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BIOLOGICAL ACTIVITY OF SELECTED NATURAL PHENOLS

DIPLOMA THESIS

Brno 2010 Zuzana Hanáková

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ÚSTAV PŘÍRODNÍCH LÉČIV

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DIPLOMOVÁ PRÁCE

Brno 2010 Zuzana Hanáková

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Abstract

36 phenolic compounds isolated from four plants used in folk medicine (*Paulownia tomentosa*, *Morus alba*, *Maclura pomifera*, *Dracaena cinnabari*) were tested for activity against trypanosomas and four bacteria strains (*Nocardia farcinica*, *Mycobacterium marinum*, *Staphylococcus aureus*, *Escherichia coli*). MICs were determined for compounds defined as active. Additionaly, cytotoxicity of phenolic compounds was determined using prostatic carcinoma cells and normal prostatic cell. Most of the tested compounds were active, some of them had MIC comparable to a positive control. The influence of molecule structures is discussed. These assays confirm the use of tested plants in traditional medicine and indicate the high potential for these compounds as antimicrobial agents.

Abstrakt

36 fenolických látek izolovaných ze čtyř rostlin používaných v lidové medicíně (*Paulownia tomentosa*, *Morus alba*, *Maclura pomifera*, *Dracaena cinnabari*) bylo testováno na aktivitu proti trypanosomám a čtyřem kmenům bakterií (*Nocardia farcinica*, *Mycobacterium marinum*, *Staphylococcus aureus*, *Escherichia coli*). U látek, které byly vyhodnoceny jako aktivní, byla dále stanovena jejich MIC. Dále byla testována cytotoxicita fenolických sloučenin za použití buněk karcinomu prostaty a běžných buněk prostaty. Většina látek byla aktivní, některé se v hodnotě MIC přiblížily pozitivní kontrole. Diskutován je i vliv molekulové struktury na aktivitu. Tyto zkoušky potvrzují použití testovaných látek v tradiční medicíně a naznačují vysoký potenciál jejich využití jako antimikrobních látek.

Key words

Natural phenols, Prenylated flavonoids, Antimicrobial activity, Antiparasitic activity, Antibacterial activity, Cytotoxicity

Klíčová slova

Přírodní fenoly, Prenylované flavonoidy, Antimikrobiální aktivita, Antiparazitární aktivita, Antibakteriální aktivita, Cytotoxicita

Proclamation

I declare that I have worked out this diploma thesis myself using only the literature stated.

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Acknowledgement

It is a pleasure to acknowledge the generous help and valuable advices of my supervisor PharmDr. Karel Šmejkal, Ph.D. (*Department of Natural Drugs*, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Brno, CR). This project was developed thanks to Freemovers UVPS Brno programme in cooperation with University of Strathclyde, Glasgow, UK. The whole work wouldn't been realized without great benevolence, admirable help and support of Carol Clements, Dr Alexander Gray, and Dr John Igoli from *Strathclyde Institute of Pharmacy and Biomedical Sciences*. Finally, I wish to thank to my family and to my friends for supporting me during my studies.

CONTENTS

1 INTRODUCTION

The usage of herbs in medicine can be dated many years backwards. In 1991, 5,300 years old frozen human body was found in Italian Alps. He carried a pouch with a fungus called *Piptoporus betulinus*, which possesses a laxative and antimicrobial effect. Later, scientists discovered the eggs of an intestinal parasite in his rectum and revealed, that the man used the fungus as a medical treatment. In 3400 B.C. (i.e. in the time of Iceman), Chinese emperor Shen Nung discovered medicinal value of many plants. He is also unwarranted author of first Chinese great herbal, *Pen Tsao Ching* (The Classic of Herbs), which contains 237 herbal prescriptions, but many authorities believe he is not the author and they date the origin of the herbal to 100 A.D. Since that, famous scholars and phycians such as Hippocrates, Dioscorides (who wrote *De Materia Medica*, the first European herbal), Galen, Avicenna, and many others were spreading knowledge about herbal treatment.[1]

Nowadays, due to the rapid development of technologies and medicinal sciences, many diseases, which were commonly letal hundread years ago, are now easily curable by routine medication. This is particularly true in diseases caused by bacteria. The invention of penicillin and other antimicrobial agents massively decreased mortality of infected people. Nevertheless, the recent abuse and misuse of antibiotics contribute to extensive increase of resistant organisms and possibly may have fatal consequences.

These facts lead to growing interest in investigating of plants used in traditional and folk medicine. *Department of Natural Drugs* of Faculty of Pharmacy in Brno participates in research of many interesting plants. Selected phenolic compounds isolated from four of them were tested for biological activity in the cooperation with *Strathclyde Institute for Drug Research* in University of Strathclyde in Glasgow. My diploma thesis summarize the results of these assays.

2 THEORETICAL PART

2.1 PLANTS

2.1.1 Paulownia tomentosa

Fig.1 *P. tomentosa* (Thunb.) Siebold et. Zucc. ex Steud.[2]

2.1.1.1 Taxonomical Classification

Kingdom	Viridiplantae
Phylum	Streptophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Lamiales
Family	Paulowniaceae
Genus	*Paulownia* Siebold et Zucc.
Species	*Paulownia tomentosa* (Thunb.) Siebold et. Zucc. ex Steud.[3]

P. tomentosa (Thunb.) Siebold et. Zucc. ex Steud. has also several synonyms, such as *P. imperialis* Siebold et Zucc., *Bignonia tomentosa* Thunb. and *P. recurva* Rehder.[4,5] Commonly

it is also known as princess-tree, empress-tree, royal paulownia, karri-tree, kiri-tree or mao pao tong.[6]

2.1.1.2 Morphology

Paulownia is a small to medium sized tree, which can reach 9 – 19 m in height. The bark is rough, with gray to brown color. Stems are hairy, brown, and flattened at the nodes. Leaves are large, broad, and oval or heart shaped, and stand opposite. Margins of leaves are entire or shallowly lobed and small plants may have even toothed leaves margins. Leaves are 15 – 40 cm long and 10 – 30 cm wide. Top surface of leaves is light-green, and beneath it is pale-green and tomentose. Blossoms are large, showy, fragrant, and emerge in upright clusters 15 – 30 cm long. Corrola is 5 cm long and pale violet. Fruits are brown, woody, 4 cm long capsules with beaked to ovoid shape. One seed contain approximately 2000 tiny winged seeds. The capsules mature in autumn, after opening they release the seeds and then they remain attached all winter.[7]

2.1.1.3 Origin and Distribution

Paulownia is native to western and central China. According to historical records, it was used in Chinese medicine and wood-carving from the third century B.C. Dutch East India Company imported *Paulownia* to Europe in the 1830s and a few years later to North America.

Paulownia trees can be found on roadsides, stream banks, and disturbed habitats, such as fire sites or forests defoliated by pests and landsides.[7]

2.1.1.4 Usage

In China, *Paulownia* has a significant medicinal use. A decoction of the leaves prevents greying of hair and also stimulates their growth.[8,9] The leaf juice can be used in the treatment of warts. The flowers are used in the treatment of skin ailments and bruises. The inner bark extract is used in the treatment of fevers and delirium. It has astringent and vermifuge effect.[8]

Paulownia fruits have also shown antibacterial,[10,11] antiradical[12] and cytotoxic effect[13]. *Paulownia* wood is easy to work with and is suitable for the manifacture of furniture,

plywood, moldings, doors, musical instruments, etc., because it is light in weight, dries easily, has a beautiful grain, and it does not warp, crack or deform easily.[14] *Paulownia* wood is widely used in Japanese joinery with products such as flower vases, braziers, boxes, chests and others.[15]

2.1.1.5 Constituents

Different constituents of *P. tomentosa* were extracted from various parts of the plant, i.e. from fruits, leaves, stem, wood, and bark. The most important isolated constituents of *P. tomentosa* are:

1) **Flavonoids**, derivates of phenylpropane unit and C_6 fragment (generated by 3 molecules of malonyl-CoA), which undergo cyclization to create a basic chalcone skelet. Modifications of chalcone give rise to flavanones, flavones, flavanols, etc.[16] This group is represented for example by apigenin, 5,4´-dihydroxy-7,3´-dimethoxyflavanone, both isolated from *P. tomentosa* flowers[17], and dihydrotricin isolated from fruit extract[18]. More interesting compounds are flavonoids substituted with geranyl group on carbon C-6. There have been isolated these compounds: tomentodiplacol, 3´-*O*-methyl-5´-methoxydiplacol, 6-isopentenyl-3´-*O*-methyltaxifoline (**1**), which does not have geranyl, but prenyl in its molecule, 3´-*O*-methyldiplacone, mimulone (**2**) and diplacone (**3**), which showed the best antiradical activity, but was the most cytotoxic too[18], then tomentodiplacone, 3´-*O*-methyl-5´-hydroxydiplacone (**4**), 3´-*O*-methyl-5´-*O*-methyldiplacone (**5**), tomentodiplacone B, 3´-*O*-methyldiplacol (**6**), among which especially compounds **4**, **5** and **6** showed moderate activity against some Gram-positive bacteria[10], and 5,7-dihydroxy-6-geranylchromon (**7**)[13]. All these geranylated compounds were isolated from fruit extract. Fifteen *C*-geranyl compounds were also isolated from the methanol extract of the viscous secretion on the surface of immature fruits of *P. tomentosa*.[19]

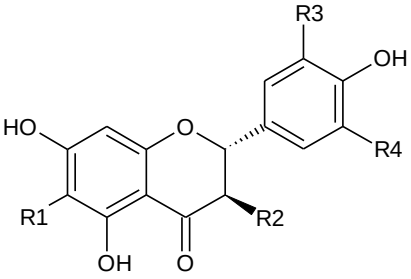
2) **Phenylpropanoid glycosides**, which are phenolic compounds made by phenylpropanoids conjugated with one or more sugars. Phenylpropanoids are derived from the amino acid phenylalanine and contain aromatic ring (C_6) and three-carbon chain (C_3).[20] Phenylpropanoid glycosides isolated from *P. tomentosa* fruit are acteoside (syn. verbascoside) (**8**), isoacteoside (syn. isoverbascoside) (**9**), campneoside II and isocampneoside II.[21] Campneosid I and martynoside were found in stem.[22] Phenylpropanoid glycoside coniferin was isolated from the bark of *P. tomentosa*.[23]

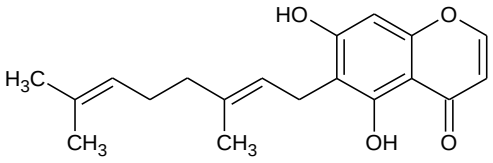
3) **Iridoid glycosides** are monoterpenoid lactones, typical by cyclopentanodihydropyran skelet, conjugated with one or more sugars.[20] In leaves extract there was found paulownioside [24]. Later, aucubin, catalpol, tomentoside, 7-hydroxytomentoside[25] and 7-*β*-hydroxyharpagide[26] were isolated from whole plant extract.

4) **Lignans** are dimers of phenylpropanoid units and in plants they are present mainly in wood and woody parts. From *P. tomentosa*, there were isolated three main lignans: (+)-sesamin (**10**), (+)-paulownin[27] and (+)-piperitol.[28]

5) **Furanoquinones**, represented by methyl-5-hydroxydinaphtol[1,2-2´,3´]furan-7,12-dione-6-carboxylate, which is isolated from the stem bark extract and showed potential antiviral effect.[29]

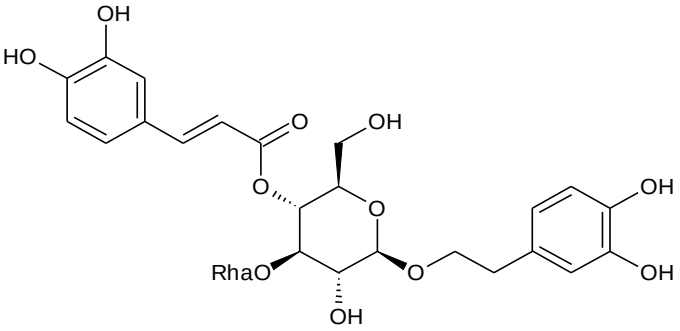
We used 10 compounds isolated from *P. tomentosa* extract – compounds **1**-**10**.





7

	R1	R2	R3	R4
1	prenyl	OH	OMe	H
2	geranyl	H	H	H
3	geranyl	H	H	OH
4	geranyl	H	OH	OMe
5	geranyl	H	OMe	OMe
6	geranyl	OH	H	OMe



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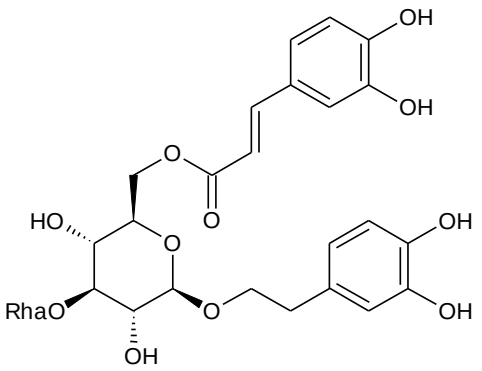

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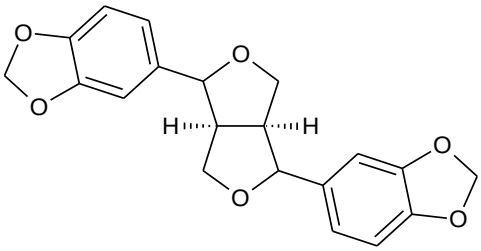

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2.1.2 Morus alba

Fig. 2 *M. alba* L.[30]

2.1.2.1 Taxonomical Classification

Kingdom	Viridiplantae
Phylum	Streptophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosids

Order	Rosales
Family	Moraceae
Genus	*Morus* L.
Species	*Morus alba* L.[31]

This plant has also some botanic synonyms, such as *M. alba* L. var. *multicaulis* (Perr.) Loudon, *M. alba* L. var. *tatarica* (L.) Ser., *M. tatarica* L.[32] and *M. intermedia* Perr.[33] and common names such as chinese white mulberry, common mulberry, Russian mulberry, silkworm mulberry, chi sang, chin sang, moral blanco[34] and tut, tutri or tut kishmishmi in Pakistan.[35]

2.1.2.2 Morphology

M. alba is a deciduous shrub or tree 9 – 15 m high and uniquely can even grow up to the hight of 24 m. The trees vary in form and can be drooping or pyramid-shaped. The bark is light-brown to gray and smooth. The leaves are thin, glossy and light-green. Their shape is not unique and they are often variously lobed even on the same plant. Some can be unlobed while the others are glove-shaped. They are 5 – 18 cm long. White mulberries generally begin to leaf-out in early spring. The small, green-coloured flowers are monoecious, so the plant is self-fertile. When the flowers are pollinated by wind, they begin to enlarge. In the end they become completely changed in texture and colour, becoming succulent, fat and full of juice. The colour of the fruit does not strictly identify the species of mulberry – white mulberries can produce white, lavender, purple or black fruit.[34,36-38]

2.1.2.3 Origin and Distribution

M. alba is native to eastern and central China. It became naturalized in Europe centruries ago and cultivated throughout the world wherever silkworms are raised. In early colonial times, white mulberry was introduced into America because of silkworm culture and then was subsequently naturalized and hybridized with the native red mulberry. White mulberry is naturalized in the urban environment and therefore can be found in these areas more likely than in disturbed forest communities.[34,36]

These trees grow well on a wide variety of soils. They prefer a warm, moist, well-drained loamy soil in a sunny position. They are quite salt tolerant and wind-resistant.[34]

2.1.2.4 Usage

White mulberry is well known because of the relation to silk. Leaves of *M. alba* are favourite food of silkworms - larvae of silkmoth, *Bombyx mori*, Bombycidae. Nowadays, they are generaly cultivated in captivity for sericulture (the silk industry) and they even lost their adaptation helpful to feeding in the wild. The larvae lost their ability to hang on plants and must be fed by humans. The larvae of *B. mori* are caterpillars 4 cm long, buff coloured with brown thoracic markings, while the adults are moths with wingspan of about 4 cm. *B. mori* produce in their silk glands a fluid, which hardens in the air and is worth to form their coccoon.[39]

The mulberries are also popular for the taste of their fruit. White mulberry fruits are generally very sweet but they often lack needed tartness. This is why the black mulberries are more popular for eating – they are large and juicy, with a good balance of sweetness and tartness.[36] Nevertheless, the fruits of white mulberry may be eaten raw or made into jellies, jams, pies, or mulberry wine. The young shoots of leaves were used as a tea substitute.[34,38]

The wood is durable, flexible and elastic and is therefore valued for sporting goods (tennis and badminton rackets, hockey sticks), and also for furniture, agricultural implements, and house and boat building materials.[34] The stem is used for making paper in Europe and China.[34]

M. alba has a traditional use in Chinese medicine, almost all parts of the plant are used. Extracts of the plant have antibacterial and fungicidal activity.[40] The leaves are antibacterial, astringent, diaphoretic, hypoglycaemic, odontalgic and ophtalmic. They are used in the treatment of sore throats, colds, eye infections, and nose bleeds.[38,42] The stems are used in the treatment of rheumatic pains and spasms.[38,43] The bark and especially the bark of the roots has expectorant, cathartic, diuretic, antiasthmatic, antihypertensive, antitussive, anti-oedema, sedative and anticonvulsant actions.[41-43] The use of fruit is in the treatment of anemia, tinnitus and vertigo[41] and they are also beneficial for diabetes, blurred vision, liver and kidneys.[43]

2.1.2.5 Constituents

Due to the traditional use of *M. alba* root bark (known as Cortex Mori) in Chinese medicine, we can observe growing interest in research of *M. alba* constituents. They are classified into several phytochemical groups, mostly possessing interesting biological activity.

1) **Prenylflavonoids**, synthesized as **Diels-Alder type adducts**. Some flavonoids substituted with one or more prenyl chains undergo a special enzymatic reaction known as Diels-Alder addition (more precisely cycloaddition). Requirements for this reaction are the presence of conjugated diene (in this case, represented by dehydroprenyl) and subsituted alkene, termed the dienophile (represented by α,β-double bond of chalcone). There have been isolated more than forty or fifty kinds of Diels-Alder type adducts from the Moraceous plants.

The first isolated active compounds showing a hypotensive effect were kuwanon G and kuwanon H. Diels-Alder type adducts may be divided into four groups according to the type of the two original substances: 1) adducts of a chalcone and a dehydroprenylflavone (e.g. kuwanons G and H), 2) adducts of a chalcone and a dehydroprenylchalcone (e.g. kuwanon I and kuwanon J), 3) adducts of a chalcone and a dehydroprenyl-2-arylbenzofuran (e.g. mulberrofuran C and mulberrofuran J) and 4) adducts of a chalcone and a dehydroprenylstilbene (e.g. kuwanon X and kuwanon Y).[44,45]

2) **Flavonoids**, which also occur commonly in other plants, again they can exist free or conjugated with one or more sugars. From the water extract of *M. alba* root bark, there were isolated oxyresveratrol-2-*O*-*β*-D-glucopyranoside, oxyresveratrol-3´-*O*-*β*-D-glucopyranoside and resveratrol-4,3´-*O*-*β*-D-glucopyranoside.[46] Steppogenin-4´-*O*-*β*-D-glucopyranoside was found in Cortex mori too.[49] Kaempferol-3-*O*-(6´´-*O*-*α*-L-rhamnosyl)-*β*-D-glucopyranoside and quercetin were found in *M. alba* extract lowering blood sugars level.[47] Ethylacetate extract of mulberry leaf involved kaempferol-3-*O*-*β*-D-glucopyranoside, quercetin-3-*O*-*β*-D-glucopyranoside, quercitrin and morin-3-*O*-*β*-D-glucopyranoside.[48]

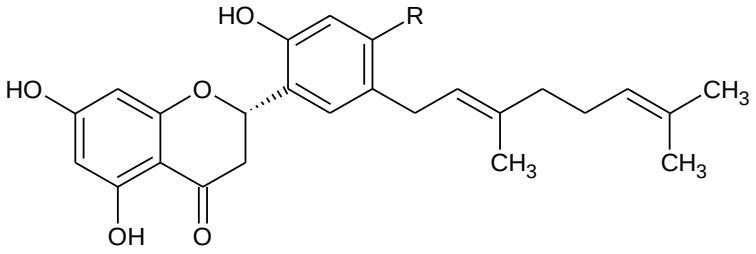
3) **Benzofurans**, with basic skelet created by fusion of benzene ring and furan ring.[20] This group is represented by moracin M, existing also in the glycosidic form: moracin-3´-*O*-*β*-D-glucopyranoside. Moracin C belongs to the group of prenylated benzofurans.[49] Many prenylated benzofurans undergo Diels-Alder type reaction, such as moracin O[49] or mulberrofuran Q.[50]

4) **Coumarines**, biosynthesized from *p*-hydroxycinnamic acid.[20] Bergapten, scopoletin and umbelliferone were isolated from *M. alba* leaves[51], whereas 7-hydroxycoumarin and 5,7-dihydroxycoumarin-7-*O*-*β*-D-glucopyranoside were found in root bark extract.[49]

5) **Alkaloids** are natural compounds which contain a nitrogen atom in their molecule, usually as a part of a heterocycle. In principle, they possess strong physiological effect and they are bitter tasting.[20] In *M. alba* extract lowering blood sugars, there were found piperidine alkaloids such as 1-deoxynojirimycin and fagomine.[47] Five nortropane alkaloids (dihydroxy and trihydroxy derivates) were isolated from the fruit extract.[52]

6) **Sterols**, sythesized from acetyl-CoA, occur generally in mulberry leaves. There have been isolated *β*-sitosterol, daucosterol[48], isofucosterol, campesterol and stigmasterol.[53]

For the experiment, we used 17 pure fractions of *M. alba* root extract, among which there were two compounds identificated as kuwanon E (**11**) and methylkuwanon E (**12**).



	R
11	OH
12	OMe

2.1.3 Maclura pomifera

Fig. 3 *M. pomifera* (Raf.) C. K. Schneid.[54]

2.1.3.1 Taxonomical Classification

Kingdom	Viridiplantae
Phylum	Streptophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosids
Order	Rosales
Family	Moraceae
Genus	*Maclura* Nutt.
Species	*Maclura pomifera* (Raf.) C. K. Schneid.[55]

The synonyms for *Maclura* are *Ioxylon pomiferum* Raf., orth. var., *Toxylon pomiferum* Raf., *Maclura aurantiaca* Nutt., *Joxylon pomiferum* Raf. and *Toxylon aurantiacum* (Nutt.) Raf.[56-58] This plant has also its homonym – *Maclura pomifera* Rob. Synonyms represent the same plant by different names, but the homonyms are the same names for different plants, generally varying in the descriptor. The common names for *M. pomifera* are osage-orange, bois d'arc, bow-wood, hedge apple, or horse apple.[58-60]

2.1.3.2 Morphology

M. pomifera is generally a medium-sized tree about 10 – 13 m high. It has irregular outline or silhouette, becoming stout and artistic with age. The twigs are brown and thick. The strong, durable wood is bright orange in colour. There are thorns present on the trunk and branches, in the place where stems meet the twig. Leaves are long-pointed, glossy and smooth, about 12.5 cm long and 6 cm wide. The leaf shape is lanceolate, oblong or ovate and the venation is pinnate. They emerge in very late spring and avoid frosts. The tree is fully dioecious. The flowers are white and inconspicuous, blooming in spring. More interesting than not too showy flowers is the fruit - round, grepfruit-sized, green hedge-apple. Its resemblance to large orange is superficial only; it is filled with the sticky white sap and plenty of seeds.[59,61]

2.1.3.3 Origin and Distribution

M. pomifera is native to North America, found originally in a small area of Texas and Oklahoma.[61] Afterwards it has become naturalized through the eastern United States and the Central Great Plains.[63] Due to its invasiveness Dana et al. include *M. pomifera* in the European list of aliens which have showed to be very invasive and which may represent a potential danger for Spanish ecosystems. In Italy, it is already considered invasive – not yet in Spain, although it was detected there.[62]

Tree grows in full sun and is really highly tolerant – it resists to wind, drought, diseases and insect, pollution and salt, and has considerable aestetic potential. Trees are safe to keep around houses.[59,61]

2.1.3.4 Usage

Osage-orange wood is hard, strong, durable, and resistant to decay. It is primarily used for fence posts. The wood contains brigtly coloured orange dyes which can be extracted and also used for food processing and pesticide manufacturing. The Osage Indians used the wood for dye and bows. The fleshy fruit is generally not eaten because of bitter taste, but some animals including squirell, fox, red crossbill, and northern bobwhite ocassionaly eat the seeds. It is said that the strong-smelling fruit repels cockroaches. Osage-orange trees are used for soil stabilisation and strip mine reclamation and also in shelterbelt plantings.[63]

An aqueous infusion has been used as a wash for sore eyes and has potential cardiovascular effects.[57] Some isoflavonoids isolated from *M. pomifera* are considered to have antioxidant, antidiabetic, antitumor[64] and cholinestherase inhibitory effects.[65]

2.1.3.5 Constituents

The most interesting parts of *M. pomifera* are the fruit and the root bark, both of them containing pigments, i.e. compounds brightly (generally yellow or orange) coloured. Prenylated flavonoids with potential biological activity were also found in leaves and stems[66]. The most explored constituents of *M. pomifera* are:

1) **Prenylated isoflavones**, isomeric to flavones, differing in aryl-substitution on carbon C-3 rather than C-2. In plants, they occur mostly in free state (not as glycosides).[20] Great portion of osage orange's fruit constituents is made by osajin (**13**)[67] and pomiferin (**14**)[68]. Scandenone and auriculasin were isolated from the fruit later.[69]

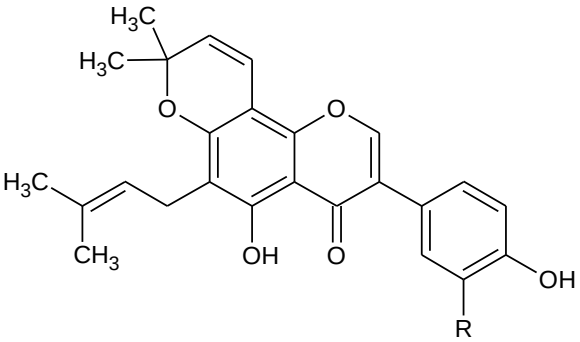
2) **Xanthones,** 9-oxo-derivates of xanthen, heterocyclic molecule made by two benzene rings and one pyran ring between them (dibenzo[*a,e*]pyran).[20] In *Maclura*, xanthones are yellow to orange pigments and are present in the root bark, e.g. macluraxanthone (**15**), osajaxanthone and alvaxanthone.[70] Xanthones were also isolated from trunk bark, such as 8-deoxygartanin, 6-deoxyjacareubin and toxyloxanthones A, B, C and D.[73]

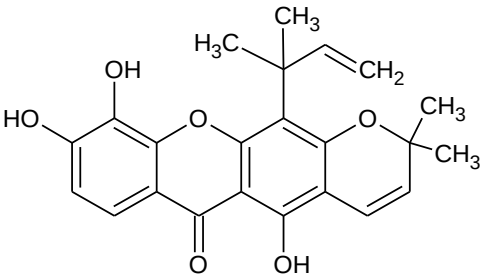
3) **Terpenoids**, synthesized from five-carbon isoprene units. Terpenoids found in *M. pomifera* fruit are elemol, α-copaene, α-cubebene, δ-cadinene, β-elemene, β-caryophyllene, α-ylangene, (*Z, E*)-farnesol.[71]

4) **Triterpenes**, lipids comprised by six isoprene units. Lurenol, lurenyl acetate, lupeol and lupeol acetate were isolated from *M. pomifera* fruit.[72]

5) **Polyphenolic compounds**, like resorcinol, and several flavonoids: kaempferol, quercetin, morin, dihydromorin, dihydrokaempferol and oxyresveratrol, were isolated from heartwood of *M. pomifera*.[73]

Compounds **13**, **14** and **15** were used for the experiment.





	R	15
13	H	
14	OH	

2.1.4 Dracaena cinnabari

Fig. 4 *D. cinnabari* Balf.f.[74]

2.1.4.1 Taxonomical Classification

Kingdom	Viridiplantae
Division	Magnoliophyta
Class	Liliopsida
Order	Asparagales
Family	Asparagaceae

Genus	*Dracaena* L.
Species	*Dracaena cinnabari* Balf.f.[75]

This plant has also a synonym *Draco cinnabari* Kuntze and it is commonly known as Socotra dragontree or Dragon's blood tree.[76- 78]

2.1.4.2 Morphology

D. cinnabari is a tree or shrub 5 to 9 m high. It has simple or compound trunk created when more trunks grow together. The tree can have an umbrella-like appearance. The growth is very slow; the tree reaches the hight of 60 - 90 cm in 10 years. Young trees have simple stem and start branching between 8–11 years of age, after past blossoming. Leaves are emerald to grey green, long, tough, stemless, growing right from the trunk. They can reach the lenght of 60 cm. Flowers are green-white with sweet smell. Fruits are cherry-sized, orange berries. Roots are smooth, beige to orange-colored.[79,80]

2.1.4.3 Origin and Distribution

D. cinnabari is endemic to the island of Socotra belonging to Yemen. That's why it is been named Socotra dragon tree. It is restricted to the zones of submontane thickets and montane grasslands.[81,82]

This tree is classified by IUCN as vulnerable.[82] Its spontaneous reproduction is not possible, because the birds that ate *Dracaena* berries and therefore were the only disseminators of the seeds died-out 400 years ago.[79]

2.1.4.4 Usage

D. cinnabari is the most famous commercial source of dragon's blood. It is a deep red liquid exuding from the bark as a result of any injury. The red resin had many uses in ancient times. Romans coloured their weapons by this resin to give them bloodily look. During antiquity it was used by many cultures as incense addition, red dye, for ritual purposes, alchemy, or as a theriac. In the 18th century it was added to varnish medium of musical instruments, to tooth

paste and dermal healing oil. Today it is used by some shamans during animistic rituals.[79] In folk medicine, *Dracaena*'s resin is used to stop hemorrhaging.[83]

2.1.4.5 Constituents

D. cinnabari resin called dragon's blood is comprised predominantly by flavonoids and terpenoids. Recently, a new constituent, rather complex substance dracophane (a metacyclophane derivate) was found in dragon's blood.[86]

1) **Flavonoids**, represented by **flavones** (e.g. 7,4´-dihydroxyflavone), **flavans** (e.g. 7-hydroxyflavan), **flavanones** (e.g. 7-hydroxyflavanone) and **chalcones** (e.g. 4,4´-dihydroxy-2´-methoxychalcone). More complex flavonoids isolated from dragon's blood are **homoisoflavonoids** (e.g. 7-hydroxy-3-(4-hydroxybenzyl)-8-methoxychromane), **biflavonoids** (e.g. cinnabarone, 2´-methoxysocotrin-5´-ol, socotrin-4´-ol and homoisosocotrin-4´-ol) and **triflavonoids** represented by damalachawin.[84,85]

2) **Terpenoids**, generally modified triterpenes, such as stigmasterol, betulin, campesterol, lophenol, cycloartanol or lanost-7-en-3-β-ol.[84]

We used six still unidentified pure fractions of *D. cinnabari* extract for the experiment.

2.2 MICROORGANISMS

2.2.1 Trypanosoma brucei brucei

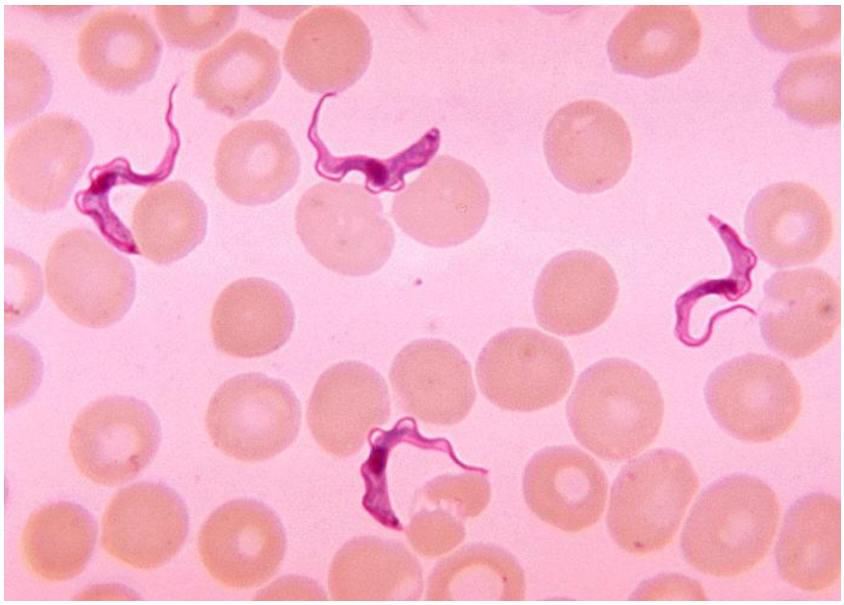


Fig. 5 *T. brucei*[87]

2.2.1.1 Classification

Superkingdom	Eukaryota
Phylum	Euglenozoa
Order	Kinetoplastida
Family	Trypanosomatidae
Genus	*Trypanosoma*
Species	*Trypanosoma brucei*
Subspecies	*Trypanosoma brucei brucei*[88]

Trypanosoma brucei brucei is subspecies of species *T. brucei* together with other subspecies *T. brucei gambiense* and *T. brucei rhodesiense*. These three subspecies do not differ in morphology and can be differentiated by some biological criteria such as host specifity, or sensitivity to human serum as well as by isoenzymatic and DNA analysis. Due to high similarity and non-infectivity to humans *T. b. brucei* is used as a model for human infections in laboratory and animal studies.[97]

2.2.1.2 Morphology, Physiology and Life Cell Cycle

Trypasonoma has the common property that during the life cycle various forms develop in involved carriers. Morphologically, we can differentiate spindly, uniflagellate stages (trypomastigote, epimastigote, promastigote) and a rounded, amastigote form. Trypomastigote is the infective stage and is present in the salivary glands of transmitting tsetse flies (*Glossina*). In this stage the following characteristic features are present: a central nucleus, a mitochondrion containing the kinetoplast, which is an area free of cristae with especially densely packed DNA. Outside of the mitochondrion there is the base of the flagellum, typical for *Trypanosoma*. It originates in the plasmatic basal body, emerges onto the surface of the organism and runs to the anterior end of the organism. During movement an „undulating membrane" is folded out – this movement is visible under a light microscope. The cell is enclosed by an elementary membrane covered by a surface coat or glycocalyx. Spiral microtubules form a cytoskeleton and are arranged along the inner cell membrane. The epimastigote and promastigote forms differ by the location of the kinetoplast and base of the flagellum, which are closer to the nucleus or more toward the anterior end. In the amastigote form, a flagellum is reduced and does not emerge onto the cell surface, and is visible only by electron microscopy.[97]

Trypanosomatidae multiply by longitudinal binary fission. In *T. b. brucei* there is evidence of genetic exchange that occurs during development within the vector.[97]

After the bite of tsetse fly the trypomastigotes enter the wound and proceed into blood and lymph, also they may invade the CNS. If an uninfected tsetse fly bites a diseased person, the trypomastigotes will find their way to fly's midgut, where they can reproduce. The organisms then migrate to the salivary glands, where an epimastigote form continues reproduction to the infective trypomastigote stage. Tsetse fly becomes infective 4 to 6 weeks after biting a diseased person.[98]

2.2.1.3 Epidemiology

T. b. brucei is not infective to humans and is transmitted only between animals. *T. b. gambiense* has a human reservoir and occurs primarily in Western Africa. *T. b. rhodesiense* has an animal reservoir (especially wild antelope) and occurs primarily in Eastern Africa. The

disease is endemic in sub-Saharan Africa, the natural habitat of tsetse fly. The disease can be transmitted by both sexes of fly.[99]

2.2.1.4 Clinical Manifestation

Although *T. b. gambiense* and *T. b. rhodesiense* are both the causative agents of sleeping sickness (African trypanosomiasis), the progress of the disease differs. That one caused by *T. b. gambiense* develops over a few years and has chronic, slow character, while *T. b. rhodesiense* causes a disease with more acute, rapid progress and which, if untreated, can often result in death within several months.[99]

The disease initially demonstrates as an indurated skin ulcer (called „trypanosomal chancre“) at the site of the fly bite. After the organisms enter the blood, systemic symptoms such as typical intermittent weekly fever and lymphadenopathy develop, together with enlargement of the posterior cervical lymph nodes (Winterbottom's sign). The further encephalitis is characterized by headache, insomnia, and mood changes, followed by muscle tremors, slurred speach, and apathy progressing to somnolence and final coma. Untreated disease is usually fatal as a result of pneumonia.[99]

2.2.1.5 Diagnosis

During the early stages, blood can be examinated by microscope and trypomastigotes can be easily revealed. An aspirate of the chancre or enlarged lymph node can be used for microscopic testing as well. The late stage with encephalitis is demonstrated by the presence of trypanosomes in the spinal fluid, combined with an elevated protein level and pleocytosis. Another possibility of making diagnosis besides microscopy is especially the ELISA tests for IgM antibody.[99]

2.2.1.6 Therapy and Prevention

Medical treatment of sleeping sickness is very problematical, due to a small number of effective drugs, serious side effects of these drugs and potential drug-resistance. In early stages, *T. b. gambiense* infections are mainly treated with pentamidine, and infections caused

by *T. b. rhodesiense* are treated with suramin. Nevertheless, these drugs are not effective in the second stage, when encephalitis develops, and in this case the arsenic compound melarsoprol, a relatively toxic substance, must be used. The worst side effect observed in 1–10 % of patients is potentially lethal encephalopathy. The late stage of the *T. b. gambiense* infection is treated by eflornithine.[97]

The prevention comprises individual protection against the *Glossina* flies, generally wearing clothing that fully covers the skin and treating uncovered skin with repellents. In highly affected areas there are also preventive programs focused on insecticide sprayings. More recently, insecticide-charged traps using attractant colours and odours are sometimes used.[97]

2.2.2 Nocardia farcinica

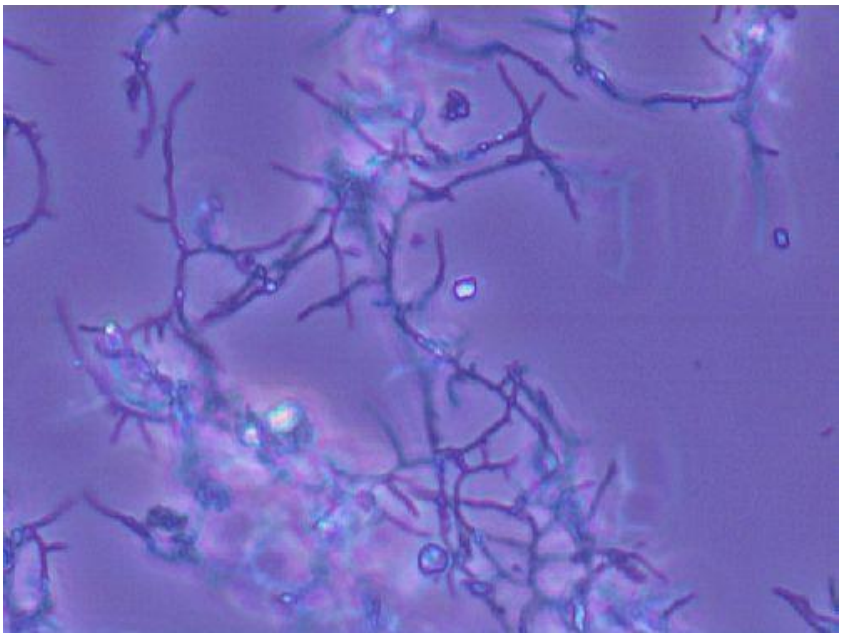


Fig. 6 *N. farcinica*[89]

2.2.2.1 Classification

Superkingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Actinomycetales
Suborder	Corynebacterineae

Family	Nocardiaceae
Genus	*Nocardia*
Species	*Nocardia farcinica*[90]

The genus *Nocardia* is Gram-positive aerobe that stain weakly acid-fast due to the medium-chain mycolic acids in their cell wall. Its natural habitat is the soil and damp biotope. The pathogens known for involvement in nocardioses include *N. asteroides*, *N. brasiliensis*, *N. farcinica*, *N. nova*, and *N. otitidiscaviarum*.[97,98]

2.2.2.2 Morphology and Physiology

Nocardiae are Gram-positive, fine, pleomorphic rods that sometimes show branching. They can be cultured on standard nutrient mediums and proliferate particularly well at 30 °C.[97] *Nocardia* is sorted by different authors to the group of obligate aerobes[97] as well as to the group of strict aerobes.[100]

Nocardia forms in tissues branched filamentous forms which look like the hyphae, and at one time *Nocardia* was thought to be a fungus.[98] Most isolates stain poorly with the Gram stain and therefore can appear to be Gram-negative. The explanation is that *Nocardiae* have a mycobacteria-like cell-wall structure, containing 10-methyl stearic acid (tuberculostearic acid), *meso*-diaminopimelic acid, arabinose, galactose, and mycolic acid. The lenght of the mycolic acids in *Nocardiae* (50 to 62 carbon atoms) is shorter than in *Mycobacteria* (70 to 90 carbon atoms).[98]

2.2.2.3 Epidemiology

Nocardia infections are exogenous.[98] Nevertheless, the organisms have been isolated in small numbers from the respiratory tract of healthy persons, but still they are not considered as members of normal flora.[100] The dramatic increase of nocardiosis disease is mainly caused by the ubiquitous presence of the organism in soil rich with organic matter and the increasing numbers of immunocompromised individuals. This increase is particularly noticeable in ambulatory patients who are infected with human immunodeficiency virus (HIV) or who are immunocompromised because they have received bone marrow or solid organ transplants.[98] Annual incidence levels range from about 0.5 to 1 case per 1 000 000 inhabitants.[97]

2.2.2.4 Clinical manisfestation

Nocardia is the causative agent of disease called nocardiosis. *Nocardia* usually gets into the macroorganism through skin wounds or from the respiratory tract.[97] The way of entry determines a form of disease – inhalation of bacteria causes the pulmonary form of disease, meanwhile the cutaneous form follows skin injury, e.g. injection by a thorn prick or a similar accident.[100]

The infection generally develops only in immunocompromised patients. Monoinfections are the rule. Clinical symptoms are rather individual, usual infections involve pyogenic inflammations with central necroses. There have been described pulmonary nocardioses, systemic nocardioses and superficial nocardioses.[97]

Bronchopulmonary disease caused by *Nocardia* cannot be simply distinguished from other pulmonary infections. *Nocardia* infections usually develop more slowly and occur almost always in immunocompromised patients. Cough, dyspnea, and fever are usual signs, which are not diagnostic, commonly cavitation and spread into the pleura can be observed.[98]

Cutaneous infections may be differentiated to primary (e.g., mycetoma, lymphocutaneous infections, cellulitis, and subcutaneous abscesses) and secondary, as the result of spread of organisms from a primary pulmonary infection. Mycetoma is a disease affecting primarily feet, characterized by localized subcutaneous swelling, suppuration, and the formation of multiple sinus tracts. It develops as painless, chronic infection. Subsequently, the underlying connective tissues, muscle, and bone can be involved and draining sinus tracts can usually open on the skin surface.[98]

2.2.2.5 Diagnosis

The diagnosis by detection of *Nocardia* by means of microscopy and culturing techniques is quite easy, because the organisms are present in great numbers throughout the lesions.[100] Cultures have to be incubated for at least one week due to the long generation time. Precise identification to differentiate pathogenic and apathogenic species is desirable, but difficult.[97]

2.2.2.6 Therapy and Prevention

Nocardia are well susceptible to sulfonamide and relatively resistant to penicillin. Cotrimoxazole (the trimetoprim – sulfamethoxazole combination) is the anti-infective agent of choice and the most widely used chemotherapeutic regimen. Various reports support clinical activity of newer β-lactams (imipenem, ceftriaxone), minocycline, and aminoglycosides, but number of these studies is limited by possible increase in resistant species. Despite the similarity of structure of *Nocardia* and *Mycobacteria* species, antituberculous agents have no activity against *Nocardia*. Antifungal agents, such as amphotericin B are not effective as well. In some cases, even surgery may be required.[97,100] There are no practicable prophylatic measures.[97]

2.2.3 Mycobacterium marinum

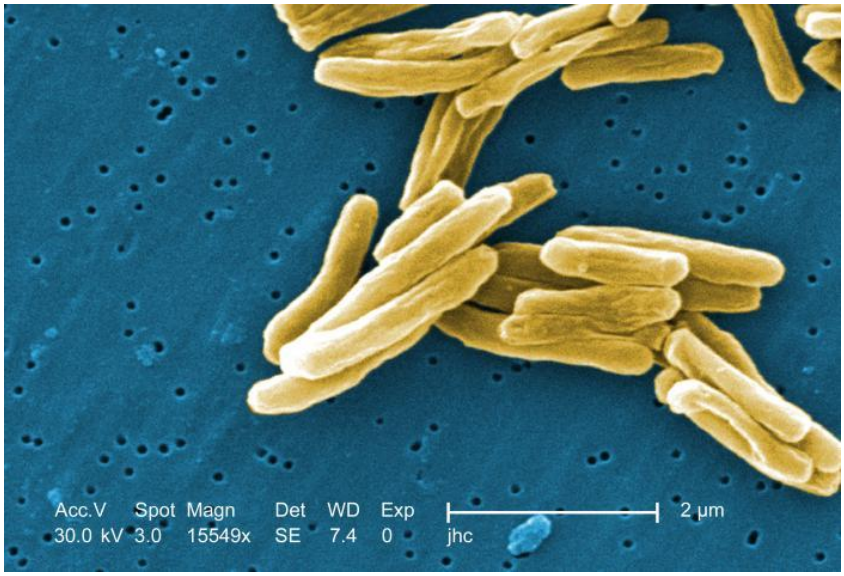


Fig. 7 *M. tuberculosis*[91]

2.2.3.1 Classification

Superkingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Actinomycetales

Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	*Mycobacterium*
Species	*Mycobacterium marinum*[92]

Mycobacterium marinum is species belonging to the genus *Mycobacterium*, which also comprises predominantly known species *M. tuberculosis* (causative agent of tuberculosis) and *M. leprae* (causative agent of leprosy). *Mycobacteria* that are neither tuberculosis nor leprosy bacteria are designated as atypical mycobacteria, nontuberculous mycobacteria (NTM) or MOTT (mycobacteria other than tubercle bacilli). *M. marinum* is used as a model for human infections in laboratory because it is genetically much close to *M. tuberculosis* species, and although it is infective to humans, it does not cause as serious disease as tuberculosis is.[97]

M. marinum causes „swimming pool granuloma", also known as „fish tank granuloma". The abrasions incurred at swimming pools or aquariums are the main causes of this disease manifesting as granulomatous and ulcerating lesions on the skin. *M. marinum* can be found in both fresh and salt water. Antibiotic agents such as minocycline or other tetracyclines are effective.[99]

2.2.3.2 Morphology and Physiology

The genus *Mycobacterium* consists of non-motile, non-spore-forming, aerobic rods that are 0.2-0.6 × 1-10 μm in size. They ocasionally form branched filaments that can be easily disrupted. *Mycobacteria* possess a complex, lipid-rich cell wall. This typical cell wall is the reason of many characteristic properties of these bacteria (e.g., acid-fastness, resistance to common antibacterial antibiotics, antigenicity, slow growth, resistance to detergents, clumping).[97] The important cell wall component is peptidoglycan which is similar to that of other Gram-positive organisms, but there is a difference in containing rather *N*-glycolylmuramic acid than *N*-acetylmuramic acid. Of particular importance are long-chain fatty acids called mycolic acids, attached to peptidoglycan. The mycolic acids, which gave name to genus *Mycobacterium*, make up more than 60 % of the total cell wall mass. Other lipid components include mycosides, sulfolipids, and lipoarabinomannan (LAM). LAM is a complex molecule extending from the plasma membrane to the surface and is structurally and functionally analogous to the lipopolysaccharide of Gram-negative bacteria. The lipids in the

cell wall make its surface hydrophobic, so *Mycobacteria* resist staining with basic aniline dyes unless they are aplied using heat, detergents, or prolonged periods of time. After succesful staining *Mycobacteria* resist decolorization with a mixture of 3% hydrochloric acid and 95% ethanol. These properties are called acid fastness or, more properly, acid-alcohol fastness, and bacteria with these properties are called acid-fast bacilli.[100]

The classification of *Mycobacteria* is based on their growth properties and colonial morphology. *M. tuberculosis* and closely related species are slow-growing bacteria, either nonpigmented or a light tan coloured. The other *Mycobacteria*, so called non-tuberculous *Mycobacteria* (NTM), were classified originally by Runyon by their grade of growth and pigmentation.[98] The pigmented mycobacteria produce intensely yellow carotenoids which may be stimulated by exposure to light (photochromogenic organisms) or produced in the absence of light (scotochromogenic organisms). The Runyon classification scheme is consisted of four groups - *M. marinum* belongs to slow-growing photochromogens.[98]

2.2.3.3 Epidemiology

M. tuberculosis is transmitted as droplet infection by respiratory aerosol and it penetrates into macroorganism generally from lungs. It is easily transmitted from person to person. In the body, it stays mainly within reticuloendothelial cells, e.g., macrophages. Humans are the natural reservoir of *M. tuberculosis*; there is no animal reservoir.[99]

Each diseased person with active tuberculosis, if untreated, infects on average 10 - 15 people every year, but infected people will not necessarily become sick with tuberculosis. The chance of becoming sick is greater in immunocompromised people, generally due to HIV. Someone in the world is newly infected with *M. tuberculosis* every second. Overall, one-third of the world's population is currently infected. 10 % of people infected with *M. tuberculosis* will become sick with active tuberculosis in lifetime. The huge majority of tuberculosis deaths is in the developing world, and more than half of all deaths occur in Asia. In 2008, there were 9.4 millions of new cases of tuberculosis, including 1.4 millions of cases among people infected with HIV.[101]

2.2.3.4 Clinical Manisfestation

Mycobacterium species comprises of many subspecies pathogenic for both humans and animals. *Mycobacteria* do not produce classic exotoxins or endotoxins. Disease process results of two successive host responses. The first, a delayed-type hypersensitivity (DTH) reaction to mycobacterial proteins, results in the destruction of non-activated macrophages containing multiplying microorganisms. The second type, cell-mediated immunity (CMI), activates macrophages, enabling them to destroy mycobacteria contained within their cytoplasm. The balance between these two responses determines the clinical response to a mycobacterial infection.[100]

There are two types of tuberculosis – primary and secondary (which is also called reactivation or postprimary tuberculosis). Primary tuberculosis can be asymptomatic or manifest by non-specific symptoms such as fever and malaise. Radiographs may show infiltrates in the mid-zones of the lung and enlarged lymph nodes, which can fibrose and calcify, and then a characteristic picture known as Ghon complex can be seen on radiograph. If uncontrolled, the primary disease merges into the reactivated type of tuberculosis, or even it disseminates into many organs to produce active miliary tuberculosis. This happens in approximately 5 % of patients. Reactivation is associated with any kind of immunosuppression, mostly malnutrition, alcoholism, diabetes, and old age. Recently, reactivation of primary tuberculosis among younger adults is commonly seen in association with AIDS and is also thought to be one of main complications of AIDS.[100]

The universal symptom is cough. It begins as dry, irritating cough, but later sputum is produced, which can be even mixed with blood (hemoptysis). As disease continues, symptoms like fever, malaise, fatigue, sweating, and weight loss progress. Reactivated tuberculosis can also spread into other organs (lymph nodes, bones, meninges, kidneys, brain, bone marrow, bowel). In these organs, disease can manifest either as a localized tumor-like granuloma called tuberculoma or as a fatal chronic meningitis. Left untreated, the progressive cough, fever, weight loss and possible organ lesion lead to final stage that usually takes 2 to 5 years to cause death. The course in patients suffering an AIDS or another type of immunodeficiency is more rapid.[100]

2.2.3.5 Diagnosis

Tuberculin skin test is the traditional test to assess the patient's response to exposure to *M. tuberculosis*. Tuberculin is purified protein derivate and contains mycobacterial antigens. Reactivity to an intradermal injection helps to differentiate between infected and non-infected people. Positive reaction usually develops 3-4 weeks after exposure to *M. tuberculosis*. Skin reactivity is measured 48 hours later. Patients infected with *M. tuberculosis* may not show a response to the tuberculin skin test if they are not reactive to antigens; particularly true in HIV-infected patients; and in this case the control antigens should always be used with tuberculin tests. Additionaly, people vaccinated with attenuated *M. bovis* (bacille Calmette-Guérin = BCG) will have a positive skin test reaction.

The microscopic detection of acid-fast bacteria is the most rapid way to confirm mycobacterial disease. The whole process involves staining with carbolfuchsin (Ziehl-Neelsen or Kinyoun methods) or fluorescent auramine-rhodamine dyes (Truant fluorochrome method), decolorizing with an acid-alcohol solution, and then counterstaining. The specimens are examined with a light microscope or a fluorescent microscope, if fluorescent dyes are used.

One disadvantage of microscopy is that it cannot identify the exact species of *Mycobacteria* involved. Therefore, there have been developed special techniques to detect nucleic acid sequences specific for *Mycobacteria*. Recently, a gene encoding a secretory protein, *Sec*A gene, has been proved to be useful for direct identification of all species of mycobacteria in clinical speciments.[98]

2.2.3.6 Treatment and Prevention

The treatment and prophylaxis of mycobacterial infections are complex and quite problematical. *Mycobacteria* are resistant to most antibiotics used in treatment other bacterial infections. In general, patients must take multiple antibiotics for an extended period (usualy for a minimum of 6 to 9 months), for there is a possibility of developing antibiotic-resistant strain. In 1990's, the first cases of multidrug-resistant *M. tuberculosis* (MDR-TB; resistant to at least isoniazid and rifampicin) were observed in patients with AIDS and in homeless. Subsequently, new strains of resistant *M. tuberculosis*, called extensively drug-resistant (XDR) TB, have emerged in every region of the world. These strains, defined as MDR-TB

resistant to fluroquinolones and at least one of the second line drugs (e.g., kanamycin, amikacin, capreomycin), are potentially untreatable.

There is a great number of treatment regimens that have been developed for drug-susceptible and drug-resistant tuberculosis. Most treatment regimens begin with 2 months of isoniazid, ethambutol, pyrazinamide, and rifampicin, followed by 4-6 months of isoniazid and rifampicin or alternative combination of drugs. It is very important to determine the drug susceptibility of the isolate and to make modifications of the treatment scheme in accordance with it.[98]

Noncompliance, which means the failure of patients to complete the full course of therapy, is a major factor in increasing of the resistant organisms. One approach to the problem of noncompliance is directly observed therapy (DOT), in which health care workers observe the patient taking the medication.[99]

An important component of prevention is screening (usually using the tuberculin skin test) to detect infected people as soon as possible. Groups that should be screened include people with HIV infection, people close to patients with active tuberculosis, alcoholics and intravenous drug users, prison inmates, and immigrants from countries with a high incidence of tuberculosis. BCG vaccine can be used, though it does not provide full resistance to tuberculosis. The vaccine is effective in preventing the clinical symptoms of tuberculosis, but does not prevent infection by *M. tuberculosis*. However, the major problem with the vaccine is its variable effectiveness, which can range from 0 % to 70 %. It is used primarily in areas of the world where the incidence of the disease is high.[99]

2.2.4 Staphylococcus aureus

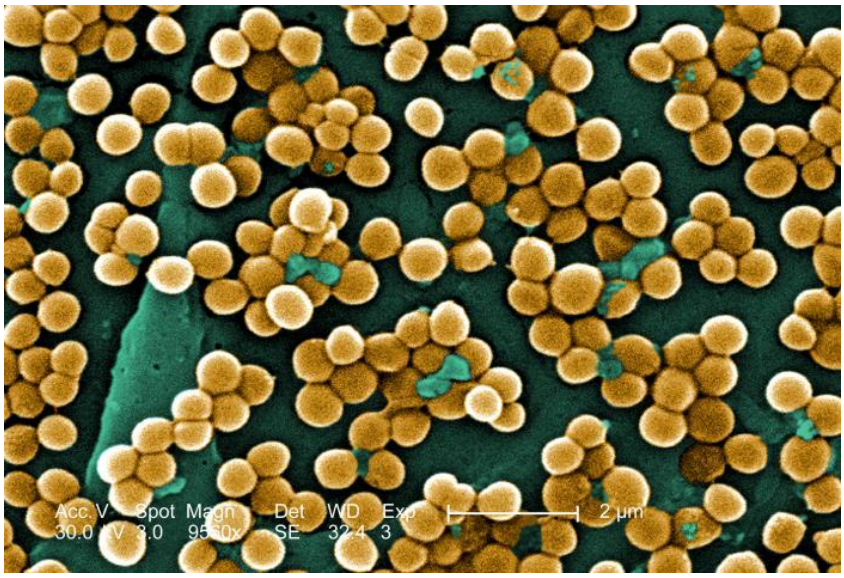


Fig. 8 *S. aureus*[93]

2.2.4.1 Classification

Superkingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Staphylococcaceae
Genus	*Staphylococcus*
Species	*Staphylococcus aureus*[94]

Staphylococci are Gram-positive cocci occuring in clusters.[97] They are non-flagellate, non-motile, and none-spore-forming. Staphylococci are facultatively anaerobic but grow best in aerobic conditions. More than one dozen of Staphylococci species colonize humans; three of these are of major importance: *S. aureus, S. epidermidis, and S. saprophyticus*.[100] The most important species in human medicine is *S. aureus*.[97]

2.2.4.2 Morphology and Physiology

Staphylococci are Gram-positive cocci with spherical shape, generally arranged in irregular grape-like clusters.[99] The cell wall of *S. aureus* consists of a typical Gram-positive peptidoglycan disseminated with molecules of a ribitol-teichoic acid, which is antigenic and relatively specific for *S. aureus*. The cell wall peptidoglycan is overlaid with specific surface proteins in most strains of *S. aureus*.[100]

In contrast to *Streptococci*, all *Staphylococci* strains produce catalase, which is an enzyme degrading H_2O_2 into O_2 and H_2O. Catalase is an important virulence factor because H_2O_2 is microbicidal, so its degradation helps *Staphylococci* to survive.[99]

S. aureus produces a carotenoid pigment that gives a golden color to its colonies. This pigment inactivates the microbicidal effect of superperoxides within neutrophils and therefore also enhances the pathogenicity of the microorganism.[99]

More than 90 % of *S. aureus* strains produce ß-lactamase, the enzyme that degrades many (but not all) penicillins. In addition, some strains of *S. aureus* have genetic changes in the penicillin-binding protein in their cell membrane, and are also resistant to the ß-lactamase-resistant penicillins (e.g. methicillin and nafcillin). These strains are commonly known as methicillin-resistant *S. aureus* (MRSA) or nafcillin-resistant *S. aureus* (NRSA).[99]

S. aureus has several important cell wall components and antigens:

1) **Protein A** is the major protein in the cell wall. It prevents the activation of complement, because it binds to the Fc portion of IgG at the complement-binding site, and so it is an imporatant virulence factor.
2) **Teichoic acids** are ribitol phosphate polymers. They help in adherence to mucosal cells and partly induce septic shock.
3) **Polysaccharide capsule** is also an important virulence factor. Producing an effective vaccine is difficult, because the capsule is only poorly immunogenic.
4) The **peptidoglycan** of *S. aureus* can stimulate macrophages to produce cytokines and activate the complement and coagulation cascades. This could be explanation of the ability of *S. aureus* to cause septic shock, although it does not produce endotoxin.[99]

S. aureus secretes numerous enzymes and toxins that determine the pathogenesis of the infections. The most important are:

1) **Plasma coagulase** is an enzyme that acts like trombin and converts fibrinogen into fibrin. Fibrin walls built around *Staphylococcus* microcolonies disallow phagocytose.

2) **α-toxin** damages membranes resulting in hemolysis, can have lethal CNS effect and in skin it is responsible for a form of dermonecrosis.
3) **Leukocidin** degranulates and damages microphages and macrophages.
4) **Exfoliatins** cause an epidermolysis.
5) There are eight serologically differentiated **enterotoxins** (A-E, H, G, and I) responsible for food poisoning symptoms. These proteins with MW 35 kDa could not be inactivated by heating to 100 °C for 15-30 minutes. *Staphylococcus* enterotoxins are superantigens.
6) About 1 % of *Staphylococcus* strains produce **toxic shock syndrome toxin-1** (TSST-1). TSST-1 is a superantigen and activates clonal expansion of many T-lymphocyte types, leading to a massive production of cytokines, which finally give rise to the clinical symptoms of toxic shock.[97]

2.2.4.3 Epidemiology

Staphylococci are commonly found in the normal human microflora. *S. aureus* is most often found in a nose and on a skin, more often in hospital staff and patients. It is also found in the vagina of approximately 5 % of women and predisposes them to toxic shock syndrome.[99]

Development of disease caused by *S. aureus* is facilitated by a heavily contaminated environment (e.g. hospitals) and a compromised immune system (reduced humoral immunity, including low levels of antibodies, complement, or neutrophils). Immune system is also weakened in patients with diabetes and in intravenous drug users, so they are more predisposed to infections by *S. aureus* as well.[99]

2.2.4.4 Clinical Manifestation

We can differentiate two groups – primary infections and then infections with manifestations caused by staphylococcal toxins.

Primary infections involve furuncules, carbuncules, chronic furunculosis, impetigo and deep lesions. The furuncle or boil is a superficial skin infection that develops by blockage of the gland duct of a hair follicle, sebaceous gland, or sweat gland. Furunculosis is often a complication of acne vulgaris. Infection at the base of the eye-lash causes the common stye. In some individuals, a chronic furunculosis can develop, in which repeated attacks of boils are caused by the same strain of *S. aureus*. Infection can spread from a furuncle and develop to

one or more abscesses in adjacent subcutaneous tissues. This lesion, known as a carbuncle, occurs most often on the back of the neck, but other skin sites may be involved too. Carbuncles are serious lesions that may result in bacteremia if *S. aureus* invades bloodstream. Strains of *S. aureus* that produce exfoliatin cause bullous impetigo, characterized by large blisters in the superficial layers of the skin. Bullous impetigo is very similar to scalded skin syndrome and can be considered to be its localized form. *S. aureus* can cause a wide variety of infections of deep tissues such as bones, joints, deep organs, and soft tissues, including surgical wounds. *S. aureus* causes more than 90 % of the cases of acute osteomyelitis in children. Staphylococcal pneumonia can develop as a secondary disease while suffering influenza, aspiration, or pulmonary edema. Serious secondary infections are also bacteremia and endocarditis.[100]

The second group involves scalded skin syndrome, toxic shock syndrome and staphylococcal food poisoning. Staphylococcal scalded skin syndrome is caused by the productions of exfoliatin in a staphylococcal lesion. The first symptoms are erythema and progressing intraepidermal desquamation of epithelial sheets. The face, axilla, and groin tend to be affected first, but the erythema, bullous formation, and subsequent desquamation can spread to all parts of the body. The disease is most common in neonates and children less than 5 years of age. Toxic shock syndrome (TSS) was firstly described in children but came to public attention during the early 1980s, when hundreds of cases were reported in young women using intravaginal tampons.[100] The main symptoms are high fever, vomiting, diarrhea, sore throat, and muscle pain. Within next 48 hours it may progress to severe shock including renal and hepatic failure. Clinical symptoms reminiscenting of scalded skin syndrome, i.e. skin rash and desquamation may develop. An ingestion of food contaminated by staphylococcal enterotoxin results in acute vomiting and diarrhea within 1- 5 hours. There is fatigue and malaise, but usually no fever. Recovery is rapid (usually by 24 hours), except in some elder patients and in those suffering another disease.[100]

2.2.4.5 Diagnosis

Laboratory diagnosis of staphylococcal infections is quite simple. Microscopic identification of *S. aureus* is easy thanks to numerous polymorphonuclear leukocytes and large numbers of Gram-positive cocci in typical grape-like clusters in acute lesions. *Staphylococci* grow overnight on blood agar incubated in aerobic conditions. In addition, easy catalase and

coagulase tests performed directly from the colonies are sufficient for identification. The increasing resistance of *S. aureus* entails also antibiotic susceptibility testing.[100]

Deep lesions which cannot be readily aspirated or surgically sampled represent specific diagnostic problem. Blood cultures are usually positive in systemic infections such as acute staphylococcal arthritis, osteomyelitis, and endocarditis but less often in localized infection such as deep abscesses.[100]

2.2.4.6 Treatment and Prevention

Most of superficial staphylococcal lesions such as furuncules or minor abscesses resolve spontaneously without a need of antimicrobial therapy. Those more expansive, deeper, or in vital organs, require a combination of surgical drainage and antibiotics. *S. aureus* is well susceptible to penicillins and cephalosporins, but due to inactivation by staphylococcal ß-lactamases, and changes in penicillin-binding proteins in cell membrane, the number of unsusceptible strains is continuously increasing. That is why penicillin G, which was always the treatment of choice for susceptible strains, is nowadays commonly replaced by the penicillinase-resistant penicillins (methicillin, nafcillin, oxacillin) and by first-generation cephalosporins. For strains resistant even to these agents or for patients with ß-lactam hypersensitivity, the acceptable alternatives are vancomycin, clindamycin, or erythromycin. Synergy between the ß-lactams and the aminoglycosides is utilisable when the *Staphylococcus* is sensitive to both types of agents. These combinations are often used in severe systemic infections when effective and rapid bacterial action is required.[100]

In patients subject to recurrent infection, e.g. to chronic furunculosis, preventive measures are important to control the infection. Clothes and bedding that may cause reinfection should be washed more frequently, at a sufficiently high temperature (70 °C or higher), or dry-cleaned. In adults, the use of bactericidal chlorhexidine or hexachlorophene soaps in showering and washing helps to reduce microorganisms in the skin.[100]

Chemoprophylaxis is required in surgical procedures such as hip or cardiac valve replacements. Methicillin, cephalosporin, or vancomycin are usually given during and shortly after surgery. In these cases, staphylococcal infection or even sepsis can have devastating consequences.[100]

2.2.5 Escherichia coli

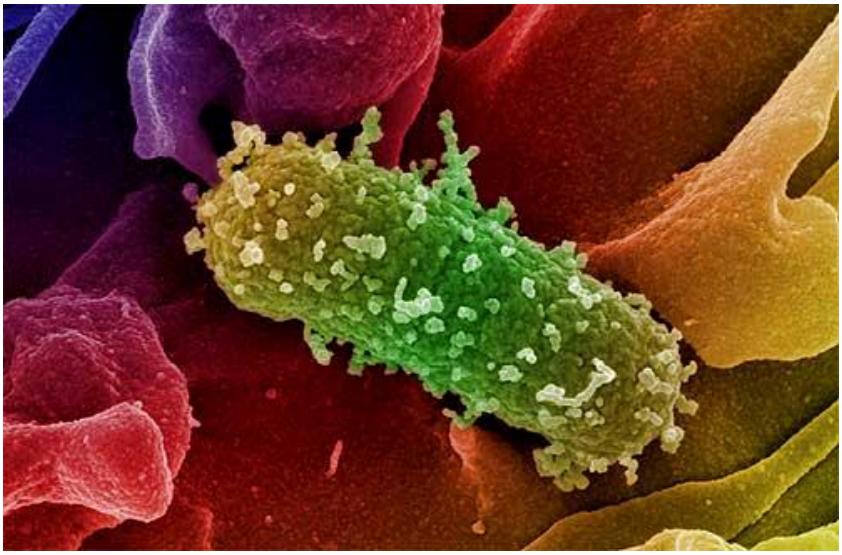


Fig. 9 *E. coli*[95]

2.2.5.1 Classification

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Escherichia coli*[96]

Escherichia coli is the most common and important member of genus *Escherichia*.[98] Hundreds of serotypes are possible – there are over 150 different O antigens and a great number of K and H antigens, all of which are designated by number. The antigenic formula for serotypes is described by three letters (O, K, or H) and number of the antigens present (e.g., O111:K76:H7).[100]

2.2.5.2 Morphology and Physiology

E. coli is Gram-negative, peritrichously flagellated, straight rod with frequently present pili (also called fimbriae) on the surface. Most *E. coli* strains ferment lactose rapidly and produce indole.[97,100]

E. coli can produce every kind of toxin that whole Enterobacteriaceae family do. The α-hemolysin is a pore-forming cytotoxin able to insert into the plasma membrane of host cells. The toxin causes leakage of cytoplasmic contents and eventually cell death.[100]

Shiga toxin is named after species *Shigella dysenteriae* and this toxin was once believed to be produced only by that species. Nowadays, it is recognized to exist in at least two molecular forms released by multiple *E. coli* and *Shigella* strains during lysis of the bacteria.[100]

Labile toxin is named after its physical property of heat lability and contrasts with the heat-stable toxin described below. This toxin permanently activates the adenylyl cyclase system in cell membrane and initializes a cascade of other events. If an enterocyte is involved, it results in the stimulation of chloride secretion out of the cell and the blockage of absorption of sodium chloride. The whole process results in the accumulation of water and electrolytes into the bowel lumen, which causes severe diarrhea. Both structure and biological effects of labile toxin are very similar to cholera toxin.[100]

Stable toxin binds to a glycoprotein receptor and activates a membrane-bound guanylyl cyclase. This activation causes secretion of water and electrolytes into the bowel lumen just like labile toxin.[100]

2.2.5.3 Epidemiology

Large numbers of *E. coli* are commonly present in the gastrointestinal tract. *E. coli* can even become opportunistic pathogen, when the intestine is perforated and the bacteria enter the peritoneal cavity. However, most *E. coli* that cause gastrointestinal and extraintestinal disease do so because they have acquired specific virulence factors. *E. coli* as pathogen is the most common Gram-negative bacterium isolated from patients with sepsis and is responsible for causing more than 80 % of all urinary tract infections, as well as of many hospital-acquired infections, and finally it is a prominent cause of gastroenteritis in developing countries.[98]

2.2.5.4 Clinical Manifestation

We can simply divide the infections caused by *E. coli* into intestinal and extraintestinal infections.

E. coli causing intestinal infections are classified in five pathovars, each with a different pathogenicity and clinical pictures:

1) **Enteropathogenic *E. coli*** (EPEC) – These bacteria cause epidemic or sporadic infant diarrheas. Nowadays it is a main contributor to infant mortality in developing countries. In industrialized countries it occurs quite rarely.
2) **Enterotoxic *E.coli*** (ETEC) – The main part in pathogenicity of these bacteria belongs to the heat-labile enterotoxin and the heat-stable toxins Sta and STb. Enterotoxic strains can produce only one or all of these toxins. The clinical picture of an ETEC infection is characterized by massive watery diarrhea. After recovering from the disease, a local immunity is acquired lasting several months.
3) **Enteroinvasive *E. coli*** (EIEC) – These bacteria are able to penetrate into the colon mucosa, where they cause inflammatory and ulcerous lesions. The pathogenesis and clinical picture of EIEC infections are just like in bacterial dysentery.
4) **Enterohemorrhagic *E. coli*** (EHEC) – These bacteria are the causative agents in the hemorrhagic colitis and hemolytic-uremic syndrome (HUS). These significant complications occur in about 5 % of infections caused by EHEC, accompanied by acute renal failure, anemia, and thrombocytopenia.
5) **Enteroaggregative *E. coli*** (EAggEC) – These bacteria cause watery and sometimes even hemorrhagic diarrhea, generally occuring in infants and small children. They produce toxin identical to Sta in ETEC.[97]

Extraintestinal infections result from relocation of *E. coli* bacteria from common environment of gastointestinal tract to unusual places where conditions for their proliferation are still advantageous.

Urinary tract infection affects only lower urinary tract (urethritis, cystitis, urethrocystitis) or, in more serious cases, the renal pelvis and kidneys (cystopyelitis, pyelonephritis). *E. coli* is the causative organism in 80 % of acute urinary tract infections and in 40 – 50 % of chronic and persistent infections. Urinary tract infections are often caused by the pathovar UPEC (uropathogenic *E. coli*).[97]

E. coli is the causative agent in about 15 % of all cases of nosocomial sepsis. An *E. coli* sepsis is frequently caused by very resistant pathovar SEPEC.[97]

The other extraintestinal infections are wound infections, infections of the gallbladder and bile ducts, appendicitis, peritonitis, meningitis in premature infants, neonates, and very old patients.[97]

2.2.5.5 Diagnosis

Extraintestinal infections are diagnosed by identifying the pathogen in relevant materials. Samples of blood, stool, urine, or other clinical material are sent for culture. Due to a frequent mistake made by contaminating material, the diagnosis of an urinary tract infection with midstream urine requires determination of the bacterial count. Counts $\geq 10^5$/mL indicates an infection, $\leq 10^3$/mL a contamination; 10^4/mL could be either infection or contamination. In recent techniques, specific gene probes are being used to easily identify an intestinal pathovar of *E. coli*.[97]

2.2.5.6 Treatment and Prevention

Treatment of most *E. coli* diarrheas is usually not necessary, because they are mild and self-limiting. Usually rehydration and supportive measures are required and sufficient elements of therapy. In the case of hemorrhagic colitis and HUS caused by EHEC, hemodialysis or hemapheresis may be required. Treatment with cotrimoxazole or quinolones moderates the diarrhea in ETEC, EIEC, and EPEC infection. Because of the risk of HUS, which may be increased by antimicrobial treatments, many physicians do not suggest this kind of treatment. Antimotility agents are contraindicated in the case that EIEC or EHEC could be possible etiologic agent.[100]

The incidence of traveller's diarrhea can be greatly reduced by eating only cooked food and peeled fruits, and drinking hot or carbonated beverages, because the infecting dose of microgranisms is relatively high. Avoiding uncertain water and food is a wise precaution when traveling in developing countries. Chemoprophylaxis against traveler's diarrhea is not generally recommended.[100]

3 INTENTION

The intention of my work was to assess biological activity of selected phenolic compounds isolated in *Department of Natural Drugs* (Faculty of Pharmacy, Brno). Some of them showed potential biological activity in many studies before. In cooperation with Carol Clements from *Strathclyde Institute of Pharmacy and Biomedical Sciences* (University of Strathclyde, Glasgow) the assays determining activity against the species *Trypanosoma brucei brucei*, *Nocardia farcinica*, *Mycobacterium marinum*, *Staphylococcus aureus* and *Escherichia coli* were performed. These assays involved MIC determination of active compounds. As a part of assessment of biological activity, cytotoxicity assays against selected cell lines (comprising prostatic carcinoma cells) were performed. Another intention was to elucidate structures of some pure fractions of *Morus alba* extract by means of NMR spectroscopy in cooperation with Dr Alexander Gray and Dr John Igoli from *Strathclyde Institute of Pharmacy and Biomedical Sciences* (University of Strathclyde, Glasgow).

4 EXPERIMENTAL PART

4.1 MATERIAL

4.1.1 Plant Material

Compounds used for biological activity testing (Table I) were provided by Department of Natural Products, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Brno. These compounds were previously isolated from corresponding plant material. The purity of all compounds tested was proved to be more than 95 % by HPLC.

4.1.2 Chemicals

DMSO (*Sigma Aldrich*)
Gentamycin (*Sigma Aldrich*)
Suramin (*Sigma Aldrich*)
Alamar Blue (*Sigma Aldrich*)
Tween 80 (*Sigma Aldrich*)
HMI-9 medium (*Invitrogen*)
MHB - Mueller Hinton broth (*Trek Diagnostics*)
Columbia agar with 5% horse blood (both *Oxoid*)
DMEM medium (*Invitrogen*)
Triton X (*Sigma Aldrich*)
Tetramethylsilan (*Sigma Aldrich*)
DMSO-δ_6 (*Sigma Aldrich*)

4.1.3 Instruments

- Biological assays:

Wallac Victor 2 (*Wallac*)

- NMR analysis:

Bruker DRX500 NMR System (*Bruker*)

Table 1

Plant		Compound	MW	Sample amount (mg)
M. pomifera	**14**	pomiferin	420.5	109
	13	osajin	404.5	105
	15	macluraxanthone	394.4	28
P. tomentosa	**5**	3´-*O*-methyl-5´-*O*-methyldiplacone	468	16
	2	mimulone	408	23
	6	3´-*O*-methyldiplacol	454	21
	8	acteoside	624	66
	9	isoacteoside	624	29
	10	sesamin	354	18
	1	6-isopentenyl-3´-*O*-methyltaxifoline	386	16
	3	diplacone	424	11
	4	3´-*O*-methyl-5´-hydroxydiplacone	454	43
	7	5,7-dihydroxy-6-geranylchromon	314	5
M. alba		9/3	-	13
		6/4	-	2
		7/1	692	30
		10/8	-	21
		10/4	422	18
	11	kuwanon E	424	32
	12	methylkuwanon E	438	20
		13	420	37
		11/5	-	4
		2/1	626	18
		2/2	626	7.5
		4	442	6
		11/2	-	3
		6/3	-	1
		11/1	-	4
		2a	580	3
		6/5	438	15
D. cinnabari		A/7	286	56
		C/3	-	25
		A/6	-	21
		3/8-3	298	37
		3/5	240	26
		3/10	242	25

4.2 METHODS

4.2.1 Biological and Cytotoxic Assays

4.2.1.1 Trypanosoma brucei brucei

Table 2

Microorganism	*T. b. brucei* S427 (blood stream form)
Incubation temperature	37 °C
Incubation time (CA slope)	3 days
Incubation time (before AB addition)	48 hours
Incubation time (until determined)	24 hours
Inoculum suspension density	3×10^4 of trypanosomas/mL
Positive control	Suramin 1mM stock solution, then 1 in 100 dilution, 1:1 serial dilution, then 20 μL in 200 μL in assay plate
Positive control range/usual MIC	1 to 0.008 μM / 0.062 μM
Negative control	DMSO
Determination	Alamar blue assay Fluorescence determination Wallac Victor (Exc 560 nm, Em 590 nm)

4.2.1.2 Nocardia farcinica

Table 3

Microorganism	*N. farcinica* ATCC.898
Incubation temperature	31 °C
Incubation time (CA slope)	~ 4 days
Incubation time (before AB addition)	~ 3 days
Incubation time (until determined)	~ 20 hours
Inoculum suspension density	0.5 McF standard then 1 in 100 dilution
Positive control	Gentamycin 50 mg/mL stock solution, then 1 in 50 dilution, 1:1 serial dilution, then 20 μL in 200 μL in assay plate
Positive control range/usual MIC	100 to 0.78 μg/mL / 25 μg/mL
Negative control	DMSO
Determination	Alamar blue assay Fluorescence determination Wallac Victor (Exc 560 nm, Em 590 nm)

4.2.1.3 Mycobacterium marinum

Table 4

Microorganism	*M. marinum* ATCC.BAA535
Incubation temperature	31 °C
Incubation time (CA slope)	~ 5 days
Incubation time (before AB addition)	~ 5 days
Incubation time (until determined)	~ 24 hours
Inoculum suspension density	0.5 McF standard then 1 in 10 dilution
Positive control	Gentamycin 50 mg/mL stock solution, then 1 in 50 dilution, 1:1 serial dilution, then 20 µL in 200 µL in assay plate
Positive control range/usual MIC	100 to 0.78 µg/mL / 6.25 µg/mL
Negative control	DMSO
Determination	Alamar blue assay Fluorescence determination Wallac Victor (Exc 560 nm, Em 590 nm)

4.2.1.4 Staphylococcus aureus

Table 5

Microorganism	*S. aureus* ATCC.29213
Incubation temperature	37 °C
Incubation time (CA slope)	~ 20 hours
Incubation time (before AB addition)	0 (time add at setup)
Incubation time (until determined)	~ 20 hours
Inoculum suspension density	0.5 McF standard then 1 in 1000 dilution
Positive control	Gentamycin 50 mg/mL stock solution, then 1 in 500 dilution, 1:1 serial dilution, then 20 µL in 200 µl in assay plate
Positive control range/usual MIC	10 to 0.08 µg/mL / 0.31 – 0.62 µg/mL
Negative control	DMSO
Determination	Alamar blue assay Fluorescence determination Wallac Victor (Exc 560 nm, Em 590 nm)

4.2.1.5 Escherichia coli

Table 6

Microorganism	*E. coli* ATCC.8739
Incubation temperature	37 °C
Incubation time (CA slope)	~ 20 hours
Incubation time (before AB addition)	0 (time add at setup)
Incubation time (until determined)	~ 20 hours
Inoculum suspension density	0.5 McF standard then 1 in 1000 dilution
Positive control	Gentamycin 50 mg/mL stock solution, then 1 in 500 dilution, 1:1 serial dilution, then 20 µL in 200 µL in assay plate
Positive control range/usual MIC	10 to 0.08 µg/mL / 0.62 µg/mL
Negative control	DMSO
Determination	Alamar blue assay Fluorescence determination Wallac Victor (Exc 560 nm, Em 590 nm)

4.2.1.6 Cytotoxicity

Table 7

Cell line	DU-145 LNCaP AS PC-3 PNT2A
Incubation temperature	37 °C
Incubation time (before AB addition)	48 hours
Incubation time (until determined)	24 hours
Cell seeding density	DU-145: 0.5×10^5 per mL LNCaP AS: 1×10^5 per mL PC-3: 1×10^5 per mL PNT2A: 0.5×10^5 per mL
Positive control	Triton X 0.1%
Negative control	DMEM (pure medium)
Determination	Alamar blue assay Fluorescence determination Wallac Victor (Exc 560 nm, Em 590 nm)

4.2.2 Identification of Compounds Tested

4.2.2.1 NMR spectroscopy

Table 8

Instrument	Bruker DRX500 NMR System
Inner standard	Tetramethylsilane
Solvent	DMSO-δ_6 (deuterated dimethyl sulphoxide)
Temperature	20 °C
Software	Bruker topspin NMR processing software

Spectral analysis used for structure determination:

1-D ^{1}H-NMR spectrum

1-D ^{13}C-J-MOD NMR spectrum

2-D HSQC spectrum

2-D HMBC spectrum

2-D COSY spectrum

5 RESULTS AND DISCUSSION

All 36 samples were examined for biological activity, involving antitrypanosomal, antibacterial and cytotoxic assays. Nine samples, all of them isolated from *M. alba*, were analysed using NMR method. However, the determination of their structures is still in progress and the results are not ready yet.

5.1 BIOLOGICAL ACTIVITY

At the beggining of the experiment, the compounds were diluted with DMSO to prepare 20 mM stock solutions. In the case of compounds of unknown molecular weight, the quantity of DMSO addition was estimated and final concentration of the solutions was determined in μg/mL (fractions 11/5 from *M. alba* and C/3 and A/6 from *D. cinnabari*). In the case of previous NMR analysis, all the amount of each sample was diluted with 750 μL of DMSO (fractions 9/3, 6/4, 10/8, 10/4, 11/2, 6/3, 11/1, 2a and 6/5 from *M. alba*). The final MICs of these twelve fractions were therefore determined in μg/mL. The molecular weights of *M. alba* fractions 10/4, 2a and 6/5 were elucidated later and their MICs are therefore determined in μM.

The cultivation of bacteria was performed using Columbia agar with 5% addition of horse blood. The passaging of trypanosomes requires HMI-9 medium. The suspension of microorganisms used for inoculation was stabilized by Tween 80.

The principle of MIC determination was similar for each assay. At first, initial screening at one concentration was made. It means that all samples were equally concentrated. This concentration was always 100 μM, except antitrypanosomal assay using 40 μM concentration. The medium used for diluting was MHB (Mueller Hinton broth), except antitrypanosomal assay using HMI-9 medium and cytotoxic assays using DMEM. After fluorescence determination, the compounds with fluorescence generally under 10 % of DMSO control were defined to be active against the microorganism. These compounds were subsequently tested again in the same procedure, but in panel of descending concentration. The highest concentration was always 100 μM, then serial 1:1 dilutions were made. The lowest concentration, at which the compound still inhibited the growth of the microorganism, was determined as MIC of the compound. The cytotoxicity results are determined as percentage of negative control. IC_{50} have not been determined yet.

All antimicrobial assays included a positive control in descending concentration (efficacious antimicrobial agent, i.e. suramin or gentamycin), negative control (DMSO) and sterility check (incubating medium without the addition of microorganisms). Cytotoxic assays included Triton X as positive control and pure DMEM medium as negative control. Each assay was performed in duplicate.

All the antitrypanosomal, antibacterial and cytotoxicity assays were performed using the REDOX indicator Alamar blue. After initial incubation, 20 μL of Alamar Blue (10 μL in cytotoxicity assays) was added to each well. After another 24 hours (in principle) the fluorescence was determined. The active component of Alamar Blue is resazurin (blue in colour) which in the presence of live parasites, bacteria or cells is reduced to the pink fluorescent resorufin.[105] Wells containing active compounds are easily identified as they remain blue in colour and have background levels of fluorescence. The fluorescence values for the test plates were measured using a microplate reader in fluorescence mode with excitation and emission wavelengths of 560 nm and 590 nm, respectively.

The assays were performed in 96-well microtiter plates. The incubation and treatment times, incubation conditions and seeding densities were optimised for each test species and cell line.[102-105]

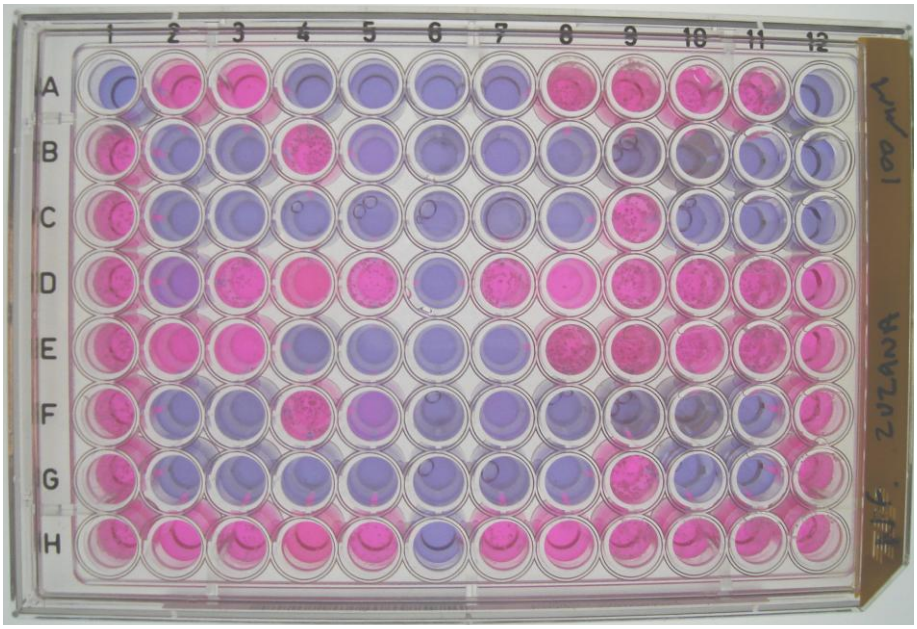


Fig. 10 The photograph above illustrates the results from a screen to test for the activity against *Nocardia*. Well A1 is the sterility check, wells B1 - H1 the DMSO control and column 12 the positive control containing a concentration range of gentamycin. The blue wells are the active compounds, pink wells are the non-active.

5.1.1 Results of Initial Biological Activity Screening

5.1.1.1 Trypanosoma b. brucei

Table 9

	Compound tested	Concentration	% of DMSO control
	M. pomifera		
14	pomiferin	40 µM	**4**
13	osajin	40 µM	**3**
15	macluraxanthone	40 µM	**25**
	P. tomentosa		
5	3´-*O*-methyl-5´-*O*-methyldiplacone	40 µM	**1**
2	mimulone	40 µM	**3**
6	3´-*O*-methyldiplacol	40 µM	**2**
8	acteoside	40 µM	**110**
9	isoacteoside	40 µM	**114**
10	sesamin	40 µM	**78**
1	6-isopentenyl-3´-*O*-methyltaxifoline	40 µM	**109**
3	diplacone	40 µM	**5**
4	3´-*O*-methyl-5´-hydroxydiplacone	40 µM	**18**
7	5,7-dihydroxy-6-geranylchromon	40 µM	**2**
	M. alba		
	9/3	34.7 µg/mL	**3**
	6/4	5.3 µg/mL	**79**
	7/1	40 µM	**31**
	10/8	56 µg/mL	**5**
	10/4	114 µM	**8**
11	kuwanon E	40 µM	**3**
12	methylkuwanon E	40 µM	**2**
	13	40 µM	**3**
	11/5	15.5 µg/mL	**0**
	2/1	40 µM	**10**
	2/2	40 µM	**43**
	4	40 µM	**-3**
	11/2	8 µg/ml	**81**
	6/3	2.7 µg/mL	**103**
	11/1	10.7 µg/mL	**68**
	2a	14 µM	**40**
	6/5	92 µM	**3**
	D. cinnabari		
	A/7	40 µM	**69**
	C/3	11.5 µg/mL	**1**
	A/6	11.7 µg/mL	**96**
	3/8-3	40 µM	**83**
	3/5	40 µM	**91**
	3/10	40 µM	**1**

The constituents of four used plants were tested against trypanosomas for the first time. 19 compounds out of 36 tested were active. In the case of these 19 compounds, futher determination of MICs followed.

From *M. pomifera* compounds, **13** and **14** exhibited much higher activity than **15**. Both **13** and **14** are prenylated isoflavones, **15** is prenylated xanthone. Antitrypanosomal activity of prenylated xanthones has been proven before in substances isolated from the root bark of plant *Garcinia livingstonei*.[106] These xanthones were substituted with prenyl group at C-3 or at C-2. Macluraxanthone (**15**) is substituted at C-4. Hence we assume that the possition of prenyl group influences antitrypanosomal activity of natural xanthones.

Non-prenylated constituents of *P. tomentosa* **8–10** belonging to the group of phenylpropanoid glycosides or lignans showed no activity. From prenylated flavonoids, compound **1** was not active and activity of **4** was too low to determine its MIC. Other constituents (**2, 3, 5-7**), each possessing a geranyl chain at C-6 of the molecule, showed very good activity in concentration used. Phenolic compounds with prenyl group in molecule have already exhibited good antitrypanosomal activity, however only prenylated derivates of *p*-hydroxycinnamic acid isolated from Brazilian propolis were tested.[107] Prenylated flavonoids were apparently tested for the first time, according to our search in literature.

The exact chemical composition of most fractions of *M. alba* is still unknown and we cannot therefore discuss the influence of molecular structure upon the biological activity. Nevertheless, we assume that in many cases these compounds are also substituted with one or more prenyl groups. Two already identified structures (compounds **11** and **12**) are flavanones geranylated on C-5´. They both possess obvious antitrypanosomal activity. Fractions 6/4, 11/2, 6/3, 11/1 and 2a were tested in quite low concentrations and we cannot deny that they would be active in slightly higher concentrations. Literature does not mention any antitrypanosomal assays in Moraceous plants ever.

Two fractions (C/3 and 3/10) out of six isolated from *D. cinnabari* were highly active, while the rest of them were rather inactive. Due to unknown structures, we cannot draw conclusions about the influence of the molecule structure upon this difference in activity. No records about earlier antitrypanosomal testing of dragon's blood or whole plant extract were found.

5.1.1.2 Nocardia farcinica

Table 10

	Compounds tested	Concentration	% of DMSO control
	M. pomifera		
14	pomiferin	100 µM	**92**
13	osajin	100 µM	**96**
15	macluraxanthone	100 µM	**0**
	P. tomentosa		
5	3´-*O*-methyl-5´-*O*-methyldiplacone	100 µM	**-1**
2	mimulone	100 µM	**-2**
6	3´-*O*-methyldiplacol	100 µM	**2**
8	acteoside	100 µM	**96**
9	isoacteoside	100 µM	**94**
10	sesamin	100 µM	**98**
1	6-isopentenyl-3´-*O*-methyltaxifoline	100 µM	**98**
3	diplacone	100 µM	**0**
4	3´-*O*-methyl-5´-hydroxydiplacone	100 µM	**-1**
7	5,7-dihydroxy-6-geranylchromon	100 µM	**100**
	M. alba		
	9/3	86.7 µg/mL	**0**
	6/4	13.4 µg/mL	**8**
	7/1	100 µM	**0**
	10/8	140 µg/mL	**-2**
	10/4	285 µM	**-1**
11	kuwanon E	100 µM	**2**
12	methylkuwanon E	100 µM	**-1**
	13	100 µM	**-2**
	11/5	38.8 µg/mL	**-2**
	2/1	100 µM	**1**
	2/2	100 µM	**1**
	4	100 µM	**0**
	11/2	20 µg/mL	**-1**
	6/3	6.7 µg/mL	**100**
	11/1	26.7 µg/mL	**-1**
	2a	35 µM	**0**
	6/5	230 µM	**68**
	D. cinnabari		
	A/7	100 µM	**99**
	C/3	28.8 µg/mL	**99**
	A/6	29.3 µg/mL	**98**
	3/8-3	100 µM	**3**
	3/5	100 µM	**98**
	3/10	100 µM	**97**

Overall, 21 compounds were efficacious in assay of activity against *Nocardiae*. In this case, compound **15** was active, while compounds **13** and **14** were not. There is no reference in literature about anti-nocardiae effect neither of isoflavonoids nor xanthones.

Inactive compounds isolated from *P. tomentosa* involved again compounds **8–10**. These compounds miss prenyl chain in their molecule. On the other hand, compounds **1** and **7**, which are prenylated at C-6, were also inactive. However, as seen in *M. pomifera* constituents, prenyl group does not seem to be determinating for the anti-nocardiae effect. The rest of the compounds showed very good activity. Comparing them with other *P. tomentosa* constituents, no crucial substitution is observed.

Overwhelming majority of *M. alba* constituents are active. The adverse result of fraction 6/3 can be accredited to its low concetration (6.7 μg/mL), while fraction 6/5 (with concentration 100 μM) is obviously inactive. *M. alba* constituents were tested against *Nocardiae* for the first time.

In the group of *D. cinnabari* constituents, only fraction 3/8-3 was active. Other fractions exhibit level of fluorescence comparable with negative control. Anti-nocardiae assays of *Dracaena* or similar plants have not been performed before.

5.1.1.3 Mycobacterium marinum

Table 11

	Compounds tested	Concentration	% of DMSO control
	M. pomifera		
14	pomiferin	100 µM	**71**
13	osajin	100 µM	**1**
15	macluraxanthone	100 µM	**2**
	P. tomentosa		
5	3´-*O*-methyl-5´-*O*-methyldiplacone	100 µM	**0**
2	mimulone	100 µM	**1**
6	3´-*O*-methyldiplacol	100 µM	**4**
8	acteoside	100 µM	**97**
9	isoacteoside	100 µM	**95**
10	sesamin	100 µM	**1**
1	6-isopentenyl-3´-*O*-methyltaxifoline	100 µM	**99**
3	diplacone	100 µM	**2**
4	3´-*O*-methyl-5´-hydroxydiplacone	100 µM	**1**
7	5,7-dihydroxy-6-geranylchromon	100 µM	**77**
	M. alba		
	9/3	86.7 µg/mL	**2**
	6/4	13.4 µg/mL	**12**
	7/1	100 µM	**1**
	10/8	140 µg/mL	**0**
	10/4	285 µM	**0**
11	kuwanon E	100 µM	**2**
12	methylkuwanon E	100 µM	**0**
	13	100 µM	**0**
	11/5	38.8 µg/mL	**0**
	2/1	100 µM	**4**
	2/2	100 µM	**16**
	4	100 µM	**2**
	11/2	20 µg/mL	**1**
	6/3	6.7 µg/mL	**80**
	11/1	26.7 µg/mL	**2**
	2a	35 µM	**61**
	6/5	230 µM	**3**
	D. cinnabari		
	A/7	100 µM	**62**
	C/3	28.8 µg/mL	**35**
	A/6	29.3 µg/mL	**25**
	3/8-3	100 µM	**2**
	3/5	100 µM	**22**
	3/10	100 µM	**0**

The greatest effect of the compounds was that anti-mycobacterial. Only 9 compounds were defined as non-active and were not futher tested for MIC determination.

Compounds **13** and **15** isolated from *M. pomifera* were active, activity of compound **14** was markedly lower. *M. pomifera* constituents have not been examined for anti-tuberculosis effect in any study yet.

The group of inactive compounds from *P. tomentosa* involves again phenylpropanoid glycosides **8** and **9** and flavonoids **1** and **7**. Compound **10** was active this time, however in literature its non-effectivity agains *Mycobacteria* was mentioned.[108] Flavonoids **2–6** were highly active. All these compounds were tested against *Mycobacteria* for the first time. Nevertheless, prenylated flavonoids from other plants were tested in previous studies. For example, in the study of antimycobacterial effect of *Eriosema chinense* constituents, four prenylated flavonoids had MICs values 12.5 μM. Higher MICs (25–100 μM) and therefore lower activity were observed in substances with one or more methoxyl substitutions. A compound with dihydroxylated prenyl chain were inactive.[109]

Results of the activity of *M. alba* constituents confirmed the traditional usage of *M. alba* bark as tuberculosis treatment in China.[110] All fractions were active apart from fractions 6/3 and 2a. Plant *M. alba* has not been tested before, but other plants from Moraceae family, such as genus *Artocarpus* or *Ficus* have been tested.[111,112] Constituents of both genus involve prenylated flavonoids. Substances isolated from *Artocarpus altilis* are even very similar to those found in *M. alba* and prenylated flavone morusin was actually found in both of them.[113] Most of *Artocarpus* compouds had very good activity. The most active substance from *Ficus* was laburnetin, which was one of two used prenylated compounds.[112]

D. cinnabari constituents were also tested for the first time. Anti-mycobacterial assays were previously performed only in constituents of *Dracaena angustifolia*. Three substances of *D. angustifolia* showed strong anti-tuberculosis activity (MICs about 1-2 μg/mL). These substances were ergosterol-5,8-endoperoxide, linoleic acid and *E*-phytol. Comparing these compounds with tested fractions, the best match was found at *E*-phytol (MW 296.5) and fraction 3/8-3 (MW 298).[114]

5.1.1.4 Staphylococcus aureus

Table 12

	Compounds Tested	Concentration	% of DMSO control
	M. pomifera		
14	pomiferin	100 µM	**105**
13	osajin	100 µM	**0**
15	macluraxanthone	100 µM	**106**
	P. tomentosa		
5	3´-*O*-methyl-5´-*O*-methyldiplacone	100 µM	**2**
2	mimulone	100 µM	**-1**
6	3´-*O*-methyldiplacol	100 µM	**6**
8	acteoside	100 µM	**103**
9	isoacteoside	100 µM	**100**
10	sesamin	100 µM	**99**
1	6-isopentenyl-3´-*O*-methyltaxifoline	100 µM	**103**
3	diplacone	100 µM	**3**
4	3´-*O*-methyl-5´-hydroxydiplacone	100 µM	**5**
7	5,7-dihydroxy-6-geranylchromon	100 µM	**83**
	M. alba		
	9/3	86.7 µg/mL	**1**
	6/4	13.4 µg/mL	**4**
	7/1	100 µM	**2**
	10/8	140 µg/mL	**0**
	10/4	285 µM	**3**
11	kuwanon E	100 µM	**5**
12	methylkuwanon E	100 µM	**1**
	13	100 µM	**1**
	11/5	38.8 µg/mL	**0**
	2/1	100 µM	**2**
	2/2	100 µM	**3**
	4	100 µM	**1**
	11/2	20 µg/mL	**1**
	6/3	6.7 µg/mL	**93**
	11/1	26.7 µg/mL	**1**
	2a	35 µM	**3**
	6/5	230 µM	**23**
	D. cinnabari		
	A/7	100 µM	**89**
	C/3	28.8 µg/mL	**85**
	A/6	29.3 µg/mL	**80**
	3/8-3	100 µM	**87**
	3/5	100 µM	**92**
	3/10	100 µM	**103**

21 compounds including 15 *M. alba* constituents and 5 *P. tomentosa* constituents were active against *S. aureus*.

Only compound **13** was active among *M. pomifera* constituents. Compound **14** differs from **13** only by hydroxylation on C-3′, so we can assume, that this hydroxylation is crucial in antimicrobial effect. Compound **15** was inactive too. This is confirmed by the study of Yimdjo et al.[115] No record of antistaphylococcal activity of **13** or **14** is available.

The activity of *P. tomentosa* constituents is explored much more. Compounds **4–6** were tested agains *S. aureus* before, all of them with MICs of 2 – 4 μg/mL.[10] The essentiality of hydroxylation or methoxylation at C-3′, C-4′ and C-5′ on ring B was discussed.[10] Nevertheless, two other active compounds, **2** and **3**, which are hydroxylated only on C-4′ (in the case of **2**) and C-3′ (in the case of **3**), exhibited comparable activity. The inactivity of compound **1** can be explained by substitution of prenyl chain, because **6** substituted with geranyl chain was much more active. Although **7** possess geranyl group on C-6, the absence of ring B apparently decreases the antimicrobial activity. Compounds **8–10** were inactive just like in the case of previous assays. In the study of the butanol extract of *P. tomentosa* stem, campneoside I was defined to be the most active compound due to its methoxy group,[22] which is absent in the structure of **8**. One antistaphylococcal substance was also isolated from the epicard of the *P. tomentosa* fruits.[116]

The advantageous presence of prenyl group in molecule structure is also confirmed by studies of activity of *M. alba* constituents (kuwanon C, morusin, sanggenon B and D).[118] Another study contributes by determination of the antistaphylococcal activity of 2-arylbenzofurans isolated from *M. alba*.[117] It seems likely that a few of these active compounds will be identical with or similar to compounds of fractions used in this experiment. From the tested fractions, 6/5 and 6/3 showed the weakest activity. However, in the case of fraction 6/3, this can be caused by low concentration of the sample.

Although Kumar et al. described significant anti-staphylococcal activity of *D. cinnabari* crude extract,[119] none of *D. cinnabari* constituents were herein defined to be active. This can be caused either by high concentration of crude extract (500 μg/mL) or by the fact that crude extract could contain substances which are more active than fractions we tested.

5.1.1.5 Escherichia coli

Table 13

	Compounds tested	Concentration	% of DMSO control
	M. pomifera		
14	pomiferin	100 µM	**101**
13	osajin	100 µM	**100**
15	macluraxanthone	100 µM	**95**
	P. tomentosa		
5	3´-*O*-methyl-5´-*O*-methyldiplacone	100 µM	**101**
2	mimulone	100 µM	**102**
6	3´-*O*-methyldiplacol	100 µM	**98**
8	acteoside	100 µM	**104**
9	isoacteoside	100 µM	**96**
10	sesamin	100 µM	**96**
1	6-isopentenyl-3´-*O*-methyltaxifoline	100 µM	**89**
3	diplacone	100 µM	**58**
4	3´-*O*-methyl-5´-hydroxydiplacone	100 µM	**51**
7	5,7-dihydroxy-6-geranylchromon	100 µM	**62**
	M. alba		
	9/3	86.7 µg/mL	**49**
	6/4	13.4 µg/mL	**62**
	7/1	100 µM	**55**
	10/8	140 µg/mL	**54**
	10/4	285 µM	**46**
11	kuwanon E	100 µM	**61**
12	methylkuwanon E	100 µM	**58**
	13	100 µM	**58**
	11/5	38.8 µg/mL	**64**
	2/1	100 µM	**54**
	2/2	100 µM	**55**
	4	100 µM	**55**
	11/2	20 µg/mL	**55**
	6/3	6.7 µg/mL	**64**
	11/1	26.7 µg/mL	**73**
	2a	35 µM	**63**
	6/5	230 µM	**65**
	D. cinnabari		
	A/7	100 µM	**69**
	C/3	28.8 µg/mL	**63**
	A/6	29.3 µg/mL	**63**
	3/8-3	100 µM	**60**
	3/5	100 µM	**67**
	3/10	100 µM	**68**

In concentration of 100 μM (or as seen on table 13), none of compounds were active enough to proceed to MIC determination. Studies mentioned above confirm that compounds or extracts of *M. pomifera*[115], *P. tomentosa*[116], *M. alba*[117,118] and *D. cinnabari*[119] showed very week activity against *E. coli* and eventually other Gram-negative bacteria, and at the same time were active against Gram-positive bacteria. The ability of medicine plants to inhibit *E. coli* is not generally very strong.[120] In principle, Gram-positive bacteria are more susceptible to natural drugs than Gram-negative ones.[121] However, several studies contradict this convention, for example Basile et al. reported that flavonoids are more active against Gram-negative bacteria and suggest that the selectivity towards Gram-positive bacteria is not special phenomenom of natural substances, but is generally observed among most antibiotics.[122]

5.1.2 MICs Results

Table 14

		T. b. brucei	*N. farcinica*	*M.marinum*	*S. aureus*
		MIC (µM)			
	M. pomifera				
14	pomiferin	**3.12**	**-**	**-**	**-**
13	osajin	**12.5**	**-**	**6.25**	**6.25**
15	macluraxanthone	**-**	**50**	**12.5**	**-**
	P. tomentosa				
5	3´-*O*-methyl-5´-*O*-methyldiplacone	**3.12**	**12.5**	**6.25**	**12.5**
2	mimulone	**6.25**	**6.25**	**6.25**	**6.25**
6	3´-*O*-methyldiplacol	**6.25**	**25**	**25**	**12.5**
8	acteoside	**-**	**-**	**-**	**-**
9	isoacteoside	**-**	**-**	**-**	**-**
10	sesamin	**-**	**-**	**50**	**-**
1	6-isopentenyl-3´-*O*-methyltaxifoline	**-**	**-**	**-**	**-**
3	diplacone	**6.25**	**50**	**100**	**50**
4	3´-*O*-methyl-5´-hydroxydiplacone	**-**	**50**	**50**	**25**
7	5,7-dihydroxy-6-geranylchromon	**12.5**	**>100**	**-**	**-**
	M. alba				
	9/3	**1.38** (µg/mL)	**5.4** (µg/mL)	**5.4** (µg/mL)	**2.8** (µg/mL)
	6/4	**-**	**6.75** (µg/mL)	**13.5** (µg/mL)	**27** (µg/mL)
	7/1	**-**	**12.5**	**25**	**6.25**
	10/8	**8.75** (µg/mL)	**-**	**4.37** (µg/mL)	**2.2** (µg/mL)
	10/4	**-**	**4.56**	**8.89**	**-**
11	kuwanon E	**1.6**	**25**	**25**	**25**
12	methylkuwanon E	**6.25**	**12.5**	**12.5**	**12.5**
	13	**1.6**	**12.5**	**6.25**	**12.5**
	11/5	**2.42** (µg/mL)	**2.42** (µg/mL)	**2.42** (µg/mL)	**1.21** (µg/mL)
	2/1	**25**	**50**	**50**	**25**
	2/2	**-**	**50**	**100**	**50**
	4	**6.25**	**50**	**50**	**25**
	11/2	**-**	**20** (µg/mL)	**10** (µg/mL)	**10** (µg/mL)
	6/3	**-**	**-**	**-**	**-**
	11/1	**-**	**3.31** (µg/mL)	**6.62** (µg/mL)	**1.65** (µg/mL)
	2a	**-**	**35**	**-**	**-**
	6/5	**28.75**	**-**	**57.5**	**-**
	D. cinnabari				
	A/7	**-**	**-**	**-**	**-**
	C/3	**3.6** (µg/mL)	**-**	**-**	**-**
	A/6	**-**	**-**	**29.3** (µg/mL)	**-**
	3/8-3	**-**	**>100**	**50**	**-**
	3/5	**-**	**-**	**>100**	**-**
	3/10	**3.12**	**-**	**50**	**-**
	suramin	**0.06**	**-**	**-**	**-**
	gentamycin	**-**	**25** (µg/mL)	**6.25** (µg/mL)	**0.31** (µg/mL)

The antimicrobial activity of the constituents of *M. pomifera*, reperesented by pomiferin (**14**), osajin (**13**) and macluraxanthone (**15**), is not much examined. Literature only mentions antibacterial activity of prenylated isoflavonoids isolated from the genus *Erythrina* and antitrypanosomal activity of prenylated xanthones from the root bark of *Garcinia livingstonei*.[106,123,124] The best antimicrobial activity was exhibited by **13**, which inhibited three microorganisms with MIC from 6.25 to 12.5 μM. **14** was efficacious only in antitrypanosomal assay, but in quite low concentration (MIC of 3.12 μM). **13** and **14** differ only in hydroxylation of C-3′. Compound **15** showed the best effect in antimycobacterial assay.

Among the compounds isolated from *P. tomentosa*, substances with prenyl group in their molecule proved obviously higher activity than unprenylated compounds. Compound **2** inhibited all microorganisms (except *E. coli*, as well as other compounds) with MIC 6.25. This substance is the least substituted one among others, hydroxylated only on C-5, C-7 and C-4′. Hence the hydroxylation of these three carbons indicates great antibacterial potential. Compound **5** showed the best antitrypanosomal effect with MIC 3.12 μM, its other MICs ranged from 6.25 to 12.5 μM. This substance is methoxylated on C-3′ and C-5′ and hydroxylated on C-4′. This may indicate that symetric substitution of ring B of flavonoids can increase antimicrobial effect. This can be also confirmed by compound **3**, which has lower activity than similar **2**. Another compound with rather high activity was **6** with MICs from 6.25 to 25 μM. This compound is very similar to **1**, which was non-active against all microorganisms. This may signify more beneficial substitution by geranyl chain rather than by prenyl chain in flavanones. Compound **4** exhibited good activity against *S. aureus*, but was less active against other microorganisms. In comparision with previous studies,[10] compounds **5** and **6** retain their high antimicrobial potential, while **4** showed less activity against microorganisms tested herein. Compounds **2** and **3** have not been examined in antibacterial assays before and especially the results of **2** indicate its high future potential. In this experiment, compounds **8–10** exhibited very low antimicrobial activity. Weak activity of these substances is also reported in several studies.[108,125] Compound **7** did not show any remarkable activity except good antitrypanosomal effect. Such worse activity can be caused by the absence of B-ring, which is important for biological activity of flavonoids.[126]

A lot of fractions isolated from *M. alba* possess significant antimicrobial activity. The most active ones are fractions 9/3, 10/8, 13, 11/5, 11/1 and also kuwanon E (**11**) and methylkuwanon E (**12**), the only compounds which have been identified untill present. **11** has outstanding activity against trypanosomes (MIC 1.6 μM), while **12** showed better activity

against other bacteria (MICs 12.5). Another high anti-trypanosomal effect can be observed in fractions 13 (MIC 1.6 μM) and 9/3 (MIC 1.38 μg/mL). Most of these fractions contein potentially prenylated flavonoids, which were isolated previously from *M. alba* extracts and which in principle showed high biological activity.[111,112,117,118] The possibility to compare MIC results is complicated by different units. This would be easier once all molecular weights are elucidated.

The fractions of *D. cinnabari* in general did not show outstanding biological activity except remarkable MICs of fractions C/3 and 3/10 in *T. b. brucei* assay. The only reference in literature related to *D. cinnabari* extracts or dragon's blood is identification of *D. angustifolia* substances, which had shown antimycobacterial effect.

5.1.3 Cytotoxicity Results

Table 15

	Compounds tested	Concentration	Cell line			
			DU-145	LNCaP AS	PC-3	PNT2A
			% of negative control			
	M. pomifera					
14	pomiferin	100 µM	**10**	**6**	**5**	**6**
13	osajin	100 µM	**0**	**-1**	**-2**	**1**
15	macluraxanthone	100 µM	**2**	**3**	**6**	**2**
	P. tomentosa					
5	3´-*O*-methyl-5´-*O*-methyldiplacone	100 µM	**62**	**56**	**86**	**70**
2	mimulone	100 µM	**24**	**25**	**26**	**12**
6	3´-*O*-methyldiplacol	100 µM	**89**	**78**	**93**	**85**
8	acteoside	100 µM	**97**	**92**	**93**	**92**
9	isoacteoside	100 µM	**95**	**88**	**93**	**92**
10	sesamin	100 µM	**104**	**100**	**112**	**98**
1	6-isopentenyl-3´-*O*-methyltaxifoline	100 µM	**94**	**86**	**98**	**91**
3	diplacone	100 µM	**11**	**11**	**13**	**12**
4	3´-*O*-methyl-5´-hydroxydiplacone	100 µM	**8**	**18**	**8**	**9**
7	5,7-dihydroxy-6-geranylchromon	100 µM	**114**	**107**	**120**	**92**
	M. alba					
	9/3	86.7 µg/mL	**46**	**2**	**16**	**36**
	6/4	13.4 µg/mL	**96**	**103**	**113**	**87**
	7/1	100 µM	**118**	**113**	**130**	**96**
	10/8	140 µg/mL	**3**	**1**	**3**	**2**
	10/4	285 µM	**2**	**7**	**15**	**2**
11	kuwanon E	100 µM	**42**	**64**	**78**	**81**
12	methylkuwanon E	100 µM	**30**	**32**	**18**	**48**
	13	100 µM	**2**	**3**	**3**	**3**
	11/5	38.8 µg/mL	**33**	**-1**	**5**	**3**
	2/1	100 µM	**109**	**109**	**105**	**92**
	2/2	100 µM	**106**	**108**	**113**	**100**
	4	100 µM	**31**	**25**	**41**	**57**
	11/2	20 µg/mL	**97**	**97**	**103**	**104**
	6/3	6.7 µg/mL	**97**	**95**	**103**	**102**
	11/1	26.7 µg/mL	**37**	**59**	**65**	**89**
	2a	35 µM	**101**	**106**	**99**	**102**
	6/5	230 µM	**80**	**66**	**100**	**81**
	D. cinnabari					
	A/7	100 µM	**89**	**87**	**82**	**84**
	C/3	28.8 µg/mL	**96**	**100**	**102**	**83**
	A/6	29.3 µg/mL	**100**	**101**	**102**	**92**
	3/8-3	100 µM	**113**	**110**	**113**	**97**
	3/5	100 µM	**99**	**108**	**106**	**100**
	3/10	100 µM	**98**	**110**	**104**	**97**
	Triton X	0.1%	**0**	**0**	**0**	**0**

Cell lines DU-145, LNCap AS and PC-3 cells are prostatic carcinoma cells, PNT2A is a normal prostatic cell line used as a reference. Ideal compounds would be active against the cancer cells and not against the normal cells. The lower percentage of negative control means the higher cytotoxicity. Because the results are determined in % of control and not as IC_{50}, we cannot make straight conclusions about cytotoxicity of tested compounds and also the comparison with results from previous studies of cytotoxicity is much complicated. All plants were earlier examined for cytotoxicity in several studies,[127-136] but prostatic cell lines were never used before. Overall, cytotoxicity assays against one similar type of cell lines do not practically assure general cytotoxic character of a compound. That's why related cytotoxic studies are mentioned herein.

In the group of *M.pomifera* constituents, all three compounds are cytotoxic against used cell lines. Compound **2** appears to be more cytotoxic than compound **1**. In the study of Son et al., osajin showed less cytotoxicity than pomiferin, which was even more cytotoxic against hepatocytes than standard (SAHA) and comparable against colon tumor cell lines (HCT-15). Pomiferin was also effective against human cholangiocarcinoma cells (HuCCA-1) by inducing apoptosis of the cells.[128] Although compound **3** seems to be herein cytotoxic against prostatic carcinoma cell lines, it was not cytotoxic against KB cells (nasopharynx cancer line).[115]

From *P. tomentosa* constituents, mimulone (**2**), diplacone (**3**) and 3′-*O*-methyl-5′-hydroxydiplacone (**4**) exhibited the highest cytotoxicity, the others are rather non-cytotoxic. Previous assessments of these compounds confirm our results. In the study of cytotoxicity against tobacco culture BY-2 and human leukemia cells K562,[13] **3** showed the best cytotoxicity against K562 cells, followed by **2** and **4**. On the other hand, acteoside (**8**) was highly effective against BY-2 cells, but in our experiment it did not exhibit any significant cytotoxicity. **5–7** and **9** were also tested, but they were not active enough, just like against prostatic cells. In the assay against epitheloid cell line (WB 344), **3** was more cytotoxic than **2**. The high cytotoxic potential of **3** and **4** was proved again in the study of cytotoxicity against breast cancer cells (MCF-7), T-lymphoblastic leukemia cells (CEM), multiple myeloma cells (RPMI 8226 and U 266), cervical cancer cells (HeLa), monocytic leukemia cell line (THP-1) and normal BJ fibroblast cell line.[129] **2, 5** and **6** did not show any remarkable activity in these assays. The most effective modifications of molecule structure are hydroxylation at C-3′ and C-4′ (**3**) or methoxylation at C-3′ and hydroxylation at C-4′ and C-5′ (**4**), and geranyl chain at C-6 (both **3** and **4**). Very low level of cytotoxicity of **10** is confirmed in the assays of effect on

breast cancer cells (MCF-7), non-small-cell lung cancer cells (NCI-H460) and CNS cancer cells (SF-460).[130]

In the case of *M. alba* fractions, we can see approximately half of the fractions are cytotoxic and half of them are not. In the study of cytotoxicity of *P. tomentosa* constituents, two compounds from *M. alba* (**11** and **12**) were tested too.[129] Their cytotoxicity levels were, same as here, lower than in the case of **2–4**. This is explained by disadvantageous position of geranyl chain at C-5´. Leaves extract of *M. alba* was cytotoxic against myeloid leukemia cells (HL60) and was even effective against few multidrug resistant sublines.[131] From the group of prenylated flavonoids isolated from *M. mongolica*, sanggenol M and sanggenon C were cytotoxic against human oral tumor cell lines (HSC-2 and HSG). Flavanones showed higher cytotoxicity rather than 2-arylbenzofurans.[132] The induction of apoptosis of leukemia cells (K562, B380) and mouse melanoma cells (B-16) was also observed in Cortex mori extract.[133]

Every fractions of *D. cinnabari* appear to be uncytotoxic to both cancer and normal prostatic cells. Nevertheless, several studies give information about cytotoxic effect of compounds isolated from the genus *Dracaena* against leukemia cells, hepatocytes, and epidermoid cells (KB, HepG2, HL-60 and A-431).[134,135] The least sensitive cells were cervix cancer cells (HeLa) and ovarial cancer cells (SK-OV-3).[135] *D. cinnabari* extract was active against human bladder cancer cell line ECV-304 in concentration < 30 μg/mL.[136]

6 SUMMARY

The intention of this work was to assess biological activity of several natural compounds isolated from four plants in *Department of Natural Drugs,* Faculty of Pharmacy, Brno. These plants have been used in folk medicine and compounds isolated from them have previously shown promising biological activity. In cooperation with Carol Clements from *Strathclyde Institute for Drug Research*, Strathclyde Institute of Pharmacy and Biomedicinal Sciences, University of Strathclyde, Glasgow, we contributed to extension of knowledge of activity of 36 natural compounds against susceptible microorganisms and we examined their cytotoxicity.

All plants involved in our assays contain compounds with antimicrobial activity. The most promising ones are *P. tomentosa* and *M. alba* constituents, especially from the group of prenylated flavonoids. These compounds exhibited wide range of antimicrobial activity with low values of MICs. Some of them have also low levels of cytotoxicity, which indicates their high potential in antimicrobial treatment, because it means they are toxic to parasites or bacteria and not toxic to human organism.

To summarize this work, our results support the use of natural phenolic compounds in traditional medicine and indicate a high potential for prenylated flavonoids as antimicrobial agents.

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8 ABBREVIATIONS LIST

1-D	One-dimensional
2-D	Two-dimensional
AB	Alamar Blue
AIDS	Acquired immune deficiency syndrome
BCG	Bacille Calmette-Guérin
CA	Columbia agar
CMI	Cell-mediated immunity
CNS	Central nervous system
CoA	Coenzyme A
COSY	Correlation spectroscopy
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOT	Directly observed therapy
DTH	Delayed-type hypersensitivity
EAggEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
Em	Emission
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxic *E. coli*
Exc	Excitation
H	Hydrogen
H_2O	Water
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
HMBC	Heteronuclear Multiple-Bond Correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear Single Quantum Correlation
HUS	Hemolytic-uremic syndrome

IgM	Immunoglobulin M
IUCN	International Union for Conservation of Nature
J-MOD	J-modulated spin-echo
LAM	Lipoarabinomannan
MDR-TB	Multidrug-resistant *M. tuberculosis*
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MOTT	Mycobacteria other than tubercle bacilli
MRSA	Methicillin-resistant *S. aureus*
MW	Molecular weight
NMR	Nuclear magnetic resonance
NRSA	Nafcillin-resistant *S. aureus*
NTM	Nontuberculous mycobacteria
O_2	Oxygen
OH	Hydroxyl
OMe	Methoxyl
REDOX	Reduction-oxidation reaction
Rha	Rhamnose
SEPEC	Septicemic *E. coli*
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
XDR-TB	Extensively drug-resistant *M. tuberculosis*