarica	Tymol (27,9) Carvacrol (45,5) p-Cymen (4,4)	0,035	*Helicobacter pylori*	37)
Thymus caramanicus	Carvacrol (68,9)	14,400	*H. pylori*	38)
Origanum minutiflorum	Carvacrol (29,2) o-Cymen (5,9)	46,000	*H. pylori*	39)
Sideritis italica	Kaur-15-en (20,1) β-Cubeben (12,6)	25,100	*H. pylori*	40)
Anacardiaceae				
Sclerocarya birrea	Terpinen-4-ol (35,83) Aromadendren (13,63)	0,060	*H. pylori*	41)
Myrtaceae				
Plinia cerrocampanensis	α-Bisabolol (42,8)	62,500	*H. pylori*	15)
Eucalyptus grandis	Alloocymen, -Pinen*	1,560	*H. pylori*	42)
Myrtus communis	α-Pinen (57-60) 1,8-Cineol (33)	0,010–2,500	*H. pylori*	43)
Apiaceae				
Apium nodiflorum	Limonen (27,7) p-Cymen (23,1)	12,500	*H. pylori*	15)
Asteraceae				
Dittrichia viscosa	3-methoxy cuminyl isobutyrát (40,0)	*	*H. pylori*	44)
Rutaceae				
Citrus lemon	Limonen (70,8) β-Pinen (13,5)	25,000	*H. pylori*	45)

*Údaje neuvedeny v použité literatuře.
MIC – minimální inhibiční koncentrace

vytváří zubní plak, který je složen ze slin, bakteriálních metabolických produktů, zbytků potravy a bakterií. Mezi bakterie tvořící plak patří *Streptococcus mutans, Streptococcus sobrinus, Streptococcus cricetus* a *Streptococcus pyogenes*. Schopnost syntetizovat extracelulární polysacharidy glukany za přítomnosti sacharózy pomocí glukosyltransferas umožňuje velmi pevnou adhezi těchto mikroorganismů na zubním povrchu a tvorbu plaku[46].

Některé silice mají schopnost inhibovat růst těchto mikroorganismů a předcházet vzniku biofilmu. Některé silice mají dokonce větší účinnost než chlorhexidin, který se používá jako antimikrobiální látka při prevenci zubního kazu. Konkrétně se jedná o silice rostliny *Rosmarinus officinalis* (Lamiaceae) a *Mentha piperita* (Lamiaceae), kde je hlavní složkou silice menthol (47,5 %), kdy hodnota MBC (minimální baktericidní koncentrace) byla pro *Satureja mutans* 4krát nižší než MIC (minimální inhibiční koncentrace) chlorhexidinu a pro *Satureja pyogenes* 2krát nižší než hodnota MIC chlorhexidinu[15].

Nikolić et al. prováděli studii pěti rostlin z čeledi Lamiaceae, kde byla porovnána MIC silic z rostlinných drog (*Mentha piperita, Mentha pulegium, Lavandula angustifolia, Satureja montana* a *Salvia lavandulifolia*) s MIC Hexoralu (obsahující chlorhexidin) a MIC Streptomycinu. Silice měly mnohem menší hodnotu MIC než Hexoral, kde byla MIC 650,0 µg/ml pro *Satureja pyogenes* a 1560,0 µg/ml pro *Satureja mutans*. Nejúčinnější byla silice ze *Satureja montana,* kde hodnota MIC pro *Satureja mutans* byla 60,0 µg/ml a pro *Satureja pyogenes* 166,7 µg/ml. Žádná z testovaných silic ovšem nedosáhla nižší hodnoty MIC než Erytromycin, kde se hodnota MIC pro *Satureja mutans* rovnala 20,0 µg/ml, pro *Satureja pyogenes* 40,0 µg/ml[47].

V tabulce 4 jsou shrnuty silice, které se mohou podílet na předcházení vzniku zubních kazů.

Silice s antibakteriální aktivitou k Mycobacterium tuberculosis

Mycobacterium tuberculosis je gram-pozitivní patogen zodpovědný za vznik tuberkulózy. Tuberkulóza (TBC) je jednou z nejsmrtelnějších infekčních onemocnění na světě. Mnohdy je brána jako nemoc minulosti, protože ve vyspělých zemích má incidence onemocnění klesající tendenci. Hlavním problémem se stávají rozvojové země například v Africe, kde je TBC vedle malárie jednou ze tří nejčastějších příčin úmrtí HIV pozitivních jedinců. Každý rok onemocní touto nemocí 8 milionů

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

Tab. 4. Silice s antibakteriální aktivitou k bakteriím způsobujícím zubní kaz

Čeleď / *název rostliny*	**Hlavní složka silice (%)**	**MIC (µg/ml)**	**Testovaná bakterie**	**Zdroj**
Lamiaceae				
Hyptis pectinata	β-Caryophylen (28,3) Caryophylen oxid (28,0)	200,0	*Streptococcus mutans*	15)
Mentha piperin	Menthol (47,5) Menthon (21,7)	603,3 630,0	*S. mutans* *S. pyogenes*	47)
Mentha pulegium	Pulegon (68,7) Piperiton (14,7)	630,0 620,0	*S. mutans* *S. pyogenes*	47)
Lavandula angustifolia	Linalool (40,3) Borneol (13,1)	1191,7 1208,3	*S. mutans* *S. pyogenes*	47)
Satureja montana	Tymol (44,6) p-Cimen (13,4)	60,0 116,7	*S. mutans* *S. pyogenes*	47)
Salvia lavandulifolia	Kafr (29,1) 1,8-Cineol (20,3)	630,0 620,0	*S. mutans* *S. pyogenes*	47)
Ocimum suave	Methyl-eugenol (82,7)	190,0	*S. mutans*	48)
Asteraceae				
Achillea ligustica	Virdiflorol (14,5) Terpinem-4-ol (13,0)	39,0	*S. mutans*	15)
Myrtaceae				
Eucalyptus globulus	1,8-Cineol (71,1) α-Pinen (8,3)	13,0	*S. mutans*	49)
Eucalyptus urograndis	1,8-Cineol (36,2) α-Pinen (17,5)	25,0	*S. mutans*	49)
Myrtaceae				
Melaleuca linarrifolia	1,8-Cineol (77,4) α-Terpineol (7,7)	250,0	*S. mutans*	34)
Eugenia calycina	Spathulenol (21,4) Bicyclogermacren (19,3) β-Caryophylen (8,6)	›400,0	*S. mutans*	50)
Ranunculaceae				
Nigella sativa	p-Cymen (49,48) α-Thujen (18,93	2130,0 4250,0	*S. mutans* *S. oralis*	51)
Phyllanthaceae				
Phyllanthus muellerianus	Isoelemicin (36,4) Caryophylen oxid (22,5)	126,0 108,0	*S. mutans* *S. pyogenes*	52)
Verbenaceae				
Lippia origanoides	Carvacrol (38,6) Tymol (18,5)	*	*S. mutans*	53)
Araucariaceae				
Araucaria cunninghamii	α-Pinen (16,2) Beyeren (34,6)	250,0	*S. mutans*	28)
Araucaria heterophylla	13-Epi-Dolabradien (42,7) Beyeren (22,2) Rimuen (13,7)	500,0	*S. mutans*	28)

*Hodnota MIC v literatuře neuvedena, antibakteriální aktivita ověřena pomocí diskové difuzní metody.
MIC – minimální inhibiční koncentrace

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

Tab. 5. Silice s antibakteriální aktivitou k Mycobacterium tuberculosis

Čeleď / ***název rostliny***	**Hlavní složka silice (%)**	**MIC (μg/ml)**	**Testovaná bakterie**	**Zdroj**
Myrtaceae				
Myrtus communis	α-Pinen (57-60) 1,8-Cineol (33)	*	*M. tuberculosis*	43)
Lamiaceae				
Thymus vulgaris	Tymol (79,2)	500,0	*M. tuberculosis*	55)
	Tymol (79,2)	400,0	*M. tuberculosis*	56)
Chenopodiaceae				
Chenopodium ambrosioides	Ascaridol** Carvacrol**	500,0	*M. tuberculosis*	55)
Euphorbiaceae				
Croton pseudopulchellus	Linalool** Caryophylen oxid**	100,0	*M. tuberculosis*	55)
Anemiaceae				
Anemia tomentosa	Epi-presilphiperfolan-1-ol (30,6)	100,0	*M. tuberculosis*	15)
Verbenaceae				
Lantana fucata	β-Elemen (27,1) Germacren D (11,6)	100,0	*M. tuberculosis*	15)
Lantana triforia	Germacren D (45,1)	80,0	*M. tuberculosis*	15)
Rutaceae				
Swinglea glutinosa	β-Pinen (49,6)	100,0	*M. tuberculosis*	15)
Asteraceae				
Achyrocline alata	Tymol (24,0)	62,5	*M. tuberculosis*	15)

*MIC ověřena pouze diskovou difuzní metodou.
**Procentuální složení silice neuvedeno v použité literatuře.
MIC – minimální inhibiční koncentrace

pacientů na celém světě a přibližně 2–3 miliony pacientů zemře.

Zdrojem nákazy TBC je v dnešní době ve většině případů infikovaný jedinec vylučující patogenní mykobakterie. Branou vstupu infekce je většinou dýchací ústrojí, velmi vzácně trávicí trakt nebo poraněná kůže. U velké části infikovaných osob se onemocnění nemusí projevit a dotyčný je přenašečem. Až když se oslabí imunitní systém, například při HIV nebo ve stáří, dostaví se klinické příznaky onemocnění.

Nemoc nejčastěji postihuje plíce, ale může postihnout i jiné části těla, například kosti, močové ústrojí a nervový systém. Mezi nejčastější příznaky onemocnění patří neutišitelný kašel, vysoké teploty, bolesti na hrudi, úbytek na váze.

V současné době byl evidován výskyt rezistentních a multirezistentních kmenů, proti kterým nezabírají běžně dostupné léky. Proto je nutné hledat nové sloučeniny, které by mohly v budoucnu pomoci v boji s tímto smrtícím patogenem[54]. Tabulka 5 shrnuje přírodní látky z řad silic, které vykazují antibakteriální aktivitu k *Mycobacterium tuberculosis.*

Lall a Meyer testovali asi 180 rostlin z jižní Afriky, které by mohly být použity v boji proti *Mycobacterium tuberculosis.* Asi u 30 % se projevila určitá antibakteriální aktivita proti tomuto mikroorganismu. Mezi těmito drogami byly i rostliny obsahující jako účinné látky silice. Antibakteriální aktivitu prokázala například silice z *Foeniculum vulgare* (Apiaceae) obsahující hlavně anethol, silice z *Heteromorpha trifoliata* (Apiaceae) obsahující hlavně α-pinen a germacren D, silice z *Artemisia afra* (Asteraceae) obsahující 1,8-cineol, α i β-thujon a kafr, silice ze *Senecio quinquelobus* (Asteraceae) obsahující 1,8-cineol, silice z *Myrothamus flabeliformus* (Myrothamnaceae) obsahující kafr, α-pinen a 1,8-cineol. U extraktů výše uvedených siličných drog však doposud nebyla stanovena MIC a není zatím ani publikováno detailní složení silice a hlavní účinná složka. Víme však, že prokazují antibakteriální aktivitu k *Mycobacterium tuberculosis*, a mohou tak být předmětem dalšího zkoumání[55].

Synergický efekt některých silic v kombinaci s antibiotiky

Velký počet silic vykazuje silný synergický efekt s běžně používanými antibiotiky. Ke stanovení synergické antimikrobiální aktivity *in vitro* se v praxi používá frakční inhibiční koncentrace (FIC). FIC pro látku A vypočítáme jako poměr MIC (A + B)/MIC (A) a FIC pro látku B poměrem MIC (B + A)/MIC (B). Pro vyjádření stupně synergismu se používá Index frakční inhibiční koncentrace (FIC_i), který se rovná součtu FIC (A) + FIC (B). Dle hodnoty FIC_i určíme míru synergismu, úplný synergismus ($FIC_i \leq 0,5$), parciální synergismus ($0,5 < FIC_i \leq 0,75$), bez efektu ($0,75 < FIC_i \leq 2$) nebo antagonismus ($FIC_i > 2$).

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

Silice ze *Zataria multiflora* (Lamiaceae) s hlavní složkou tymol (38,7 %) vykazuje synergismus s vancomycinem k MRSA s FIC_i (0,185) a k MRSA s FIC_i (0,320). Směs silice a vancomycinu by tedy mohla vést k vývoji nové účinné antibakteriální kombinace k léčbě MRSA[16].

Další studie ukázala synergický efekt silice z *Thymus maroccanus* (Lamiaceae) s hlavní složkou carvacrol (76,35 %) s několika běžně užívanými antibiotiky (ciprofloxacin, gentamicin, pristinamycin, cefixim) proti různým patogenům (*Escherichia coli, Salmonella* sp.*, Enterobacter cloacae, Klebsiella pneumoniae, Vibrio cholerae, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus*, *Micrococcus luteus, Staphylococcus aureus*). Kombinace silice s ciprofloxacinem vykazuje u všech patogenů úplný synergismus, kombinace s gentamicinem vykazuje úplný synergismus kromě patogenů *Salmonella* sp.*, Vibrio cholerae* a *Micrococcus luteus*, kde je synergismus parciální. Parciální synergismus vykazuje kombinace s pristinamycinem u *Vibrio cholerae, Pseudomonas aeruginosa* a *Staphylococcus aureus.* Bez efektu zůstala kombinace s cefiximem u *Enterobacter cloacae, Klebsiella pneumoniae* a *Micrococcus luteus*. Žádná z kombinací nevykazuje antagonismus a hodnoty MIC jsou až 16krát menší než hodnoty samotné antimikrobiální látky[57].

Diterpeny silice z *Lycopus europaeus* (Lamiaceae) mají aditivní antibakteriální efekt v kombinaci s tetracyclinem k *Staphylococcus aureus*. Totarol, ferulenol jako hlavní složky silice z *Ferula communis* (Apiaceae) působí synergicky s hydrazidem kyseliny isonikotinové na patogeny rodu *Mycobacterium* (*M. intracellulare, M. smegmatis, M. xenopei, M. Chelonei*). Kyselina isopimaricová z *Pinus nigra* (Pinaceae) působí s reserpinem aditivně proti MRSA[58]. U rostliny *Pelargonium graveolans* (Geraniaceae) s hlavní složkou silice citronellolem a geraniolem se zkoumal synergický efekt s Norfloxacinem, testováno bylo pět bakteriálních kmenů (*Bacillus cereus, Bacillus subtilis, Escherichia coli, Staphylococcus aureus* ATCC 6538 a *Staphylococcus aureus* ATCC 29213). U tří z pěti bakteriálních kmenů tato kombinace vykazuje úplný synergismus, a to u *Staphylococcus aureus* ATCC 29213 s indexem FIC_i 0,38, u *Staphylococcus aureus* 6538 s indexem FIC_i 0,37 a u *Bacillus cereus* s indexem FIC_i 0,50, u zbylých dvou bakteriálních kmenů se jedná o synergismus částečný[59].

Velmi výhodná je také kombinace Amoxicilinu a silice z *Tetraclinis artuculata* (Cupressaceae) s hlavní složkou oleje α-campholenalem (16,34 %) a trans-pinocarveolem (15,45 %). Toto spojení silice a antibiotika vykazuje nejvyšší aktivitu proti *Listeria monocytogenes* (FIC_i = 0,5), *Staphylococcus aureus* (FIC_i = 0,2) a *Salmonella enterica* (FIC_i = 0,5), proti *Escherichia coli* a *Pseudomonas aeruginosa* nevykazuje tato kombinace efekt žádný, ale proti žádnému testovanému bakteriálnímu kmenu není prokázán antagonismus[60]. Další velmi významný aditivní antibakteriální efekt vykazuje spojení silice *Lippia origanoides* (Verbenaceae) s aminoglykosidy neomycinem a amikacinem. Kombinace byla zkoušena na MRSA a výsledky jsou obdivuhodné, spojení silice s neomycinem snížilo hodnotu MIC 10krát MIC 2500 μg/ml klesla na MIC 248 μg/ml a kombinace s amikacinem snížila minimální inhibiční koncentraci ze 788 μg/ml na 78 μg/ml[61].

Závěr

Rezistence bakterií k dostupné léčbě se stává globálním problémem. Je proto velmi důležité zaměřit se na studium a vývoj nových účinných léčiv. Jednou z variant je studium přírodních látek, které se pro své léčivé účinky používaly již v dobách, kdy lidstvo nemělo ani povědomí o tom, co je to bakterie či účinná látka. Rostliny obsahují velké množství sekundárních metabolitů, které využívají mimo jiné i k ochraně proti infekcím.

Cílem této práce bylo vyhledání nejnovějších poznatků o antibakteriální aktivitě silic. Jednotlivé silice jsou seřazeny do tabulek a na základě hodnot MIC a MBC můžeme orientačně porovnávat jejich aktivitu. Velké množství silic vykazuje antibakteriální účinek proti různým původcům infekčních onemocnění. Studie se však zabývají hlavně aktivitou *in vitro*, a tak tyto přírodní sloučeniny, než dostanou své místo ve farmakoterapii, čeká ještě dlouhá cesta testování. Problémem není jen nedostatek informací o toxicitě, farmakodynamice a farmakokinetice, ale také značná nestabilita silice. Tím se zvyšují nároky na extrakci, skladování a uchovávání.

Silice v porovnání s dostupnými antibiotiky většinou vykazují nižší antibakteriální aktivitu, ale jelikož často mají odlišný mechanismus účinku, vykazují účinek i k rezistentním bakteriálním kmenům. Spousta studií prokazuje synergický efekt silice s běžně užívanými antibiotiky, což by mohlo vést ke snížení jejich spotřeby a zmenšení rychlosti nárůstu bakteriální rezistence.

Práce byla podpořena grantovým projektem IGA ČZU Praha, FŽP-20154247/2015.

Střet zájmů: žádný.

Literatura

1. **Hassan S. T. S., Masarčíková R., Berchová K.** Bioactive natural products with anti-herpes simplex virus properties. J. Pharm. Pharmacol. 2015; 67(10), 1325–1336.
2. **Pidot S. J., Coyne S., Kloss F., Hertweck C.** Antibiotics from neglected bacterial sources. Int. J. Med. Microbiol. 2014; 304(1), 14–22.
3. **Tomko J., a kol.** Farmakognózia. 2. vydanie. Martin: Osveta 1999.
4. **Nowak A., Kalemba D., Krala L., Piotrowska M., Czyzowska A.** The effects of thyme (Thymus vulgaris) and rosemary (Rosmarinus officinalis) essential oils on Brochothrix thermosphacta and on the shelf life of beef packaged in high-oxygen modified atmosphere. Food Microbiol. 2012; 32(1), 212–216.
5. **Votava M.** Lékařská mikrobiologie obecná. 1. vydání. Brno: Neptun 2001.
6. **Fadli M., Chevalier J., Saad A., Mezrioui N. E., Hassani L., et al.** Essential oils from Moroccan plants as potential chemosensitisers restoring antibiotic activity in resistant Gram-negative bacteria. Int. J. Antimicrob. Agents 2011; 38(4), 325–330.
7. **Kalemba D., Kunicka A.** Antibacterial and antifungal properties of essential oils. Curr Med. Chem. 2003; 10(10), 813–829.
8. **Warnke P. H., Becker S. T., Podschun R., Sivananthan S., Springer I. N., et al.** The battle against multi-resistant strains: renaissance of antimicrobial essential oils as a promising force to fight hospital-acquired infections. J. Craniomaxillofac. Surg. 2009; 37(7), 392–397.

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

9. **Haba E., Bouhdid S., Torrego-Solana N., Marqués A. M., Espuny M. J., et al.** Rhamnolipids as emulsifying agents for essential oil formulations: Antimicrobial effect against Candida albicans and methicillin-resistant Staphylococcus aureus. Int. J. Pharm. 2014; 476(1–2), 134–141.
10. **Polatoğlu K., Demirci B, Demirci F, Gören N, Başer K. H. C.** Biological activity and essential oil composition of two new Tanacetum chiliophyllum (Fisch. & Mey.) Schultz Bip. var. chiliophyllum chemotypes from Turkey. Ind. Crops Prod. 2012; 39, 97–105.
11. **Dung N. T., Kim J. M., Kang S. C.** Chemical composition, antimicrobial and antioxidant activities of the essential oil and the ethanol extract of Cleistocalyx operculatus (Roxb.) Merr and Perry buds. Food Chem. Toxicol. 2008; 46(12), 3632–3639.
12. **Tohidpour A, Sattari M, Omidbaigi R, Yadegar A, Nazemi J.** Antibacterial effect of essential oils from two medicinal plants against Methicillin-resistant Staphylococcus aureus (MRSA). Phytomedicine 2010; 17(2), 142–145.
13. **Thomsen N. A., Hammer K. A., Riley T. V., Van Belkum A, Carson C. F.** Effect of habituation to tea tree (Melaleuca alternifolia) oil on the subsequent susceptibility of Staphylococcus spp. to antimicrobials, triclosan, tea tree oil, terpinen-4-ol and carvacrol. Int J Antimicrob Agents. 2013; 41(4), 343–351.
14. **Moon T., Wilkinson J., Cavanagh H. M. A.** Antibacterial activity of essential oils, hydrosols and plant extracts from Australian grown Lavandula spp. Int. J. Aromather. 2006; 16(1), 9–14.
15. **Lang G., Buchbauer G.** A review on recent research results (2008–2010) on essential oils as antimicrobials and antifungals. A review. Flavour Fragr. J. 2012; 27: 13–39.
16. **Mahboubi M., Bidgoli F. G.** Antistaphylococcal activity of Zataria multiflora essential oil and its synergy with vancomycin. Phytomedicine 2010; 17(7), 548–550.
17. **Chang S. T., Chen P. F., Chang S. C.** Antibacterial activity of leaf essential oils and their constituents from Cinnamomum osmophloeum. J. Ethnopharmacol. 2001; 77(1), 123–127.
18. **Wu J. G., Peng W., Yi J., Wu Y. B., Chen T. Q.** Chemical composition, antimicrobial activity against Staphylococcus aureus and a pro-apoptotic effect in SGC-7901 of the essential oil from Toona sinensis (A. Juss.) Roem. leaves. J. Ethnopharmacol. 2014; 154(1), 198–205.
19. **Srisukha V., Tribuddhara C., Nukoolkarn V., Bunyapraphatsara N., Chokephaibulkit K., et al.** Antibacterial activity of essential oils from Citrus hystrix (makrut lime) against respiratory tract pathogens. Science Asia 2012; 38, 212–217.
20. **Djabou N., Lorenzi V., Guinoiseau E., Andreani S., Giuliani M. C., et al.** Phytochemical composition of Corsican Teucrium essential oils and antibacterial activity against foodborne or toxi-infectious pathogens. Food Control. 2013; 30(1), 354–363.
21. **Martins Mdo R., Arantes S., Candeias F., Tinoco M. T., Cruz-Morais J.** Antioxidant, antimicrobial and toxicological properties of Schinus molle L. essential oils. J. Ethnopharmacol. 2014; 151(1), 485–492.
22. **Sieniawska E., Los R., Baj T., Malm A., Glowniak K.** Antimicrobial efficacy of Mutellina purpurea essential oil and α-pinene against Staphylococcus epidermidis grown in planktonic and biofilm cultures. Ind. Crops Prod. 2013; 51, 152–157.
23. **Gao C., Tian C., Lu Y., Xu J., Luo J., et al.** Essential oil composition and antimicrobial activity of Sphallerocarpus gracilis seeds against selected food-related bacteria. Food Control. 2011: 22(3–4), 517–522.
24. **Sassi A. B., Harzallah-Skhirib F., Chraiefc I., Bourgougnond N., Hammami M., et al.** Chemical composition and antimicrobial activities of the essential oil of (Tunisian) Chrysanthemum trifurcatum (Desf.) Batt. and Trab. Flowerheads. Comptes Rendus Chimie 2008; 11(3), 324–330.
25. **Hernandez T., Canales M., Teran B., Avila O., Duran A., et al.** Antimicrobial activity of the essential oil and extracts of Cordia curassavica (Boraginaceae). J. Ethnopharmacol. 2007; 111(1), 137–141.
26. **Rather M. A., Dar B. A., Dar M. Y., Wani B. A., Shah W. A., et al.** Chemical composition, antioxidant and antibacterial activities of the leaf essential oil of Juglans regia L. and its constituents. Phytomedicine 2012; 19(13), 1185–1190.
27. **Espina L., Somolinos M., Lorán S., Conchello P., García D., et al.** Chemical composition of commercial citrus fruit essential oils and evaluation of their antimicrobial activity acting alone or in combined processes. Food control 2011; 22(6), 896–902.
28. **Verma R. S., Padaliaa R. C., Goswamia P., Vermab S. K., Chauhan A., et al.** Chemical composition and antibacterial activity of foliage and resin essential oils of Araucaria cunninghamii Aiton ex D.Don and Araucaria heterophylla (Salisb.) Franco from India. Ind. Crops Prod. 2014; 61, 410–416.
29. **Farjam M. H.** Comparative study of the antimicrobial activity of essential oil and two different extract from Salvia urmiensis Bunge. Asian Pac. J. Trop. Biomed. 2012; 2(3), 1680–1682.
30. **Cardile V., Russo A., Formisano C., Rigano D., Senatore F., et al.** Essential oils of Salvia bracteata and Salvia rubifolia from Lebanon: Chemical composition, antimicrobial activity and inhibitory effect on human melanoma cells. J. Ethnopharmacol. 2009; 126(2), 265–272.
31. **Lertsatitthanakorn P, Taweechaisupapongb S, Aromdeea C, Khunkittia W.** In vitro bioactivities of essential oils used for acne control. Int. J. Aromather. 2006; 16(1), 43–49.
32. **Scopel R, Falcão M. A., Lucas A. M., Almeida R. N., Gandolfi P. H. K., Cassel E., et al.** Supercritical fluid extraction from Syzygium aromaticum buds: Phase equilibrium, mathematical modeling and antimicrobial activity. J. Supercrit. Fluids 2014; 92, 223–230.
33. **van Vuuren S. F., Docrat Y., Kamatou G. P. P., Viljoen A. M.** Essential oil composition and antimicrobial interactions of understudied tea tree species. S. Afr. J. Bot. 2014: 92, 7–14.
34. **Padalia R. C., Vermaa R. S., Chauhana A., Goswamia P., Vermab S. K., et al.** Chemical composition of Melaleuca linarrifolia Sm. from India: a potential source of 1,8-cineole. Ind. Crops Prod. 2015; 63, 264–268.
35. **Chen X., Zhang Y., Zu Y., Fu Y., Wang W.** Composition and biological activities of the essential oil from Schisandra chinensis obtained by solvent-free microwave extraction. LWT-Food Science and Technology 2011; 44(10), 2047–2052.
36. **Santos G. K. N., Dutrab K. A., Barrosa R. A., da Cãmarab C. A. G., Lira D. D., et al.** Essential oils from Alpinia purpurata (Zingiberaceae): Chemical composition, oviposition deterrence, larvicidal and antibacterial activity. Ind. Crops Prod. 2012; 40, 254–260.
37. **Falsafi T., Moradi P., Mahboubi M., Rahimi E., Momtaz H., et al.** Chemical composition and anti-Helicobacter pylori effect of Satureja bachtiarica Bunge essential oil. Phytomedicine 2015; 22(1), 173–177.
38. **Pročke M.** Současný pohled na eradikaci Helicobacter pylori. Zdravi.e15.cz [online]. 2010, 5. http://zdravi.e15.cz/clanek/priloha-lekarske-listy/soucasny-pohled-na-eradikaci-helicobacter-pylori-450196
39. **Ozen F., Ekinci F. Y., Korachi M.** The inhibition of *Helicobacter pylori* infected cells by Origanum minutiflorum. Ind. Crops Prod. 2014; 58, 329–334.
40. **Basile A., Senatore F., Gargano R., Sorbo S., del Pezzo M., et al.** Antibacterial and antioxidant activities in *Sideritis italica* (Miller) Greuter et Burdet essential oils. J. Ethnopharmacol. 2006; 107(2), 240–248.
41. **Njume C., Afolayan A. J., Green E., Ndip R. N.** Volatile compounds in the stem bark of Sclerocarya birrea (Anacardiaceae) possess antimicrobial activity against drug-resistant strains of Helicobacter pylori. Int. J. Antimicrob. Agents 2011; 38(4): 319–324.
42. **Adeniyi B. A., Onwubuchea B. C., Anyiama F. M., Ekundayob O, Mahad G. B.** Anti-Helicobacter pylori activities of Eucalyptus grandis: Effects on susceptibility, urease activity and cell surface hydrophobicity. Pharm. Biol. 2009; 47(1), 13–17.
43. **Aleksic V., Knezevic P.** Antimicrobial and antioxidative activity of extracts and essential oils of Myrtus communis L. Microbiol. Res. 2014; 169(4), 240–254.
44. **Miguel G., Faleiro L., Cavaleiro C., Salgueiro L., Casanova J.** Susceptibility of Helicobacter pylori to essential oil of Dittrichia viscosa subsp. revoluta. Phytother. Res. 2008; 22(2), 259–263.

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 4.3.2016

45. **Rozza A. L., Moraes Tde M., Kushima H., Tanimoto A., Marques M. O., et al.** Gastroprotective mechanisms of Citrus lemon (Rutaceae) essential oil and its majority compounds limonene and β-pinene: involvement of heat-shock protein-70, vasoactive intestinal peptide, glutathione, sulfhydryl compounds, nitric oxide and prostaglandin E. Chem. Biol. Interact. 2011; 189(1–2), 82–89.
46. Zubní kaz – odborný článek. In: Nechcikazy.cz [online] 2012. http://www.nechcikazy.cz/zubni-kaz-odborny-clanek. (20.2.2015)
47. **Nikolić M., Jovanović K. K., Marković T., Marković D., Gligorijević N., et al.** Chemical composition, antimicrobial, and cytotoxic properties of five Lamiaceae essential oils. Ind. Crops Prod. 2014; 61, 225–232.
48. **Runyoro D., Ngassapa O., Vagionas K., Aligiannis N., Graikou K., et al.** Chemical composition and antimicrobial activity of the essential oils of four Ocimum species growing in Tanzania. Food Chem. 2010; 119(1), 311–316.
49. **Goldbeck J. C., Nascimento J. E. D., Jacob R. G., Fiorentini A. M., da Silva W. P.** Bioactivity of essential oils from Eucalyptus globulus and Eucalyptus urograndis against planktonic cells and biofilms of Streptococcus mutans. Ind. Crops Prod. 2014; 60, 304–309.
50. **Sousa R. M. F., de Morais S. A. L., Vieira R. B. K., Napolitano D. R., Napolitano V. B., et al.** Chemical composition, cytotoxic, and antibacterial activity of the essential oil from Eugenia calycina Cambess. leaves against oral bacteria. Ind. Crops Prod. 2015; 65, 71–78.
51. **Harzallah H. J., Kouidhi B., Flamini G., Bakhrouf A., Mahjoub T.** Chemical composition, antimicrobial potential against cariogenic bacteria and cytotoxic activity of Tunisian Nigella sativa essential oil and thymoquinone. Food Chem. 2011; 129(4), 1469–1474.
52. **Brusotti G., Cesari I., Gilardoni G., Tosi S., Grisoli P., et al.** Chemical composition and antimicrobial activity of Phyllanthus muellerianus (Kuntze) Excel essential oil. J Ethnopharmacol. 2012; 142(3), 657–662.
53. **Oliveira D. R., Leitão G. G., Bizzo H. R., Lopes D, Alviano D. S., et al.** Chemical and antimicrobial analyses of essential oil of Lippia origanoides H.B.K. Food Chem. 2007; 101(1), 236–240.
54. **Potrepčiaková S., a kol.** Tuberkulóza. Practicus.eu [online] 2008, 4. http://web.practicus.eu/Documents/Practicus-04-2008/24-tuberkuloza.pdf (20. 2. 2015).
55. **Lall N., Meyer J. J.** In vitro inhibition of drug-resistant and drug-sensitive strains of Mycobacterium tuberculosis by ethnobotanically selected South African plants. J. Ethnopharmacol. 1999; 66(3), 347–354.
56. **Sartoratto A., Machado A. L. M., Delarmelina C., Figueir G. M., Duarte M. C. T., et al.** Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. Braz. J. Microbiol. 2004; 35, 275–280.
57. **Fadli M., Saad A., Sayadi S., Chevalier J., Mezrioui N. E., et al.** Antibacterial activity of Thymus maroccanus and Thymus broussonetii essential oils against nosocomial infection-bacteria and their synergistic potential with antibiotics. Phytomedicine 2012; 19(5), 464–471.
58. **Hemaiswarya S., Kruthiventi A. K., Doble M.** Synergism between natural products and antibiotics against infectious diseases. Phytomedicine 2008; 15(8), 639–652.
59. **Rosato A., Vitali C., de Laurentis N., Armenise D., Antonietta Milillo M.** Antibacterial effect of some essential oils administered alone or in combination with Norfloxacin. Phytomedicine 2007; 14(11), 727–732.
60. **Djouahri A., Saka B., Boudarene L., Benseradj F., Aberrane S., et al.** In vitro synergistic/antagonistic antibacterial and anti-inflammatory effect of various extracts/essential oil from cones of Tetraclinis articulata (Vahl) Masters with antibiotic and anti-inflammatory agents. Ind. Crops. Prod. 2014; 56, 60–66.
61. **Barreto H. M. de Lima I. S, Coelho K. M. R. N., Osório L. R., Mourčo R. D. A., et al**. Effect of Lippia origanoides H.B.K. essential oil in the resistance to aminoglycosides in methicillin resistant Staphylococcus aureus. Eur. J. Integr. Med. 2014; 6(5), 560–564.

NOVÉ KNIHY

Lukáč M., Devínsky F. **Organická syntéza, Laboratórny manuál.** Bratislava: Univerzita Komenského 2015, 144 s. ISBN 978-80-223-3913-1

Recenzované dielo je učebnou pomôckou na výučbu organickej chémie pre študentov farmácie a obsahuje sedem kapitol.

V prvej kapitole autori popisujú prácu v chemickom laboratóriu, v druhej chemickú literatúru, v tretej izolačné techniky, v štvrtej sa zaoberajú identifikáciou a charakterizáciou vlastností organických zlúčenín, piatu tvorí preparatívna časť, šiesta je venovaná príprave bezvodých rozpúštadiel a siedma problematike chladiacich zmesí. Celkove je v ňom popísaná príprava 231 zlúčenín.

Analýza obsahu ukazuje, že dielo je napísané nielen logicky, stručne a jasné, ale keďže sa zaoberá prípravou organických zlúčenín potrebných pre prípravu liečiv, ktoré majú presné návody na ich prípravu, je napísané tak, že text spĺňa hlavne kritérium spočívajúce v tom, že jednotlivé návody sa dajú reprodukovať.

Cennou devízou tohto textu je ďalej, že autori v plnej miere dodržali kritéria kladené na názvoslovie organických zlúčenín a ich klasifikáciu, definície jednotlivých fyzikálno-chemických vlastností, analytických metód potrebných na objasnenie a potvrdenie ich chemickej štruktúry. Veľkým kladom je skutočnosť, že autori pri popise niektorých syntéz použili viaceré moderné katalyzátory, separačné techniky a všetky bežné identifikačné metódy.

I keď je dielom primárne určené pre výučbu na farmaceutických fakultách, som pevne presvedčený, že je ho možné využiť i na prírodovedeckých a chemických fakultách.

J. Čižmárik

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 10.6.2016

REVIEW ARTICLE

Antimicrobial, antiparasitic and anticancer properties of *Hibiscus sabdariffa* (L.) and its phytochemicals: *in vitro* and in *vivo* studies

Antimikrobiální, protiparazitické a protinádorové vlastnosti *Hibiscus sabdariffa* (L.) a jeho sloučeniny: *in vitro* a *in vivo* studie

Sherif T. S. Hassan • Kateřina Berchová • Miroslava Šudomová

Received 25 November 2015 / Accepted 16 December 2015

Summary

In the last few decades, *Hibiscus sabdariffa* L. (Malvaceae; *H. sabdariffa*) has gained much attention in research field because of its potentially useful bioactivity as well as a great safety and tolerability. For decades, microbial, parasitic and cancer diseases remain a serious threat to human health and animals as well. To treat such diseases, a search for new sources such as plants that provide various bioactive compounds useful in the treatment of several physiological conditions is urgently needed, since most of the drugs currently used in the therapy have several undesirable side effects, toxicity, and drug resistance. In this paper, we aim to present an updated overview of *in vitro* and *in vivo* studies that show the significant therapeutic properties of the crude extracts and phytochemicals derived from *H. sabdariffa* as antimicrobial, antiparasitic, and anticancer agents. The future directions of the use of *H. sabdariffa* in clinical trials will be discussed.
Key words: *Hibiscus sabdariffa* L. • antimicrobial agents • cancer preventive agents • antiparasitic drugs • natural products

Souhrn

V posledních několika desetiletích si získal *Hibiscus sabdariffa* L. (Malvaceae, *H. sabdariffa*) velkou pozornost v oblasti výzkumu kvůli svému potenciálu biologické aktivity stejně jako pro svou velkou bezpečnost a snášenlivost. Po celá desetiletí zůstávají mikrobiální, parazitární a rakovinotvorná onemocnění vážnou hrozbou pro lidi a zvířata. K léčbě těchto chorob je velmi nutné naléhavě hledat nové zdroje – rostliny, které poskytují různé biologicky aktivní sloučeniny použitelné při léčbě některých onemocnění, protože v současné době většina léků používaných při léčbě má několik nežádoucí vedlejší účinků, toxicitu a rezistenci. V tomto článku se snažíme představit aktualizovaný přehled o *in vitro* a *in vivo* studiích, které ukazují významné léčebné vlastnosti surových extraktů a fytochemikálií – odvozené z *H. sabdariffa* jako antimikrobiální, protiparazitické a proti - nádorové látky. Možnosti využití a zkoumání *H. sabdariffa* v klinických studiích budou teprve diskutovány.
Klíčová slova: *Hibiscus sabdariffa* L. • antimikrobiální látky • protinádorové látky • protiparazitické látky • přírodní látky

Ing. Sherif T. S. Hassan (✉)
Department of Natural Drugs, Faculty of Pharmacy
University of Veterinary and Pharmaceutical Sciences
Palackého tř. 1946/1, 612 42 Brno, Czech Republic
e-mail: sherif.hassan@seznam.cz

S. T. S. Hassan • K. Berchová
Department of Applied Ecology, Faculty of Environmental Sciences
Czech University of Life Sciences, Prague, Czech Republic

M. Šudomová
Department of Archeology and Museology, Faculty of Arts
Masaryk University, Brno, Czech Republic

Introduction

Hibiscus sabdariffa L. (Malvaceae; *H. sabdariffa*) is a medicinal plant which has a long history of herbal and edible uses across the world and is mainly cultivated in tropical and subtropical regions of Africa and Asia[1–3]. It is an annual or perennial plant or woody-based shrub with serrate leaves, red calyces and red stems[3, 4]. The phytochemical and pharmacological activities of various parts of *H. sabdariffa* have been evaluated including antioxidant, antidiabetic, anti-inflammatory, antimicrobial, and anticancer properties[5–7]. Nowadays, cancer, microbial, and parasitic diseases have become a global concern worldwide, since these diseases have threatened

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 10.6.2016

human and animal health[8–12]. This review aims to provide a brief overview of the *in vitro* and *in vivo* studies that present the therapeutic potential of *H. sabdariffa* extract (HSE) and its bioactive substances in the treatment of cancer, bacterial, fungal, and parasitic diseases.

Phytochemical profile

H. sabdariffa has a long tradition as it contains a rich bioactive profile responsible for its therapeutic efficacy such as anthocyanins, flavonoids, polysaccharides, and organic acids including malic, ascorbic, hydroxycitric, and *Hibiscus* acids[13–15]. Furthermore, HSE is rich in minerals such as iron and calcium with a low content of glucose, and 18 volatile compounds were identified via GC and GC-MS analyses[16]. It has been reported that *H. sabdariffa* seeds contain a large amount of polyunsaturated fatty acids, tocopherol, and the major fatty acids of seeds were found to be oleic 37.92%, linoleic 35.01% and palmitic 19.65% acids[16, 17]. Another studies have explored that 1g of aqueous extract of *H. sabdariffa* contains anthocyanins (56.5 mg/g delphinidin-3-*O*-sambubioside and 20.8 mg/g cyanidin--3-*O*-sambubioside), 3.2 mg/g quercetin, 2.1 mg/g rutin and 2.7 mg/g chlorogenic acid, while ethanol was revealed as the best solvent for the extraction of anthocyanins (ranged from 17.3 to 32.2 mg of cyanidin--3-glucoside/g dry weight in the pigmented varieties)[18, 19].

Antimicrobial and antiparasitic properties

Numerous studies have described the potential use of HSE and its phytochemicals as significant antimicrobial and antiparasitic agents in the treatment of various infections.

Antibacterial activities

Recently, Alshami and Alharbi[20] explored the effective potential of HSE to prevent recurrent urinary tract infections (UTIs). HSE was found to exhibit bacteriostatic effect with potent inhibition of the growth of six *Escherichia coli* (*E. coli*) and two *Klebsiella pneumoniae* isolates (collected from patients with recurrent UTIs) and remarkable inhibition of biofilm production of all isolates. MIC (Minimum Inhibitory Concentration) values ranged from 0.5 to 4 mg/mL, and MBC (Minimum Bactericidal Concentration) ranged from 8 to 64 mg/mL. The effectiveness of aqueous extracts of *H. sabdariffa* were investigated for antimicrobial activity against *E. coli* and *Staphylococcus aureus* (*S. aureus*) strains in a microbiological medium and ultrahigh-temperature-processed milk with various fat percentages. The results showed that extracts treated by heat revealed higher antimicrobial activity than in microbiological medium[21]. A methanol extract of *H. sabdariffa* was found to inhibit effectively the growth of *E. coli* O157:H7 (at a concentration of 10%) isolates from food, veterinary, and clinical samples, as determined by disk diffusion method[22]. Liu et al. reported that aqueous extract of *H. sabdariffa* and *H. sabdariffa* protocatechuic acid (PCA) at a concentration of 5 mg/mL inhibited notably the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. In addition, PCA (in a dose-dependent manner) exerted higher antibacterial activity against tested pathogens in broth than in human plasma. Moreover, the antibacterial effect was independent from temperature, when treated by a heat[23]. The antibacterial effects of aqueous and ethanol extracts, and PCA of *H. sabdariffa* were examined against food spoilage bacteria *Bacillus cereus*, *Salmonella typhimurium* DT104, *E. coli* O157:H7, *Listeria monocytogenes*, and *S. aureus*. MICs of aqueous, ethanol extracts, and PCA against tested bacteria were in the range of 112–144, 72–96, and 24–44 µg/mL, respectively. The results revealed that ethanol extract exhibited greater antibacterial effects than aqueous extract[24]. Jung et al.[16] evaluated the antimicrobial properties of aqueous and ethanol extracts of *H. sabdariffa* against *Bacillus subtilis*, *S. aureus*, and *E. coli*. The results showed that ethanol extract against *Bacillus subtilis* and *S. aureus* explored higher activity than that of aqueous extract, while aqueous extract at concentrations of 25 and 50 mg/mL inhibited potently the growth of *E. coli* via the paper disc method. Olaleye[25] investigated *in vitro* the inhibitory effect of aqueous-methanol extract of dried *H. sabdariffa* calyx against nine bacterial pathogens such as *Clostridium sporogenes*, *S. aureus*, *Bacillus stearothermophilus*, *Micrococcus luteus*, *Serratia mascences*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, and *Bacillus cereus*. The inhibitory effect was observed potently against all tested pathogens. Several studies have also demonstrated that HSE exerted antibacterial activity against bacteria causing oral cavity infections such as. *Streptococcus mutans* with MIC value of 2.5 mg/mL, while at concentrations ranging from 96–152 µg/mL exhibited also significant inhibitory activity against *Campylobacter coli* and *Campylobacter fetus*, which contaminate beef, pork, and chicken meat[26, 27]. The crude extracts of *H. sabdariffa* seeds were tested for inhibitory activity against three types of Gram-negative bacteria species *Enterobacter*, *Salmonella*, and *Shigella*. The higher antibacterial activity was observed against *Salmonella* sp. at a concentration of 200 mg/L[28].

Antifungal activities

The anticandidal activity of methanol extract of *H. sabdariffa* fruit (at a concentration of 10 mg/mL) was evaluated against six pathogenic *Candida* species such as *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*. The inhibitory activity was detected only against *C. albicans*[29]. El-Nagerabi et al.[30] examined the inhibitory effect of aqueous extract of *H. sabdariffa* calyx at concentrations of 5, 7.5, 10 and 12.5 g/100 mL on the growth and aflatoxin B1 production by two fungal strains *Aspergillus parasiticus* (CBS 921.7) and *Aspergillus flavus* (SQU 21). The results indicated that no inhibitory effect was observed on the growth of both fungal strains, while the inhibition of aflatoxin B1 production by the different concentrations of *H. sabdariffa* calyx ranged between 91.5–97.9% and 87.1–93.3% for *A. flavus* and *A. parasiticus* strains, respectively. In addition, the study confirmed the metabolic effect of aqueous extract of *H. sabdariffa* calyx

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 10.6.2016

on aflatoxin biosynthesis pathway of both *Aspergillus* species, and a beneficial use in food industry as an effective biocontrol and non-toxic biopreservative agent.

Antiparasitic activities

Human lymphatic filariasis, a vector-borne disease, is distributed in tropical, subtropical regions, causing a public health problem. Saxena et al.[31] determined antifilarial activity of ethanolic extract of *H. sabdariffa* leaves by *in vitro* motility and MTT methods. The results showed that the extract affected both the adult worms and microfilariae of *Brugia malayi*. The butanol fraction exhibited remarkable inhibitory effect, which was related to anthocyanin-glycosides. Animal trypanosomosis, a parasitic disease is still the main factor of decreasing the growth of livestock in Africa. Umar and colleagues[32] investigated *in vivo* the effect of aqueous extracts of *H. sabdariffa* calyces on the heamatological profile and organ pathological changes in *Trypanasoma congolense*-infected rats. The results showed that consumption of the extract (9.94 mg/100g/day) enhanced the pathological changes in blood and organs of *T. congolense*-infected rats.

Anticancer activity

The crude extracts and isolated substances from *H. sabdariffa* were found to be potential cancer chemopreventive agents. For instance, *Hibiscus* anthocyanins (HAs) exhibited an ability to promote cancer cell apoptosis, particularly in gastric cancer and leukemia, while PCA was found to suppress the carcinogenic action of various substances in different tissues of rat models *in vivo*[33, 34]. Tsai and co-workers[35] have recently evaluated the protective effect of HAs on N-nitrosomethylurea (NMU)-induced leukemia of rats *in vivo*. The results indicated that oral administration of HAs (0.2%) significantly suppressed progression of NMU--induced leukemia by approximately 33.3% in rats. Several studies have also evaluated the effectiveness of HAs including delphinidin-3-sambubioside on human leukemia cells. Interestingly, HAs effectively induced apoptotic cell death in human promyelocytic leukemia cells via the p38-FasL and Bid pathway, and ROS--mediated the mitochondrial dysfunction pathway[36, 37]. Lo and colleagues[38] also reported that HAs induced apoptosis of the proliferating smooth muscle cell via activation of P38 MAPK and p53 pathway. PCA, a phenolic acid was found to exert *in vitro* protective effects against cytotoxicity and genotoxicity of hepatocytes induced by tert-butylhydroperoxide (t-BHP). Mechanism of PCA's protective effect may be related to its ability to inhibit DNA repair synthesis caused by t-BHP and by scavenging free radicals as well[39]. Tseng et al.[40, 41] presented in two studies that PCA exhibited remarkable inhibition of 12-*O*-tetradecanolyphorbol-13--acetate (TPA)-induced skin tumor formation in female CD1-mice and the survival of human promyelocytic leukemia HL-60 cells. In their studies, they revealed that the mechanism by which PCA utilized anticancer activities is due to its ability to induce antitumor activities through DNA fragmentation, GI arrest, apoptosis, and decreasing reactive oxygen species (ROS). The apoptosis-inducing activity was implicated with the phosphorylation and degradation of RB and the suppression of Bcl-2 protein. Lin and colleagues[42] studied the apoptotic effect of PCA on human gastric carcinoma (AGS) cells. The results suggested that the apoptotic effect may be mediated via p53 signaling and p38 MAPK/FasL cascade pathway. Olvera-Garcia and co-workers[43] reported that PCA inhibited the mutagenicity of 1-nitropyrene and checked the proliferation of HeLa cells, both in a dose-response manner in human stomach adenocarcinoma AGS cells. In addition, the effect of *Hibiscus sabdariffa* extract induced cytotoxicity and apoptosis of the cancer cells in dose-dependent manner through JNK/p38 signaling cascade-mediated apoptosis. Saeed at al.[44] reported that ethanolic extract of *H. sabdariffa* exerted moderate proliferative activity in cell-culture using estrogen-responsive breast cancer cell lines (MCF-7). Moreover, the results revealed that *H. sabdariffa* extract was found to be the richest in quercetin and daidzein as phytoestrogens among the other tested plants used in the study. Lin et al.[45] studied the anticancer properties of *H. sabdariffa* leaf extracts against various human prostate cancer (CaP) cells *in vitro* and *in vivo*. The study explored that anti-apoptotic activity was mediated via both intrinsic (Bax/cytochrome c-mediated caspase 9) and extrinsic (Fas-mediated caspase 8/t-Bid) pathways and by inhibiting the growth of prostate tumor xenograft in athymic nude mice as well. The results suggested that leaf extracts contained higher amounts of polyphenolic compounds than extracts from calyces and hence, *Hibiscus* polyphenolic compounds provide effective anticancer agents. Chiu and co-workers[46] evaluated *in vitro* anticancer activity of *Hibiscus* leaf polyphenolic (HLP) extract in melanoma cells. It has been found that HLP is rich in epicatechin gallate (ECG) and other polyphenols. The results explored that anticancer effect of HLP was associated with ECG by inducing the caspases cleavages, Bcl-2 family proteins regulation, and Fas/FasL activation in A375 cells. The apoptotic activity was determined by DAPI stain, cell-cycle analysis, and acidic vascular organelle (AVO) stain. Eventually, the study suggested that HLP could be a potential antimelanoma agent.

Conclusion and future directions

In summary, *H. sabdariffa* exerted various beneficial activities with no remarkable genotoxic effects as well as great tolerability. *Hibiscus sabdariffa* was found to be a great target as a source of many chemotherapeutic agents useful in food industry and drug discovery development. The most bioactive compounds in HSE that have been found to have significant therapeutic properties against microbial, parasitic and cancer diseases were PCA, HAs, and polyphenols. In this review, we summarized exclusively the potential use of HSE and its phytochemicals in the treatment of the most serious diseases which affect human and animal health such as cancer, bacterial, fungal, and parasitic diseases. *H. sabdariffa* has been examined both in *in vitro* and *in vivo* studies but more robust, randomized, and controlled

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 10.6.2016

clinical trials with well-characterized HSE preparations would be needed in future research to confirm the therapeutic potential.

Conflict of interest: none.

This study was funded by Internal Grant Agency (IGA) of the Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Czech Republic. Project No. 20154247/2015.

References

1. **de Arruda A., Cardoso C. A., Vieira M. D., Arena A. C.** Safety assessment of Hibiscus sabdariffa after maternal exposure on male reproductive parameters in rats. Drug Chem Toxicol. 2015; 16, 1–6.
2. **Wang J., Cao X., Jiang H., Qi Y., Chin K. L., Yue Y.** Antioxidant activity of leaf extracts from different Hibiscus sabdariffa accessions and simultaneous determination five major antioxidant compounds by LC-Q-TOF-MS. Molecules 2014; 19(12), 21226–21238.
3. **Nyam K. L., Leao S. Y., Tan C. P., Long K.** Functional properties of roselle (Hibiscus sabdariffa L.) seed and its application as bakery product. J Food Sci Technol. 2014; 51(12), 3830–3837.
4. **Ramírez-Martínez D., Alvarado-Méndez E., Trejo-Durán M., Vázquez-Guevara M. A.** Nonlocal nonlinear refraction in Hibiscus Sabdariffa with large phase shifts. Opt Express 2014; 22(21), 25161–25170.
5. **Mihaljev Z., Zivkov-Balos M., Cupić Z., Jaksić S.** Levels of some microelements and essential heavy metals in herbal teas in Serbia. Acta Pol Pharm. 2014; 71(3), 385–391.
6. **Pérez-Ramírez I. F., Castaño-Tostado E., Ramírez-de León J. A., Rocha-Guzmán N. E., Reynoso-Camacho R.** Effect of stevia and citric acid on the stability of phenolic compounds and in vitro antioxidant and antidiabetic capacity of a roselle (Hibiscus sabdariffa L.) beverage. Food Chem. 2015; 172, 885–892.
7. **Ademiluyi A. O., Oboh G.** Aqueous extracts of Roselle (Hibiscus sabdariffa Linn.) varieties inhibit -amylase and -glucosidase activities in vitro. J Med Food. 2013; 16(1), 88–93.
8. **Chen Z., Lu W.** Roles of Ubiquitination and SUMOylation on Prostate Cancer: Mechanisms and Clinical Implications. Int J Mol Sci. 2015; 16(3), 4560–4580.
9. **Hassan S. T. S., Masarčíková R., Berchová K.** Bioactive natural products with anti-herpes simplex virus properties. J Pharm Pharmacol. 2015; 67(10), 1325–1336.
10. **Giannini G., Battistuzzi G., Vignola D.** Hydroxamic acid based histone deacetylase inhibitors with confirmed activity against the malaria parasite. Bioorg Med Chem Lett. 2015; 25(3), 459–461.
11. **Da-Costa-Rocha I., Bonnlaender B., Sievers H., Pischel I., Heinrich M.** Hibiscus sabdariffa L. – a phytochemical and pharmacological review. Food Chem. 2014; 165, 424–443.
12. **Sogo T., Terahara N., Hisanaga A., Kumamoto T., Yamashiro T., Wu S., Sakao K., Hou D. X.** Anti-inflammatory activity and molecular mechanism of delphinidin 3-sambubioside, a Hibiscus anthocyanin. Biofactors 2015; 41(1), 58–65.
13. **Gurrola-Díaz C. M., García-López P. M., Sánchez-Enríquez S., Troyo-Sanromán R., Andrade-González I., Gómez-Leyva J. F.** Effects of Hibiscus sabdariffa extract powder and preventive treatment (diet) on the lipid profiles of patients with metabolic syndrome (MeSy). Phytomedicine 2010; 17(7), 500–505.
14. **Pérez-Torres I., Ruiz-Ramírez A., Baños G., El-Hafidi M.** Hibiscus sabdariffa Linnaeus (Malvaceae), curcumin and resveratrol as alternative medicinal agents against metabolic syndrome. Cardiovasc Hematol Agents Med Chem. 2013; 11(1), 25–37.
15. **Mohamed R., Fernández J., Pineda M., Aguilar M.** Roselle (Hibiscus sabdariffa) seed oil is a rich source of gamma-tocopherol. J Food Sci. 2007; 72(3), S207–211.
16. **Jung E., Kim Y., Joo N.** Physicochemical properties and antimicrobial activity of Roselle (Hibiscus sabdariffa L.). J Sci Food Agric. 2013; 93(15), 3769–3776.
17. **Akinoso R., Suleiman A.** Heat treatment effects on extraction of roselle (Hibiscus sabdariffa L) seed oil. Eur J Lipid Sci Technol. 2011; 113, 1527–1532.
18. **Alarcón-Alonso J., Zamilpa A., Aguilar F. A., Herrera-Ruiz M., Tortoriello J., Jimenez-Ferrer E.** Pharmacological characterization of the diuretic effect of Hibiscus sabdariffa Linn (Malvaceae) extract. J Ethnopharmacol. 2012; 139(3), 751–756.
19. **Camelo-Méndez G. A., Ragazzo-Sánchez J. A., Jiménez-Aparicio A. R., Vanegas-Espinoza P. E., Paredes-López O., Del Villar-Martínez A. A.** Comparative study of anthocyanin and volatile compounds content of four varieties of Mexican roselle (Hibiscus sabdariffa L.) by multivariable analysis. Plant Foods Hum Nutr. 2013; 68(3), 229–234.
20. **Alshami I., Alharbi A. E.** Antimicrobial activity of Hibiscus sabdariffa extract against uropathogenic strains isolated from recurrent urinary tract infections. Asian Pac J Trop Dis. 2014; 4(4), 317–322.
21. **Higginbotham K. L., Burris K. P., Zivanovic S., Davidson P. M., Stewart C. N. Jr.** Antimicrobial activity of Hibiscus sabdariffa aqueous extracts against Escherichia coli O157:H7 and Staphylococcus aureus in a microbiological medium and milk of various fat concentrations. J Food Prot. 2014; 77(2), 262–268.
22. **Fullerton M., Khatiwada J., Johnson J. U., Davis S, Williams L. L.** Determination of antimicrobial activity of sorrel (Hibiscus sabdariffa) on Escherichia coli O157:H7 isolated from food, veterinary, and clinical samples. J Med Food 2011; 14(9), 950–956.
23. **Liu K. S., Tsao S. M., Yin M. C.** In vitro antibacterial activity of roselle calyx and protocatechuic acid. Phytother Res. 2005; 19(11), 942–945.
24. **Chao C. Y., Yin M. C.** Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Pathog Dis. 2009; 6(2), 201–206.
25. **Olaleye M. T.** Cytotoxicity and antibacterial activity of Methanolic extract of Hibiscus sabdariffa. J. Med. Plants Res. 2007; 1(1), 9–13.
26. **Afolabi O. C., Ogunsola F. T., Coker A. O.** Susceptibility of cariogenic Streptococcus mutans to extracts of Garcinia kola, Hibiscus sabdariffa, and Solanum americanum. West Afr J Med. 2008; 27(4), 230–233.
27. **Yin M. C., Chao C. Y.** Anti-Campylobacter, anti-aerobic, and anti-oxidative effects of roselle calyx extract and protocatechuic acid in ground beef. Int J Food Microbiol. 2008; 127(1–2), 73–77.
28. **Nwaiwu N. E., Mshelia F, Raufu I. A.** Antimicrobial activities of crude extract of Moringa Oleifera, Hibiscus sabdariffa and Hibiscus esculentus seeds against some enterobacteria. J. Appl. Phytotechnol. Environ. Sanit. 2012; 1(1), 11–16.
29. **Rukayadi Y., Shim J. S., Hwang J. K.** Screening of Thai medicinal plants for anticandidal activity. Mycoses 2008; 51(4), 308–312.
30. **El-Nagerabi S. A. F., Al-Bahry S. N., Elshafie A. E., AlHilali S.** Effect of Hibiscus sabdariffa extract and Nigella sativa oil on the growth and aflatoxin B_1 production of Aspergillus flavus and Aspergillus parasiticus strains. Food Control 2012; 25, 59–63.
31. **Saxena K., Dube V., Kushwaha V., Gupta V., Lakshmi M., Mishra S., et al.** Antifilarial efficacy of Hibiscus sabdariffa on lymphatic filarial parasite Brugia malayi. MedChem Res. 2011; 20, 1594–1602.
32. **Umar I. A., Maryoms N. G., Daikwo E., Gidado A., Buratai L. B., Igbokwe I. O., Ibrahim M. A.** The effect of aqueous extracts of Hibiscus sabdariffa (Sorrel) calyces on heamatological profile and organ pathological changes in Trypanasoma congolense – infected rats. Afr J Tradit Complement Altern Med. 2009; 6(4), 585–591.
33. **Patel S.** Hibiscus sabdariffa: An ideal yet under-exploited candidate for nutraceutical applications. Biomedicine & Preventive Nutrition 2014; 4, 23–27.
34. **Kakkar S., Bais S.** A review on protocatechuic Acid and its pharmacological potential. ISRN Pharmacol. 2014; 2014, 952943.
35. **Tsai T. C., Huang H. P., Chang Y. C., Wang C. J.** An anthocyanin-rich extract from Hibiscus sabdariffa linnaeus inhibits N-nitrosomethylurea-induced leukemia in rats. J Agric Food Chem. 2014; 62(7), 1572–1580.

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni

www.prolekare.cz | stazeno: 10.6.2016

36. **Chang Y. C., Huang H.P., Hsu J. D., Yang S. F., Wang C. J.** Hibiscus anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cells. Toxicol Appl Pharmacol. 2005; 205(3), 201–212.
37. **Hou D. X., Tong X., Terahara N., Luo D., Fujii M.** Delphinidin 3-sambubioside, a Hibiscus anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway. Arch Biochem Biophys. 2005; 440(1), 101–109.
38. **Lo C. W., Huang H. P., Lin H. M., Chien C. T., Wang C. J.** Effect of Hibiscus anthocyanins-rich extract induces apoptosis of proliferating smooth muscle cell via activation of P38 MAPK and p53 pathway. Mol Nutr Food Res. 2007; 51(12), 1452–1460.
39. **Tseng T. H., Wang C. J., Kao E. S., Chu H. Y.** Hibiscus protocatechuic acid protects against oxidative damage induced by tert-butylhydroperoxide in rat primary hepatocytes. Chem Biol Interact. 1996; 101(2), 137–148.
40. **Tseng T. H., Hsu J. D., Lo M. H., Chu C. Y., Chou F. P., Huang C. L., Wang C. J.** Inhibitory effect of Hibiscus protocatechuic acid on tumor promotion in mouse skin. Cancer Lett. 1998; 126(2), 199–207.
41. **Tseng T. H., Kao T. W., Chu C. Y., Chou F. P., Lin W. L., Wang C. J.** Induction of apoptosis by hibiscus protocatechuic acid in human leukemia cells via reduction of retinoblastoma (RB) phosphorylation and Bcl-2 expression. Biochem Pharmacol. 2000; 60(3), 307–315.
42. **Lin H. H., Huang H. P., Huang C. C., Chen J. H., Wang C. J.** Hibiscus polyphenol-rich extract induces apoptosis in human gastric carcinoma cells via p53 phosphorylation and p38 MAPK/FasL cascade pathway. Mol Carcinog. 2005; 43(2), 86–99.
43. **Olvera-García V., Castaño-Tostado E., Rezendiz-Lopez R. I., Reynoso-Camacho R., González de Mejía E., Elizondo G., Loarca-Piña G.** Hibiscus sabdariffa L. extracts inhibit the mutagenicity in microsuspension assay and the proliferation of HeLa cells. J Food Sci. 2008; 73(5), T75–81.
44. **Saeed I. A., Ali L., Jabeen A., Khasawneh M., Rizvi T. A., Ashraf S. S.** Estrogenic activities of ten medicinal herbs from the Middle East. J Chromatogr Sci. 2013; 51(1), 33–39.
45. **Lin H. H., Chan K. C., Sheu J. Y., Hsuan S. W., Wang C. J., Cheng J. H.** Hibiscus sabdariffa leaf induces apoptosis of human prostate cancer cells in vitro and in vivo. Food Chemistry 2012; 132(2), 880–891.
46. **Chiu C. T., Hsuan S. W., Lin H. H., Hsu C. C., Chou F. P., Chen J. H.** Hibiscus sabdariffa Leaf Polyphenolic Extract Induces Human Melanoma Cell Death, Apoptosis, and Autophagy. J Food Sci. 2015. doi:10.1111/1750-3841.12790.

Tento clanek podleha autorskemu zakonu a jeho vyuziti je mozne v souladu s pravnim prohlasenim: www.prolekare.cz/prohlaseni