CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF PHARMACEULITAL BOTANY AND ECOLOGY

DIPLOMA THESIS

DETERMINATION OF AMYGDALIN CONTENT IN TRADE STONE FRUITS AND ITS BIOLOGICAL ACTIVITY IN CULTURED CANCER CELLS

Stanovení obsahu amygdalinu v tržních peckovinách a jeho biologická aktivita v buněčných kulturách karcinomu

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Hradec Králové, April 2015 Marie Janatová

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DECLARATION

"I declare that this work is my original work. All literature and other sources, that I used during my work are stated in the literature list and cited properly. This work has not been used to achieve same or another degree."

PROHLÁŠENÍ

"Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu."

V Hradci Králové, duben 2015 Marie Janatová

CONTENTS

I. LIST OF ABREVIATIONS

ACN	acetonitrile
AH	amygdalin hydrolase
AMG	amygdalin
ATB	antibiotic antimycotic solution
ATP	adenosin tri phosphate
BA	benzaldehyde
B-G	b-glucosidase
BOH	benzyl alcohol
Ca^{2+}	calcium ion
CAM	complementary and alternative medicine
CAS	Chemical Abstract Service registration number
CO2	carbon dioxide
Co-EDTA	cobalt ethylenediamine tetraacetic acid
COX-1	cyclooxygenase - 1
COX-2	cyclooxygenase -2
CV	coefficient of variation
CYP71	cyclophilin71 enzyme from cytochrome P450
CYP79	cyclophilin79 enzyme from cytochrome P450
3-CQA	3-O-caffeoylquinic acid
4-CQA	4-O-caffeoylquinic acid
CZE	Czech Republic
4-DMAP	4-dimethylaminophenol
DMEM	Duibeco`s Modified Eagle`s Medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco`s Phosphate Buffered Saline
EDTA	ethylendiamin
ESP	Spain
EtOH	ethanol
FBS	foetal bovine serum
GER	Germany
Glc	glucose
GS	Gas Chromatography

GT1	UDPG-mandelonitrile glycosiltransferase
GT2	UDPG-prunasin glycosyltransferase
H_2SO_4	sulphuric acid
HCN	cyanide acid
HDL	high-density lipoprotein
HeLa	cervical cancer cell type
HOAc	acetone
HPLC	High-performance Liquid Chromatography
HSCCC	High-Speed Countercurrent Chromatography
ICBN	International Code of Botanical Nomenclature
$KMnO_4$	Potassium permanganate
L	Carl Linnaeus
LDL	low-density lipoprotein
MCF-7	breast cancer cell type
MDL	mandelonitrile lyase
MEKC	Micellar Electronic Chromatography
MeOH	methanol
Mg^{2+}	Magnesium ion
Mill	Miller Philip
MP	mobile phase
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	sodium hydroxide
NaPB	sodium phosphate buffer
n-BuOH	n-butyl alcohol
PBS	phosphate buffer saline
PC-3	prostate cancer cell type
pH	potential of hydrogen
PH	prunasin hydrolase
PK	Pakistan
PPARs	peroxisome proliferator-activated receptors
PR	prunasin
R_F	retention factor
RPMI	RPMI-1640 medium

Rt	retention time
Scop	Scopoli Giovanni Antonio
SD	standard deviation
SRCCC	Slow Rotary Counter-Current Chromatography
TAG	triacylglyceride
TLC	Thin Layer Chromatography
UDPG	uridine diphosphate glucose
USA	United States of America
UV	ultra-violet
v/v	volume/volume ratio
Ω-6	omega-6 fatty acids
Ω-9	omega-9 fatty acids

II. INTRODUCTION

Some of the diseases which used to be lethal can nowadays be treated quite easily thanks to knowledge of the pathogen and its mechanism of action and effective medication hand in hand with complementary treatment. Nevertheless, there are still severe diseases where any absolutely effective cure is not familiar and many patients suffer.

Actual possibilities of classic cancer therapy are radiation, chemotherapy, and surgery. Combination of these can be successful in many cases and it is getting more efficient over time but it still is unpopular thanks to many adverse effects that threaten the patients. One of the problems of the classic therapy is that these methods do not target cancer cells only. To discover any medicament or a way how to treat cancer specifically is difficult because of the similarity of cancer and stem cells. All known medicaments kill these cells too and cause severe side effects. To avoid this, people are increasingly looking for complementary and alternative medicine with the hope for less side effects and more significant therapeutic benefit. Many types of alternative treatment were used across a history, some of them turned out to be useful, some of them not. One of these alternatives is a use of vitamin B17.

Because of its content of amygdalin, Vitamin B17 is called extract from apricot seeds by some dietary supplement producers. Amygdalin is a cyanogenic glycoside which can be found mostly in kernels of bitter apricots and almonds. It was isolated for the first time in 1830 by Robiquet and Boutron. More than hundred years afterwards, Laetrile, an acronym for laevorotatory and mandelonitrile, used to describe a purified form of amygdalin, was synthetized as a miraculous substance for cancer treatment that kill only tumorous cells. (1) Since then, many researches, studies and ideas came to light. There are many supporters and opponents of amygdalin cancer treatment. Some of the studies showed antitumor activity (Guidetti (1995), Navarro (1964), Morrone (1962)), but none of the studies gave detailed information on follow-up. (1) In these days, Laetrile is used by some metabolism clinics as a complementary treatment of cancer (2) and it can be bought as a dietary supplement. In the Czech Republic there is B17 APRICARC® produced by Terezia company made from apricots seeds containing amygdalin.

One of the problems connected with effectiveness of Laetrile is that, amygdalin may be confused with its substances. Considerable confusion concerning the relationship between the structure and nomenclature of laetrile and amygdalin is present. These names are often used interchangeably, although they do not describe the same product. Amygdalin is a cyanogenic glycoside plant compound, meanwhile Laetrile is an acronym of laevorotatory and mandelonitrile, used to describe a purified form of amygdalin. The chemical composition of the US-patented laetrile (D-mandelonitrile-β-glucuronide), a semisynthetic derivative of amygdalin, is different from the laetrile/amygdalin produced by Mexican manufacturers (D-mandelonitrile-β-gentiobioside), which is made from crushed apricot pits. (1)

All these ambiguities tend to veil the truth. Does amygdalin have only toxic effect on cells or could be used as medication? Is there a significant difference between the effects of amygdalin, US-patented Laetrile (D-mandelonitrile-β-glucuronide) and laetile/amygdalin produced by Mexican manufactures (D-mandelonitrile-β-gentiobioside) (1) on tumorous cells? Where is the margin of safety? Apricots seeds are eaten in numerous countries, is it risky? And how about the almonds?

III. AIM OF WORK

The aim of this study is:

- To familiarize reader with the case of Amygdalin - Laetril cancer treatment from theoretical point of view.
- To determine content of cyanogenic glycoside amygdalin in trade stone fruits with different geographic origin, with different fruit maturity and also to test amygdalin content in the dietary supplement B17 APRICARC® by Terezia company with TLC and HPLC chromatography.
- To test biological activity of amygdalin stand-alone and amygdalin activated by enzyme β-glycosidase from almonds on three types of cancer cells: cervical cancer cells HeLa, prostate cancer cells PC-3 and breast cancer cells MCF-7.
- To test the progress and time dependence of degradation of amygdalin by enzymatic hydrolysis with β-glycosidase from almonds.

IV. TEORETICAL PART

1. BOTANICAL DESCRIPTION

The Rosales is a very extensive order with many taxonomic treatments thanks to different phylogenetic studies. I used classification according to Takhtajan (3) in this work.

DIVISION: Magnoliophyta
CLASS: Magnoliopsida
ORDER: Rosales
FAMILY: Rosaceae
SUBFAMILY: Amygdaloideae
TRIBE: Amygdaleae

1.1. Family Rosaceae

The family Rosaceae was firstly named by Michael Adanson, althought the ICBN now accepts Antoine Laurent de Jussieu as the author. Jussieu combined the Linnaean concept of binominal nomenclature with Adanson`s methodology for defining groups based on multiple characteristics. The name "Rose" comes from Latin - rosa and the origin may be from Greek Rhodia, rhodon. (4)

Rosaceae family is the core eudicot group. It includes herbs, shrubs or trees which can be either rhizomatous, climbing or thorny. The herbs are mostly perennials but some annuals also exist. Plant hairs are simple or stellate, and can be present with prickles. Leaves are generally arranged spirally, but have an opposite arrangement in some species. They are usually alternate and are simple to palmately or pinnately compound. Spines may be present on midrib of leaflets and rachis of compound leaves. (4) The leaf margin is most often serrate. Pared stipules are usually present independently. In many groups of Amygdaloideae they are undeveloped. The stipules are sometimes adnate to the petiole. Glands or extrafloral nectaries may be present on leaf margin or on petiole. (5) Flowers are often showy, bisexual or infrequently unisexual. They are actinomorphic (i.e. radially symmetrical). The hypanthium ranges from flat to cupshaped or cylindrical and it is either free form or adnate to the carpels, often enlarging in fruit. Sepals are usually pentamerous, less often alternating with epicalyx lobes. Petals are usually pentamerous, while stamens are

usually 15 or more spirally arranged, sometimes 10 or fewer. Filaments are distinct or basally fused to the nectar disk. Pollen grains are tricolpate. Carpels are many or there may be only one present. They are distinct or connate, though sometimes adnate to the hypanthium. The ovary varies from superior to inferior depending on the genus. The styles are present in the same number as carpels. The fruit can be a follicle, achene, pome, drupe (Amygdaleae), aggregate or accessory with drupelets or achenes, or rarely a capsule. Endosperm is usually absent from the seed. The occurrence of numerous stamens and the absence of endosperm has been the key structural apomorphies for systematic classification. (4)

Tannins, triterpenes and triterpenoid saponins, ellagic acid, very often sorbitol as a transport carbohydrate and fruit acids are usually present in order Rosales and some tribes produce cyanogenic glycosides. (6)

The Rosaceae is the 19th largest family of plants. It includes 95 or more genera and 2830 - 3100 species. (4) The family was traditionally divided into six subfamilies: Rosoideae, Spiraeoideae, Maloideae, Amygdaloideae, Neuradoidae, and Chrysobalanoidae primarily diagnosed by the structure of fruits. (5) Nowadays, thanks to more modern views and based on genetic analysis, grouping into subfamilies and tribes is not unanimous. For example, Potter *et al.* (7) recognizes three subfamilies in Rosaceae: Rosoideae, Dryadoideae, and Spiraeoideae (Amygdaloideae), including all the genera previously assigned to Amygdaloideae and Maloideae, meanwhile Takhtajan (3), whose division is used in this thesis, divides Rosaceae into eleven subfamilies of which I will mention Spiraeoideae, Ruboideae, Rosoideae, Potentilloideae, Pyroideae (Maloideae) and Amygdaloideae.

1.2. Subfamily Amygdaloideae

Amygdaloideae contains mostly shrubs and trees. Leaves are alternate, simple or stipulate. In solitary carpel are two ovaries. Carpels are free from the floral tube. Stylodium can be present in lateral, terminal or subbasal form. Fruits are characterized mostly like drupes with one seed. Mesocarp can be fleshy indehiscent to dryish and dehiscent and indehiscent exocarp hard or coriaceous. Base chromosome number x is 8 and plants can form ectomycorrhizal association. Amygdaloideae contains four tribes: Osmaronieae, Exochordeae, Prinsepieae and Amygdaleae. (3)

1.3. Tribe Amygdaleae

The tribe Amygdaleae, with more than 450 species spread worldwide, differs from others by having a drupe, a fleshy fruit with a stony endocarp. Seeds are without endosperm. These deciduous or sometimes evergreen shrubs or trees have opposite entire leaves with petioles, deciduous stipules. Flowers are bisexual and symmetrical. The number of petals and sepals is generally 5. Extrafloral nectaries, and 10 - 20 or more free-standing stamens in one or two circles are present. Apocarpic gynoecium with single carpel is placed at the bottom of hypanthium which is free from ovaries. The colour of flowers is mostly white or pink. The Amygdaleae are in ectomycorrhizal association and their base chromosome number x is 8. (7) (8) (9)

Ellagitannins are not produced but instead of them polyphenols and cyanogenic glycosides are generated. Cyanogenic glycosides can be catalyzed by enzyme emulsin (β-glycosidase) to form cyanide. (6)

The genera *Amygdalus, Armeniaca, Cerasus, Laurocerasus, Padus*, and *Prunus* are often treated at the subgeneric or sectional level within the genus *Prunus*. (10) *Pygeum* is usually treated as a distinct genus but has sometimes also been included within *Prunus*. The three cherry genera (*Cerasus, Laurocerasus*, and *Padus*) are probably more closely related to each other than they are to the other genera in the Amygdaleae. Recent phylogenetic studies based on molecular data show that none of these three cherry genera are monophyletic, and their separation is probably not justified. (11)

1.3.1. Genus Amygdalus L.

Genus includes deciduous trees or shrubs with unarmed or spiny branches. Plants have axillary 2 or 3 winter buds, central one leaf buds, lateral ones flower buds and terminal winter buds. Deciduous stipules are present. Leaves are simple and alternate or sometimes fascicled on short branchlets, conduplicate when young with petiole. Nectaries are present at the petioles or at base of usually serrate leaf blade margin. Inflorescences are borne on axillary short branches, 1 or 2 -flowered. Bisexual, regular flowers open before or rarely with leaves. Pedicel or nearly absent or short and only rarely long. Flowers form 5 imbricate sepals and 5 white, pink or red imbricate petals inserted on rim of hypanthium. 15 up to many free-standing stamen inserted with petals have filiform

filaments. Hairy superior ovary has one locule with 2 collateral pendulous ovules and terminal, elongated style. Fruit is a hairy drupe seldom glabrous with a conspicuous longitudinal groove. Mesocarp can be fleshy which does not split or dry one which splits when it is mature. Globose or ellipsoid endocarp is hard, usually compressed with two valves. Surface of endocarp is furrowed, pitted, rugose, or smooth. (11)

The genus includes about 40 species originating from Mediterranean region till China. The species *Amygdalus* (*Prunus*) *communis* occure in varieties. Genus *Amygdalus* is grown mostly for its seeds and seed oil used in food, cosmetic and pharmaceutical industries. Var. dulcis with white flowers and amara with pink flowers differ in the amount of cyanogenic glycosides in its seeds and also in the sweet or bitter taste. (9)

1.3.2. Genus *Armeniaca* Scop.

The genus *Armeniaca* on contrary to the genus *Amygdalus* form mostly deciduous trees, rarely shrubs with unarmed or only exceptionally spiny branches. Winter bud is axillary but not terminal. Stipules, young leaves are convoluted, leaf blade margin is singly or doubly serrate. Inflorescences are formed by 1 to 3 white to pink flowers. Stamens are periginous in number 15 - 45. Ovary is one or two. Drupe is commonly laterally compressed, hairy, rarely glabrous in colour white, yellow or orange, often tinged red. Mesocarp is usually fleshy and not splitting when ripe. Its colour is yellow to orangish yellow. Endocarp compressed on both sides with smooth, scabrous, or with reticulate surface, rarely pitted. Endocarp is separate from or adnate to mesocarp. Seeds can be bitter or sweet. (11)

In this genus there are about 11 species in central and eastern Asia, and in Caucasus. Apricots are cultivated for their delicious fruit full of vitamins and other compounds and some of them also for its seeds with or without amygdalin. (9)

1.3.3. Genus *Cerasus* Mill.

Trees or shrubs of the genus *Cerasus* have unarmed branches. Axillary winter buds are 1 or 3, terminal winter buds present. Stipules soon caducous have often gland-tipped teeth. Leaf blade margin is singly or doubly serrate, rarely serrulate. Inflorescences are axillary, fasciculate-corymbose or formed by one or two flowers. Their involucre is often formed from floral bud scales. Flowers are big white or pink and have persistent scales or conspicuous bracts. The shape of hypanthium is campanulate or tubular. Sepals

are reflexed or errected. They have 15 - 50 stamens inserted on or next to rim of hypanthium. Stigma of the carpel is emarginate. Drupes which are coloured from yellowish, pale red to purplish black do not have a longitudinal groove and are glabrous and not glaucous. Succulent yellowish mesocarp, does not split. Globose to ovoid endocarp is smooth or rugose. (11)

There are about 150 species mostly in northern hemisphere. Sweet cherries *C. avium* and sour cherries *C. vulgaris,* probably formed from species *C. avium* and *C. fruticosa,* occur in many cultivars with differences in size, shape, colour and taste of their drupes. They are grown for their fruit, leaves, flowers and wood. Seeds are small and without utilization. (9) (4)

1.3.4. Genus *Persica* Mill.

Shrubs or small trees of this genus have big pink to reddish flowers, usually grown solitary. Pedicel is very short. Hypanthium is green with a red tingle, Sepals are just as long as hypanthium. Stamens are 20 - 32. Drupe colour varies from greenish white to orangish yellow with red tinge on exposed side, densely pubescent, rarely glabrous. Mesocarp can be white, greenish white, yellow, orangish yellow or red. Endocarp is uneasily separable from mesocarp, seed is laterally compressed on both sides, bitter, rarely sweet. (11) (9)

Originally, there were about 5 species of *Persica* spread in central Asia, China but now became one of the most favourite fruit grown worldwide. Cultivars differ according to whether the fruit is compressed or not, fruit surface is pubescent or glabrous and whether it has free or adnate endocarp to mesocarp. Nectarine is a peach with smooth skin mutation. Fruits contain many vitamins, sugars and fruit acids. There is a saying that the fruits with yellow mesocarp are the richest in amount of vitamins from stone-fruits. (9) (11) (4)

1.3.5. Genus *Prunus* L.

Branches of this trees or shrubs are sometimes spine-tipped. Solitary axillary winter bud is ovoid, terminal bud is not present. Membranous stipules caducous soon. Young leaves are convolute or conduplicate, petiolate or sessile. Leaf blade margin is variously crenate or coarsely serrate. Inflorescences can be solitary or in a fascicle formed by one to three flowers. Small bracts caducous soon. Hypanthium has campanulate shape. Petals are white or sometimes purple-veined, seldom greenish. 20 - 30 stamens are present in two whorls.

Filaments are unequal and anthers have yellow to reddish colour. Ovary can be sometimes villous. Fruit is a black, purple, red, green or yellow, glabrous drupe, often glaucous. Fleshy green mesocarp does not split, endocarp is laterally compressed, smooth, rarely grooved or rugose. (11)

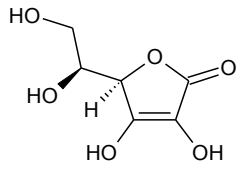
The largest genus, *Prunus* L., contains more than 40 species in Asia, Europe and in the North America. (12) Cultivated plums *P.domestica*, probably a hybrid between *P.cerasifera* and *P. spinosa,* and *P.salicina* are principal economically important species with great world production and consumption. Nowadays there are many cultivars spread out the world. (4)

2. PHYTOCHEMICAL DESCRIPTION - Amygdaleae

Plants in Amygdaleae tribe are mostly grown to harvest their fruits. Tasty fruits contain a lot of active compounds beneficial for human health. These stone fruits are full of vitamins and minerals, carbohydrates (mainly sugar alcohol sorbitol), organic acids, enzymes, carotenoids and polyphenols. Contained metabolites that have received more attention include ascorbic acid, tocopherols and tocotrienols, carotenoids and phenolics. Thanks to their antioxidant activity and ability to alleviate chronic diseases, a significant interest is also in polyphenols. There is also a focus on another group of compound, cyanogenic glycosides. (13) Amount of these phytochemicals varies not only species by species but also in one particular specie according to location of growth, current season, environmental conditions, post-harvest management and its processing. In the view of Vicetnte *et al*. (14) the highest antioxidant capacity and the richest amount of phenolic compounds can be found in black plum varieties, followed by cherries and almonds. Apricots are high in carotenoids, while peaches and nectarines have moderate level of them. (14) Cyanogenic glycosides are present mostly in bitter varieties.

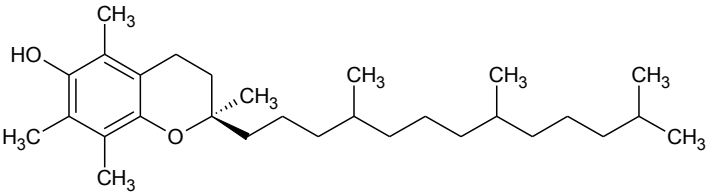
2.1. Ascorbic acid, tocopherols

Except the watersoluble antioxidant ascorbic acid (Fig.1) there is also vitamin E (tocopherols and tocotrienols) present in stone fruits. This liposoluble antioxidant is mostly represented by α-tocopherol (Fig.2). The amount of α-tocopherol in fruits range from 0.07 to 26 mg/100 g. Almonds are extremely rich in vitamin E. (14)



Ascorbic acid

Fig.1

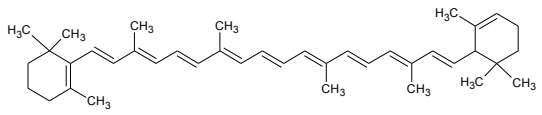


α–Tocopherol

Fig.2

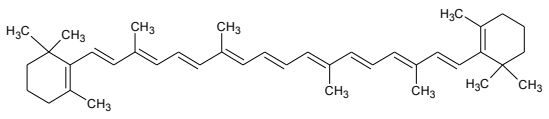
2.2. Carotenoids

Carotenoids are liposoluble terpenoids responsible for yellow and orange colour of the pulp and peel of most of *Prunus* species. It protects against photo-oxidation. There are two groups of carotenoids: one contains only carbon and hydrogen-α-carotene (Fig.3), β-carotene (Fig.4), lycopene while the other group contains its oxygenated derivates - xanthophylls such as lutein, cryptoxanthin, zeaxanthin, and violaxanthin. (14) The most common carotenoid in Amygdalae fruits is β-carotene. Also lower amount of α-carotene can be found in apricots. From xantophylls is the most common lutein (Fig.5) followed by β-cryptoxanthin (Fig.6). The highest levels of carotenoids and provitamin A show apricots, followed by cherries. The concentration is smaller in case of peaches, nectarines and plums. (14)



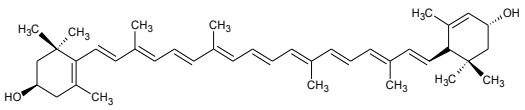
α-Carotene

Fig.3



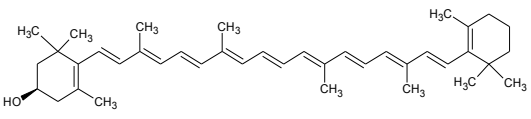
β-Carotene

Fig.4



Lutein

Fig.5

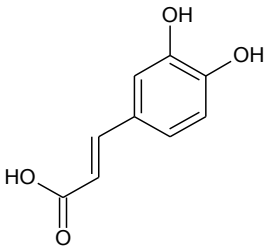


β–Cryptoxanthin

Fig.6

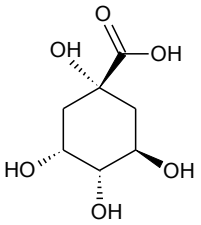
2.3. Phenolic compounds

Phenolic compounds and their glycosylated forms act as great antioxidants in plants. There is a high amount of phenolics in red and black plum varieties, cherries (in sour cherries more than in sweet ones) and almonds. Phenolic acids are derivates of hydroxybenzoic acid or hydroxycinnamic acid. Caffeic acid (Fig.7), a type of hydroxycinnamic acid, is the most frequently present in fruits where it can combine with quinic acid (Fig.8) to form ester chlorogenic acid. The most abundant isomer in cherries is 3-O-caffeoylquinic acid, (3-CQA) (Fig.9), in prunes it is the 4-CQA (Fig.10). (14)



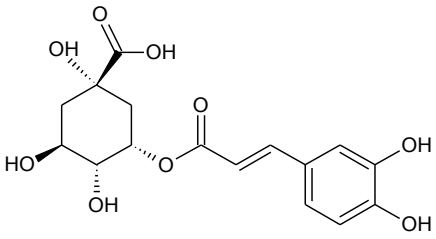
Caffeic acid

Fig.7



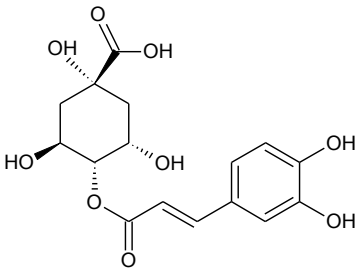
Quinic acid

Fig.8



3-CQA

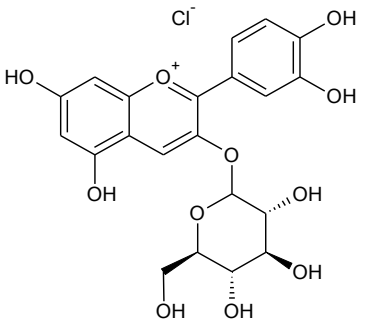
Fig.9



4-CQA

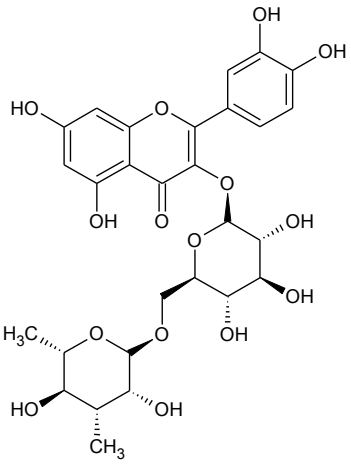
Fig.10

From flavonoids are present anthocyanins, flavones, flavonols, flavanones, and flavan-3-ols in stone fruits. Anthocyanins are responsible for red, blue, and purple colours. The most common anthocyanin found in Amygdalae is gycosylated form of cyanidin called cyanidin-3-glucoside (Fig.11) with its highest antioxidant activity between the anthocyanins. The richest stone fruit in cyanidin are cherries followed by "Black Diamond" plums and red-flesh plums. In further Amygdaleae species is the content of anthocyanins low. From flavonols, quercetin 3-rutinoside (Fig.12) is commonly found in the stone fruits, and from flavan-3-ols (+)-catechin (Fig.13) and (-)-epicatechin (Fig.14) with their gallic acid esters are present. Almonds contain low concentrations of flavanones eridictyol and naringenin. Plums and almonds are rich in proanthocyanidins, the polyphenols. (14)



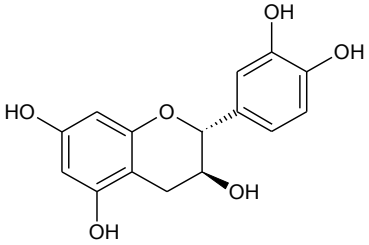
Cyanidin-3-glucoside

Fig.11



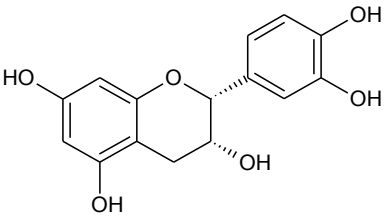
Quercetin-3-rutinoside

Fig.12



(+)-Catechin

Fig.13



(-)-Epicatechin

Fig.14

2.4. Other substances

Seed oil is rich in amino acids, proteins, minerals like calcium and magnesium, fiber, vitamin B, but mainly in saturated fatty acids like myristic, palmitic, stearic and also in significant amount of unsaturated oleic acid (Ω-9) and linoleic acid (Ω-6). (15) (16)

2.5. Cyanogenic glycosides

Cyanogenic glycosides are produced by many families (Fabaceae, Poaceae, Araceae, Euphorbiaceae, ...) including Rosaceae family too. In Amygdaleae taxa cyanogenic glycosides like prunasin which predominates in vegetative organs, and amygdalin which accumulates in the seeds can be found. (17) (18)

2.5.1. Biosynthesis of prunasin and amygdalin

Zhao (19) claims that cyanogenic glycosides are present in every part of a plant. When fruit gets mature, the highest amount of cyanogenic glycosides (mainly amygdalin) is reached in kernels of bitter varieties. (19) Prunasin is present in the vegetative parts like roots, leaves and kernels of sweet and bitter varieties. Amygdalin content is different in sweet and bitter varieties. In bitter ones higher amount of prunasin is transformed into amygdalin during fruit ripening. (20) In the view of Sánchez-Peréz *et al.* (20), biosynthesis of amygdalin begin at Phenylalanine which is catalyzed by two membrane-bound cytochrom P450 enzymes (CYP79, CYP71) to form Z-Phenylacetaldoxime and consequently Mandelonitrile. Conversion of the labile mandelonitrile into prunasin is catalyzed by soluble UDPG-mandelonitrile glycosyltransferase (GT1) and into Amygdalin by another enzyme called UDPG-prunasin glycosyltransferase (GT2). The monosaccharide combined with the α-hydroxynitrile is almost always β-D-glucose, which may itself be linked to a second monosaccharide (Fig.15). (17)

2.5.2. Degradation of prunasin and amygdalin

Amygdalin degradation starts by enzyme β-glucosidase, amygdalin hydrolase (AH) (EC 3.2.1.117), in which prunasin is formed and one molecule of Glc released. Prunasin is than hydrolized by another β-glucosidase, prunasin hydrolase (PH) (EC 3.2.1.21), to form mandelonitrile and Glc. Mandelonitrile decomposes nonenzymatically at neutral or alkaline pH and partly enzymatically by mandelonitrile lyase (MDL) (EC 4.1.2.10) into benzaldehyde with its characteristic odour of mazapan and HCN (Fig.15). (20) (21) All of these enzymes are usually present in Amygdaleae species, mostly in the kernels and become active during the mechanical damage and in aquatic environment. There is a question if this enzymatic hydrolysis is made by one enzyme β-D-glucosidase with two or three enzymatic steps or if each step is catalyzed by another enzyme. Haisman *et al.* (21) tackles this issue in their work with the result of two enzymes. Amygdalin can be also decomposed nonenzymatically by acid hydrolysis during which the glycosidic bonds break randomly, so not only Glc but also disacharide gentiobiose is produced. (21)

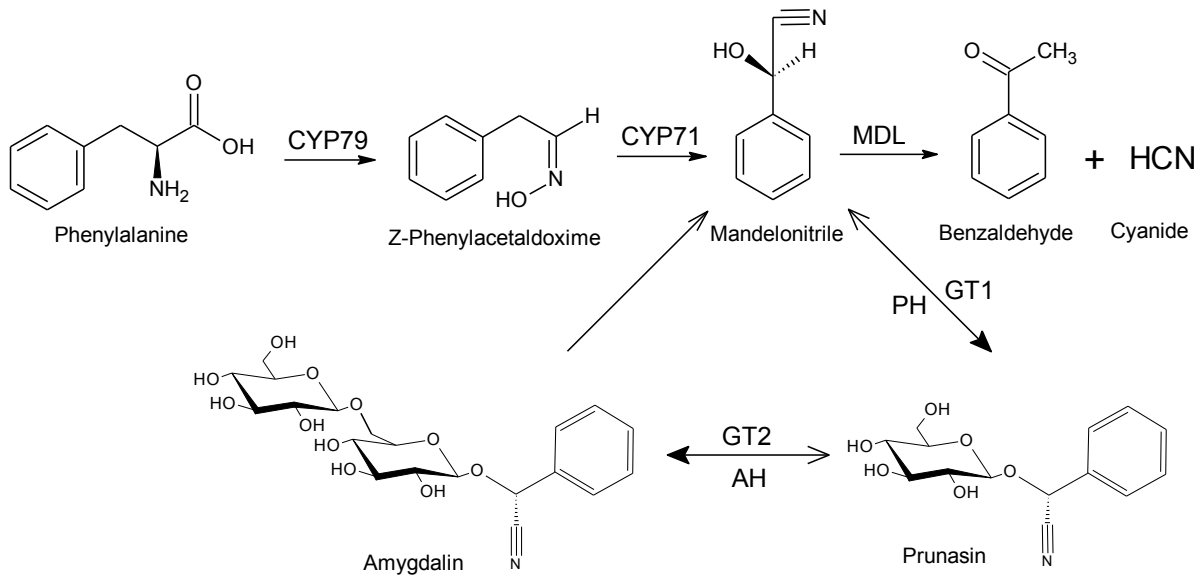


Fig.15

2.5.3. Extraction and determination of amygdalin

Cyanogenic glycosides are easily dissolved in water or in alcohol. Amygdalin is mostly extracted by reflux extraction, Soxhlet extraction or in some cases by ultrasonic extraction. (22) Amygdalin is hydrolyzed by β-glycosidase into benzaldehyde. Part of the amygdalin convertes into its epimer neoamygdalin (L-mandelonitrile-β-D-gentiobioside) when using water for extraction. Conversion of amygdalin to neoamygdalin can be inhibited by lowering the pH. This is done usually by water acidification with 0.1% citric acid. (23) To minimize hydrolyzation by enzyme present (mostly) in the inner part of seeds, sizes larger than a half of seeds should be used. (24) Methanol as an extractant does not need any treatment to protect amygdalin from degradation or epimerization. In general, the extraction efficiency tends to increase as the cutting size decreases. It meant that it is better to extract from powder than from larger pieces in the case of extraction in methanol. (25)

Cyanogenic glycosides can be analyzed directly by extraction and separation with or without mass spectroscopy, or indirectly where products like cyanide or glucose are measured degradatively. (26) The most frequent method for determination of amygdalin is by use of reversed-phase hight performance liquid chromatography (HPLC) with UV detection.

Further methods for amygdalin detection are Micellar electronic chromatography (MEKC), High-Speed Counter-Current Chromatography (HSCCC), Slow Rotary Counter-

Current Chromatography (SRCCC), potenciometry, Raman spectroscopy and also other types of spectroscopy. Gas chromatography (GS) and its combination with mass spectrometry (MS) have been used in amygdalin analysis from plasma. Thin layer Chromatography (TLC) is mostly a supplementary method showing more qualitative terms than quantitative. It can be used as a comparison method.

Another possibility how to determine cyanogenic glycosides is to decompose them into benzaldehyde and volatile cyanide and determine the cyanide content by measurement of absorbance. (27)

3. BIOLOGICAL ACTIVITIES, TOXICITY - Amygdaleae

3.1. Antioxidant activity

Oxidative stress is an imbalance between the oxidants and reductants in the organism, where oxidative forms exceed, and cause toxic effects and damages cells, lipids, proteins and DNA. During the oxidative stress the level of body`s own antioxidant like malondialdehyde, glutathion, catalase, superoxide dismutase and glutathione peroxidase increases and it consequently reduces oxidised forms. When they are saturated, the oxidised forms grow and problems occur. Thanks to the great amount of plant antioxidant - phenolic components in *Prunus* species, the level of antioxidant is enhanced and is able to reduce oxidised forms and protects organs against damage. Oxidative stress is involved in the development of cancer, atherosclerosis and heart diseases, infections, asthma... and also antioxidants can play significant role in these diseases. (28)

3.2. Antitussic, antiasthmatic activity

In official medicine the bitter almond water is used to calm cough. The enzyme emulsin and the pepsin of gastric juice can hydrolyze amygdalin to produce a small quantity of cyanic acid, HCN, which can stimulate the respiratory centre reflexively and produce a kind of tranquilizing effect. (29)

3.3. Anti-metabolic syndrome and anti-diabetes (Type 2) activity, cardioprotective activity

Studies of Seymour *et al.* (30), Munoz-Espada *et al.* (31) and Xia *et al.* (32) suggested that anthocyanins modify lipid metabolism and can reduce hyperlipidaemia by intensifying the activity of peroxisome proliferator-activated receptors (PPARs). Transcription factor PPAR can alter blood lipids, and fat metabolism in tissues and improve insulin resistance. Anthocyanins extract improves insulin sensitivity by increasing expression of PPAR, reduces plasma concentrations of LDL and TAG and elevates plasma levels of HDL and also positively influences incidence of Type 2 diabetes and cardiovascular diseases. (30) Cardioprotective activity is also related to the antioxidant phenolic compounds and their effect in decreasing the blood pressure and the peroxidation of lipids in the heart and in venous. (28)

3.4. Antinociceptive and antiinflammatory activity

Hwang *et al.* (33) shows that the intramuscular injection of amygdalin significantly reduces the formalin-induced tonic pain in both early and late phases. Anthocyanins from fruits (mostly in cherries sp.) inhibit the action of cyclooxygenase (COX-1 and COX-2) which reduces inflammatory process and the feeling of pain. Some clinical trials have confirmed that consumption of stone fruits containing anthocyanins lowers circulating concentrations of inflammation markers in healthy men and women. (14)

3.5. Other activity

Anthocyanins and other phenolic compounds (lutein) protect from neurodegenerative activities and diseases like dementia, Alzheimer disease, amnesia, moreover they support hepatoprotection (mostly *Prunus* sp.) and gastric protection, photo-protection and anti ageing effects. (28) Fruits and dry fruits are used as supplements in traditional medicine and they are often recommended to women with iron deficiency anaemia because of their high content of iron. They also evaluate the level of haemoglobin in blood and modify haematopoiesis. Bark of most species has astringent

effect which is used in cosmetics and also against haemorrhoids. Fruits act as laxative because of their high sorbitol content and the bark may stop the diarrhoea. (17)

3.6. Anticancer activity

This activity is connected with triterpenoids like betulinic acid and chlorogenic acid which showed potent antiproliferative activity toward human breast cancer cells probably by inhibition human LDL oxidation, quercetin and kaempferol as a suppressors of lung and prostate tumour cell growth. (28) Lea *et al.* (34) concluded that phenolic compounds can influence growth and differentiation in human colon cancer cells. (28) (35) Cyanogenic glycoside amygdalin is said by someone to have antitumor activity. The idea is that amygdalin is hydrolyzed by enzyme existing mostly in cancer cells to form active unstable aglycon. It breaks down and by releasing cyanide, it kills the cell. (36)

3.7. Toxicity

β-glucosidase is one of the enzymes that catalyzes the release of cyanide from amygdalin. It is present in small intestine of human and it is also found in a variety of common foods. (37) The enzyme complex emulsin from intestinal bacteria degrades the amygdalin into hydrocyanic acid, benzaldehyde, prunasin, and mandelonitrile, which are absorbed into the lymph and portal circulations. (38) The enzymatic breakdown of amygdalin occurs most rapidly in alkaline conditions. (39) The β-glucosidase may be activated in the acid environment of a gut. Amygdalin is metabolized by body to produce cyanide. (37)

Cyanide affects mitochondria where it inhibits oxydative phosphorylation. It binds reversibly to the ferric ion in mitochondrial cytochrome oxidase a_3 which normally causes reduction of oxygen to water in the fourth complex of oxidative phosphorylation and so it production of ATP by oxidative way. With cyanide poisoning anaerobic metabolism prevails and causes lactic production. Some cyanide is also bound in ferric form of haemoglobin and it makes it incapable of transporting oxygen. (40) Hydrocyanic acid is violent poison but oral intake of cyanogenic drugs does not always cause severe intoxication. This is because the range of dangerous concentrations (0.5 - 3.5 mg/kg) can only be achieved by rapid and massive ingestion of plant parts rich in cyanogenic glycosides.

In addition, the glycosides must be hydrolyzed in digestive tract. Moreover, the human organism is known to have the ability to fairly rapidly detoxify cyanides to thiocyanates using a thiosulfate sulfurtranspherase (=rhodanese). The resulting thiocyanates are eliminated in urine (30 - 60 mg/h). (17) Subletal intoxication symptoms are headache, nausea, anxiety, irritation of mucous membranes, salivation, vomitus, shortness of breath, mydriasis, lower level of consciousness, and coma. If the dose is small enough, it does not cause death. An appropriate treatment must be applied expeditiously: stomach pumping, oxygen therapy, amyl nitrite, chelation of cyanide ions by hydroxycobalamin infusion, and stimulation of detoxification mechanisms (with sodium thiosulfate). (17) So, as an antidote to cyanide overdosing hydroxycobalamin, amyl nitrite, sodium thiosulfate, 4-DMAP (4-dimethyl-aminophenol) or Co-EDTA (cobalt ethylenediamine tetraacetic acid) are used. (41) (40)

V. EXPERIMENTAL PARTS AND RESULTS

1. EXTRACTION AND DETERMINATION OF AMYGDALIN IN TRADE-STONE FRUITS

1.1. BIOLOGICAL MATERIAL

Almonds

1. *Amygdalus communis* L. convar. *dulcis* cultivar ˈMoncayoˈ - ripe (Mallorca, ESP)
2. *Amygdalus communis* L. convar. *dulcis*- unripe (Mallorca, ESP)
3. *Amygdalus communis* L. convar. *dulcis*- ripe (Mallorca, ESP)
4. *Amygdalus communis* L. convar. *dulcis*- overripe (Mallorca, ESP)
5. *Amygdalus communis* L. convar. *dulcis*- trade sort (CZE)
6. *Amygdalus communis* L. convar. *amara*- trade sort (Reformhaus Paul, Zittau, GER)

Apricots

1. *Armeniaca vulgaris* Lam.- trade sort (South Moravia, CZE)
2. *Armeniaca vulgaris* Lam.- trade sort (CZE)
3. *Armeniaca vulgaris* Lam.- trade sort (ESP)
4. *Armeniaca vulgaris* Lam.- trade sort (unknown)
5. *Armeniaca vulgaris* Lam.- ripe (PK)

Cherries

1. *Cerasus avium* L. *duracina*- unripe (Světlá pod Ještědem, North Bohemia, CZE)
2. *Cerasus avium* L. *duracina*- ripe (Světlá pod Ještědem, North Bohemia, CZE)
3. *Cerasus avium* L. *duracina*- overripe (Světlá pod Ještědem, North Bohemia, CZE)
4. *Cerasus avium* L. *juliana*- ripe (Světlá pod Ještědem, North Bohemia, CZE)
5. *Cerasus avium* L. *duracina*- unripe (Postřelmov, North Moravia, CZE)
6. *Cerasus avium* L. *duracina*- ripe (Postřelmov, North Moravia, CZE)
7. *Cerasus avium* L.- ripe (South Bohemia, CZE)
8. *Cerasus avium* L.-wild, ripe (South Bohemia, CZE)
9. *Cerasus avium* L. *juliana*- ripe (South Bohemia, CZE)
10. *Cerasus avium* L.- ripe (Jablůnka, North Moravia, CZE)

1. *Cerasus vulgaris* Mill- ripe (South Moravia, CZE)
2. *Cerasus vulgaris* Mill- ripe (Dobruška, East Bohemia, CZE)

Peaches

1. *Persica vulgaris* Mill.- ripe (Mallorca, ESP)
2. *Persica vulgaris* Mill.- ripe (Světlá pod Ještědem, North Bohemia, CZE)
3. *Persica vulgaris* Mill.- ripe (Roudnice nad Labem, Central Bohemia, CZE)
4. *Persica vulgaris* Mill.- ripe (ESP)
5. *Persica vulgaris* Mill.- wild, ripe (Světlá pod Ještědem, North Bohemia, CZE)
6. *Persica vulgaris* Mill. cultivar ˈRedhavenˈ - unripe (Turnov, North Bohemia, CZE)
7. *Persica vulgaris* Mill. cultivar ˈRedhavenˈ - ripe (Turnov, North Bohemia, CZE)
8. *Persica vulgaris* Mill. cultivar ˈRedhavenˈ - overripe (Turnov, North Bohemia, CZE)
9. *Persica vulgaris* Mill.- unripe (Plzeň, West Bohemia, CZE)
10. *Persica vulgaris* Mill.- ripe (Plzeň, West Bohemia, CZE)
11. *Persica vulgaris* Mill.- overripe (Plzeň, West Bohemia, CZE)

1n. *Persica vulgaris* Mill. var. *nucipersica* (Suckow) Holub- trade sort (unknown)

Plums

1. *Prunus domestica* L.- unripe (Světlá pod Ještědem, North Bohemia, CZE)
2. *Prunus domestica* L.- ripe (Světlá pod Ještědem, North Bohemia, CZE)
3. *Prunus domestica* L.- overripe (Světlá pod Ještědem, North Bohemia, CZE)
4. *Prunus domestica* L.- ripe (Jablůnka, North Moravia, CZE)
5. *Prunus domestica* L.- ripe (Příbram, Central Bohemia, CZE)
6. *Prunus domestica* L.- ripe (Dobruška, East Bohemia, CZE)

AP. B17 APRICARC® - dietary supplement (Terezia Co., Prague, CZE)

1.2. CHEMICALS

1.2.1. Solvents

acetone p. a. (Penta s.r.o., Prague, CZE) - HOAc

acetonitrile HPLC grade (Sigma-Aldrich Co., Saint Luis, USA)

dichloromethane p. a. (Penta s.r.o., Prague, CZE)

ethanol p. a. (Penta s.r.o., Prague, CZE) - EtOH

methanol p. a. (Penta s.r.o., Prague, CZE) - MeOH

n-butyl alcohol p. a. (Lach-Ner, Neratovice, CZE) - n-BuOH

1.2.2. Chemicals

sulphuric acid 96% (Lachema a.s.) - H_2SO_4

vanillin (Fluka Chemical Co.)

1.2.3. Standard

Amygdalin from apricot kernel ≥99%, (Sigma-Aldrich Co., Saint Luis, USA)

[(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy](phenyl)acetonitrile, CAS 29883-15-6

1.2.4. Chromatographic mobile phases (MP)

MP_{TLC}: n-BuOH : HOAc : H_2O (6:3:1) (42)

MP_{HPLC}: solvent A: H_2O (A)

solvent B: acetonitrile (B)

gradient elution: 0-2 min 5% B, 2-5 min 5-30% B, 5-8 min 30% B, 8-9 min 30-70% B, 9-11 min 70% B, 11-13 min 70-90% B, 13-19 min 90% B, 19-20 min 90-5% B, 20-24 min 5% B.

1.2.5. Detection reagents

D_{TLC}: 1% vanillin in EtOH sol. : H_2SO_4 9.6% (1:1)

D_{HPLC}: UV detection λ=215 nm (22)

1.2.6. Chromatographic material

TLC Silica gel 60 matrix F254 (Merck, Darmstadt, GER)

1.3. INSTRUMENTS

drying oven

heating mantle

rotary vacuum evaporator Büchi R-114

Soxhlet extractor

UV lamp Camag λ=254, 366 nm

HPLC Agilent 1260 Infinity

pump: G1311C

autosampler: G1329D

detector: G1365D UV/VIS

column: LiChroCART 250-4 LiChrospher 100 RP-18e, 5 µm (Merck) (22)

1.4. METHODOLOGY

Kernels from trade stone fruits were collected during the year 2012 mostly from different places around the Czech Republic or in case of almonds from Mallorca. Some of them were purchased from common street markets. If there was a possibility, collection was realized by harvesting unripe, ripe and overripe fruits. Flesh was released and seeds in endocarp were dried in an open space in a shadow for at least one month. Endocarp was broken and removed from seeds in the day of the extraction.

1.4.1. Defatting of seeds

Samples for extraction were prepared in the form of crude powder with retained seed coat. Test of the importance of defatting was used before the extraction. 2 g of apricot seed crude powder sample (S1) was purged of lipid compounds in the Soxhlet extractor by 250 mL dichlormethane for 12 cycles (approx. 2 h) in a heating mantle at about 60°C. Then the dry lipid-free crude powder sample was Soxhlet extracted using 100 mL methanol for 20 cycles (approx. 3.5 h) in a heating mantle at about 90°C. Obtained methanol extract was diluted to 100 mL. 10 mL of it were stored in a refrigerator to conserve it. 2 g of seed crude powder (the same apricot sample) (S2) was equally Soxhlet extracted by methanol and stored too. These two samples were analyzed by HPLC and results were compared. Theoretical weigh of nondefatted sample without lipid compounds had to be counted

for comparing equal units. Mean value of lipid content (41.1%), investigated from defatted samples, was used for calculations. Assuarance of this value was controlled via literature (Manzoor *et al.* (43)*,* Bachheti *et al.* (15)). Because the counted concentration of amygdalin was higher in nondefatted sample, (Tab.1) seeds defatting was not included in sample extractions.

Sample	Weigh (g)	Weigh ^{2} (g)	Area	Height	RT	Area/weigh ^{2}
S1	2.0058	2.0058*	559.5	142.8	10.209	274.29
S2	2.0284	1.1189	327.7	82	10.201	292.87

Tab. 1 Importance of defatting. Weigh ^{2}- dry lipid free crude powder weigh; (*) theoretical weigh of a nondefatted sample without lipid compounds counted from mean value of lipid content from defatted apricots samples (41.1%), (RT) retention time

1.4.2. Extraction, samples preparation

2 g of crude powder samples were Soxhlet extracted by 100 mL methanol for 20 cycles (approx. 3.5 h) in a heating mantle at about 90°C. Obtained methanol extracts were diluted to 100 mL and 10 mL of these stored in a refrigerator to conserve it. (22)

Recommended daily dosage of dietary supplement B17 APRICARC® (Terezia Co.) is 1 - 2 capsules twice a day. Four capsules (1.6 g) were extracted with the same method like samples above for analysis.

These extracts were used without any further treatment for identification of presence of amygdalin by thin layer chromatography (TLC) and it`s quantitative determination was done by reversed-phase high performance liquid chromatography (rp-HPLC).

1.4.3. Preparation of standard solution

Stock solution of amygdalin standard was prepared by dissolving 5.0 mg of amygdalin and making it up to volume 5.0 mL with MeOH in a volumetric flask.

1.4.4. Analysis

1.4.4.1. Amygdalin identification by TLC

Standard solution of amygdalin (1.0 mg/mL) prepared prior to analysis and extracted samples were positioned on the silica gel plate in the same volume. Ascending development was made in saturated chromatographic chamber with n-BuOH : HOAc : H_20 (6 : 3 : 1) as a mobile phase. The detection was investigated by scanning under the UV lamp with

wavelength 254 and 366 nm after drying of the sample without any significant visible results. Successful visualization was reached by spraying 1% ethanolic solution of vanillin with sulphuric acid 9.6% (1 : 1) and heating in 100°C until marks appeared.

1.4.4.2. Determination of amygdalin by HPLC

A calibration curve was constructed using six different concentrations of standard solution containing 0.01; 0.2; 0.4; 0.6; 0.8 and 1 mg/mL of amygdalin. These were diluted with MeOH from stock solution of amygdalin (1 mg/mL) and used as the HPLC samples. The calibration curve was constructed according to the peak area and the concentration of amygdalin from two measurements. (22)

Two HPLC determination were carried out for every extract sample. Samples were injected after dilution with MeOH (1 : 4) in cases of cherries, plums, apricots, peaches and bitter almonds. Sweet almond and B17 APRICARC® samples were injected without any further modification or dilution.

From every 100 mL of extract only 0.2 mL was used in HPLC analysis. 1 mL of 100 mL of extract was used in case of sweet almond and B17 APRICARC® samples because in these samples a lower content of amygdalin was presumed. The dilution used in calculation was 500 and 100.

Chromatographic condition

Chromatograph:	HPLC Agilent 1260 Infinity
Pump:	G1311C
Autosampler:	G1329D
Detector:	G1365D UV/VIS
Column:	LiChroCART 250-4 LiChrospher 100 RP-18e, 5 µm
Mobile phase:	H_20, acetonitrile
Elution:	gradient
Flow rate:	1 mL/min
Temperature:	30°C

1.5. RESULTS

1.5.1. TLC results

R_F of amygdalin standard was 0.53 ± 0.02. In chromatographs can be seen: presence of amygdalin in some samples, sizable marks with R_F = 0.27 ± 0.01 in every cases except the standard, uncertain spots with R_F = 0.80 ± 0.02 and others.

Chromatogram records are listed bellow (Fig.16 - Fig.22)

Mobile phase: n-BuOH : HOAc : H_2O (6 : 3 : 1)

Stationary phase: Silica gel MERCK 60 F254

Detection: 1% vanillin in EtOH sol. : H_2SO_4 9.6% (1:1), heating at 100°C

Samples were numbered like in chapter 1.1. BIOLOGICAL MATERIAL

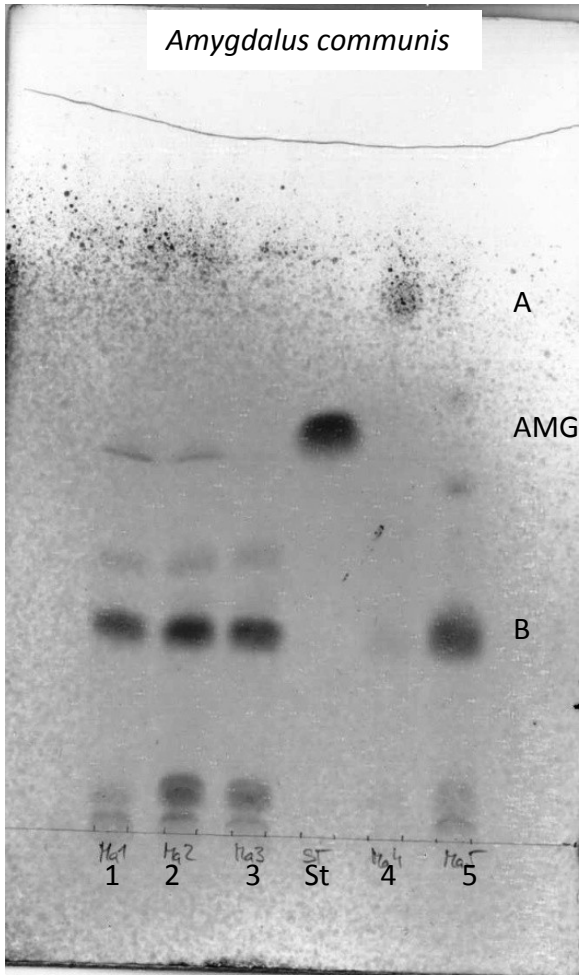


Fig.16 TLC *Amygdalus communis* var. *dulcis*
1. Moncayo ripe; 2. unripe (Mallorca); 3. ripe (Mallorca)
4. overripe (Mallorca); 5. trade sort (CZE)
A; B - might be Prunasin or Glc

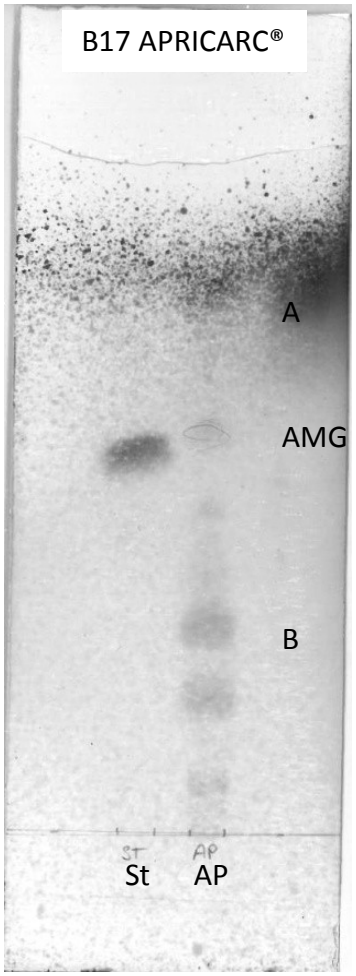


Fig.17 TLC B17 APRICARC®
AP. B17 APRICARC®
A; B - might be Prunasin or Glc

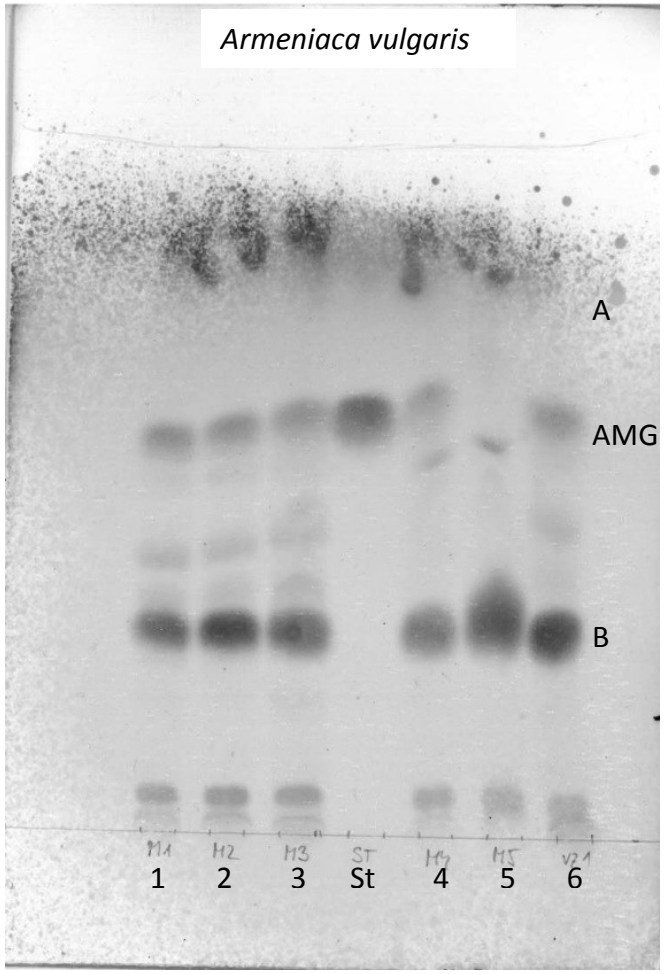


Fig.18 TLC *Armeniaca vulgaris*
1. trade sort (S. M., CZE); 2. trade sort (CZE); 3. trade sort (ESP)
4. trade sort (unknown); 5. trade sort (PK)
A; B - might be Prunasin or Glc

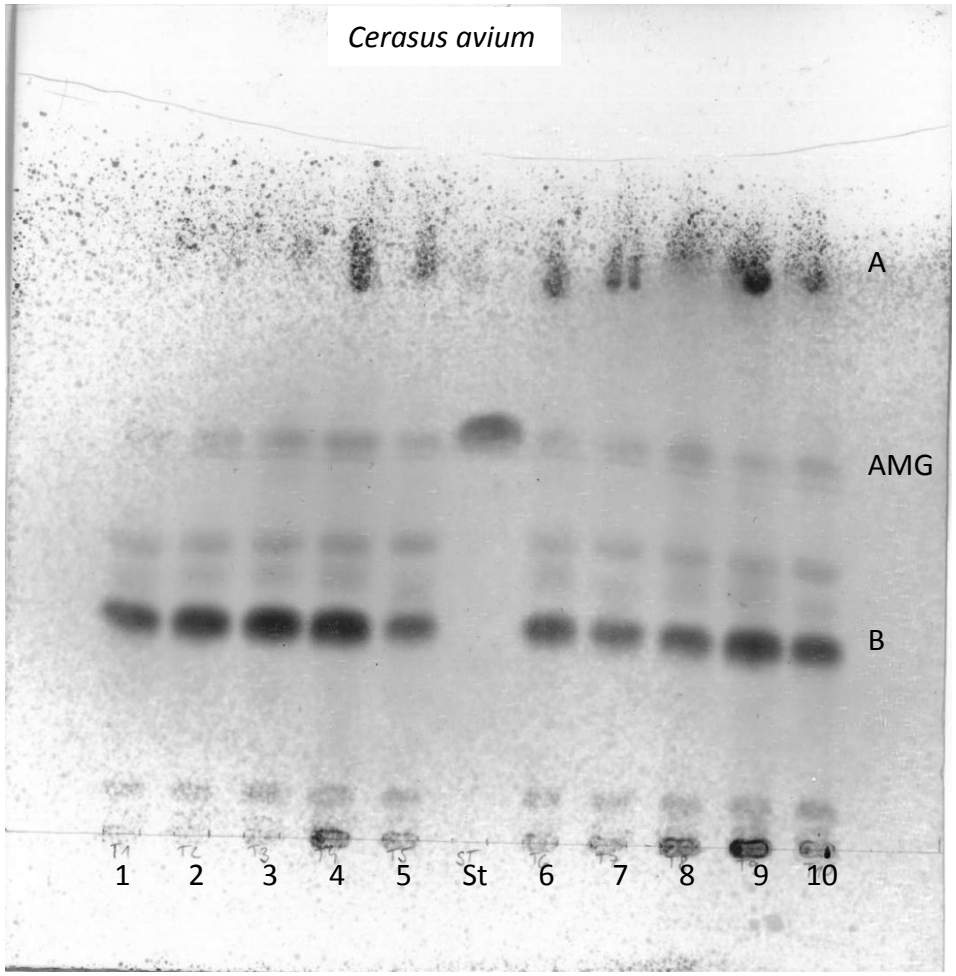


Fig.19 TLC *Cerasus avium*

1. *dur.*, unripe (Světlá p. J.); 2. *dur.*, ripe (Světlá p. J.), 3. *dur.*, overripe (Světlá p.J.);

4. *jul.*, ripe (Světlá p. J.); 5. *dur.,* unripe (Postřelmov); 6. *dur.*, ripe (Postřelmov); 7. ripe (S.B., CZE);

8. wild, ripe (S.B., CZE); 9. *jul.*, ripe (S.B., CZE); 10. ripe (Jablůnka); A; B - might be Prunasin or Glc

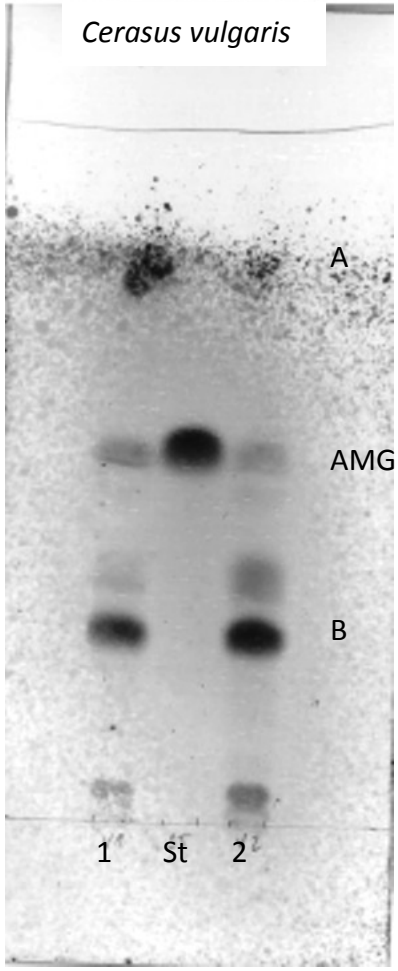


Fig.20 TCL *Cerasus vulgaris*

1. ripe (S.M., CZE); 2. ripe (Dobruška)

A; B - might be Prunasin or Glc

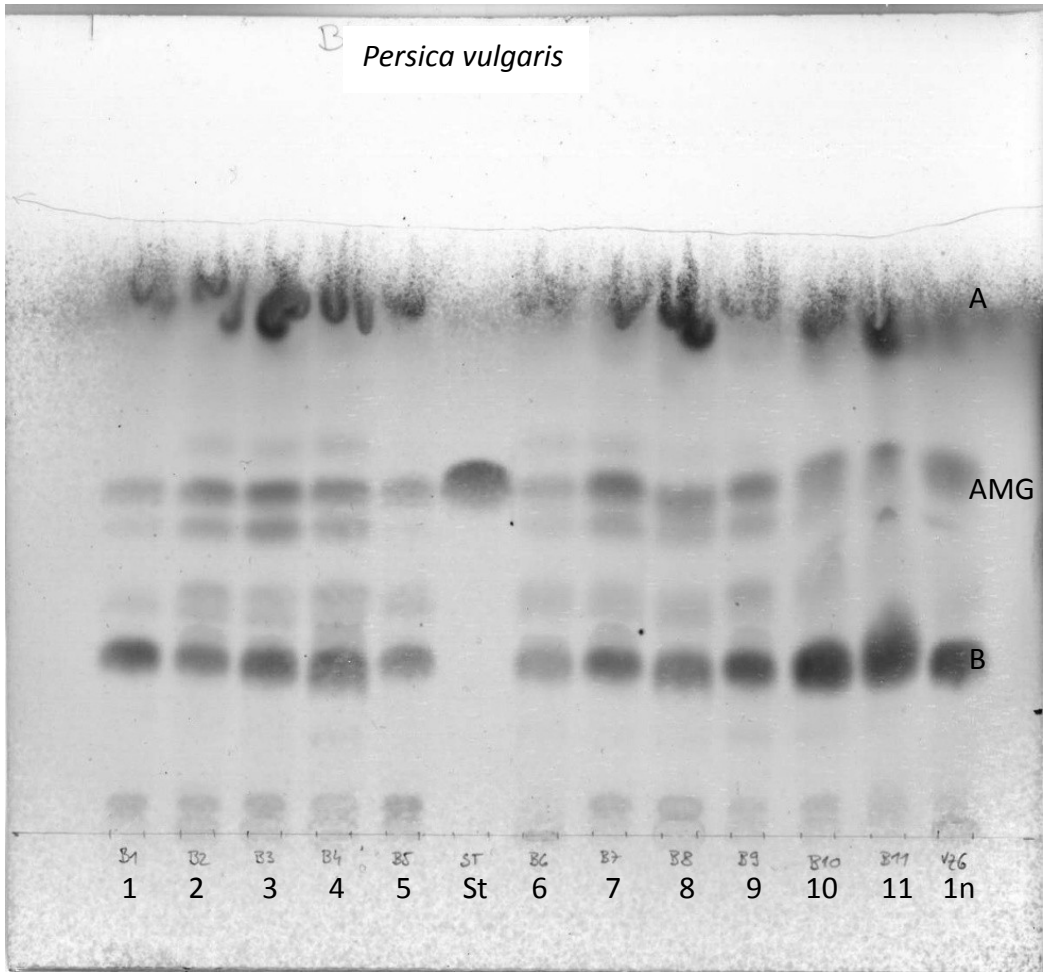


Fig.21 TLC *Persica vulgaris* A; B - might be Prunasin or Glc

1. ripe (Mallorca); 2. ripe (Světlá p. J.); 3. ripe (Roudnice n. L.); 4. ripe (ESP); 5. wild, ripe (Světlá p.J.)

6. ˈ Redhavenˈ , unripe (Turnov); 7..ˈ Redhavenˈ , ripe (Turnov); 8. ˈ Redhavenˈ , overripe (Turnov);

9. unripe (Plzeň); 10. ripe (Plzeň); 11. overripe (Plzeň); 1n. *nucipersica,* trade sort (unknown);

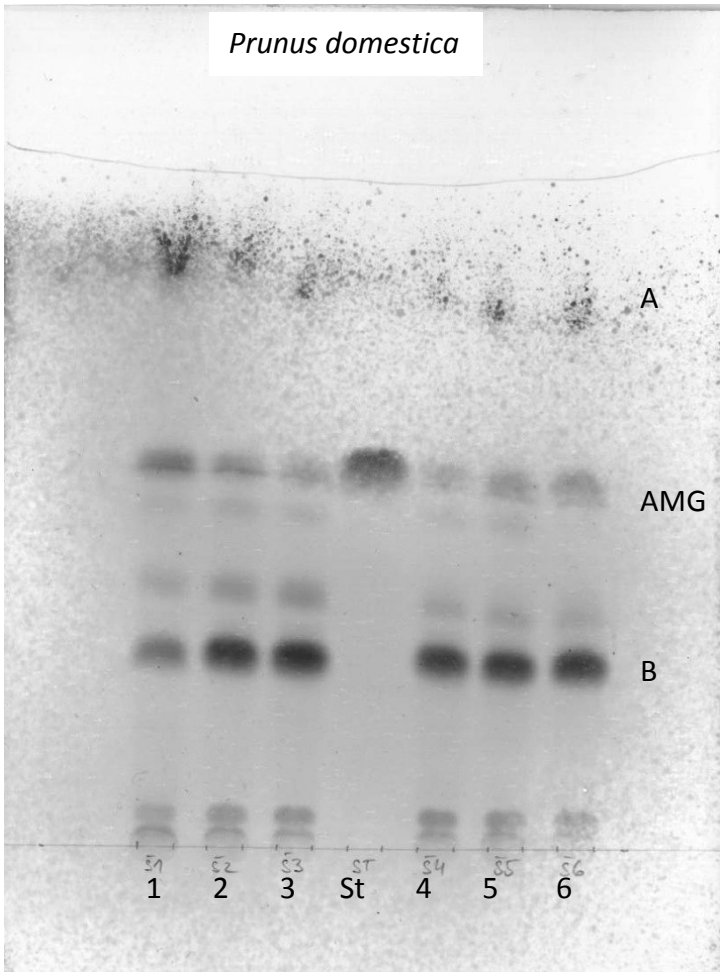


Fig.22 TLC *Prunus domestica*

1. unripe (Světlá p. J.); 2. ripe (Světlá p. J.);

3. overripe (Světlá p. J.);4. ripe (Jablůnka); 5. ripe (Příbram);

6. ripe (Dobruška); A; B - might be Prunasin or Glc

1.5.2. HPLC results

Calibration curve

215 nm	c (mg/mL)	Area	Area	Area ø	SD	CV
1	0.01	52.0	35.7	43.83	11.50	26.23
2	0.2	761.8	775.4	768.59	9.64	1.25
3	0.4	1607.7	1525.4	1566.55	58.20	3.72
4	0.6	2306.6	2298.0	2302.29	6.07	0.26
5	0.8	3114.0	3036.6	3075.32	54.75	1.78
6	1	3711.6	3721.6	3716.60	7.07	0.19

Tab.2 HPLC determination of amygdalin standards for calibration curve construction. (SD) standard deviation; (CV) coefficient of variation; (Area ø) mean values from two measurements

$$y = 3743x + 34.23$$

$$R^2 = 0.9999$$

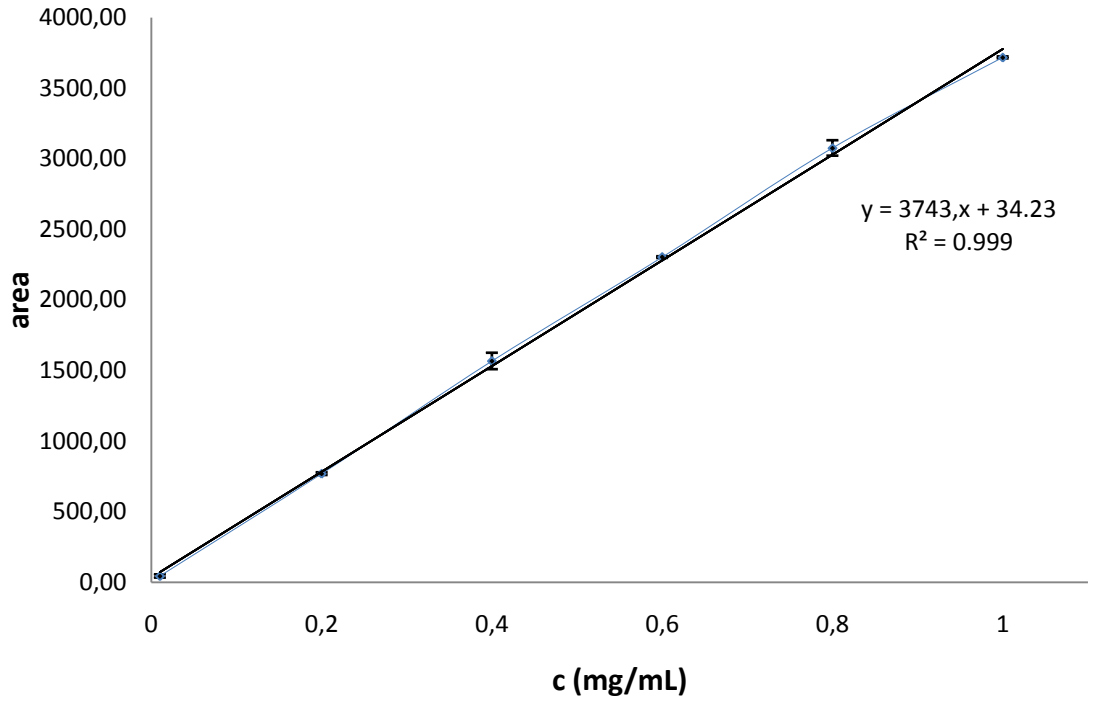
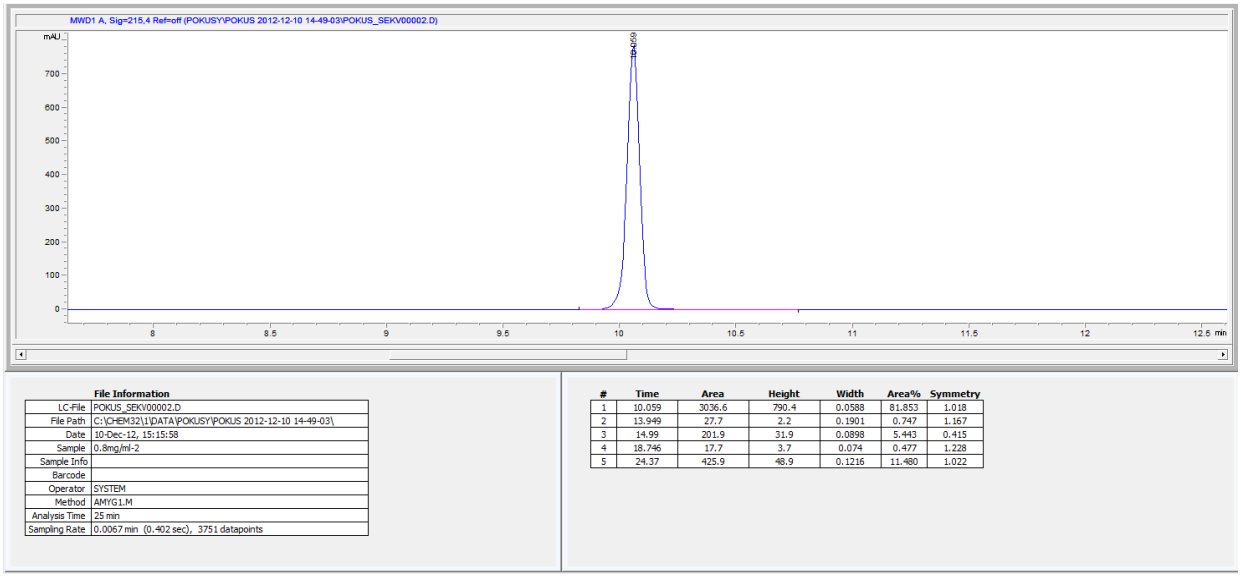


Fig. 23 Calibration curve - Amygdalin standard; (R^2) coefficient of determination; standard deviation is represented by vertical bars in graph; values are means from two measurements



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Date	10-Dec-12, 15:15:58
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Sample Info	
Barcode	
Operator	SYSTEM
Method	AMYG1.M
Analysis Time	25 min
Sampling Rate	0.0067 min (0.402 sec), 3751 datapoints

#	Time	Area	Height	Width	Area%	Symmetry
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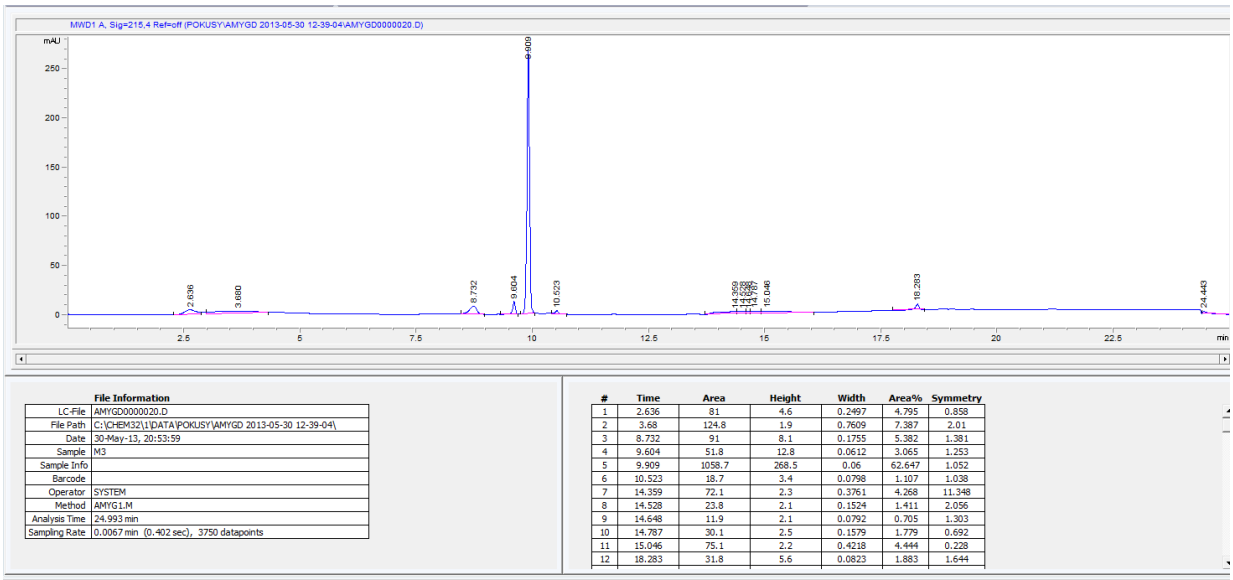
2	13.949	27.7	2.2	0.1901	0.747	1.167
3	14.99	201.9	31.9	0.0898	5.443	0.415
4	18.746	17.7	3.7	0.074	0.477	1.228
5	24.37	425.9	48.9	0.1216	11.480	1.022

Fig.24 HPLC Amygdalin standard. RT = 10.0 (retention time)



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Sample	M3
Sample Info	
Barcode	
Operator	SYSTEM
Method	AMYG1.M
Analysis Time	24.993 min
Sampling Rate	0.0067 min (0.402 sec), 3750 datapoints

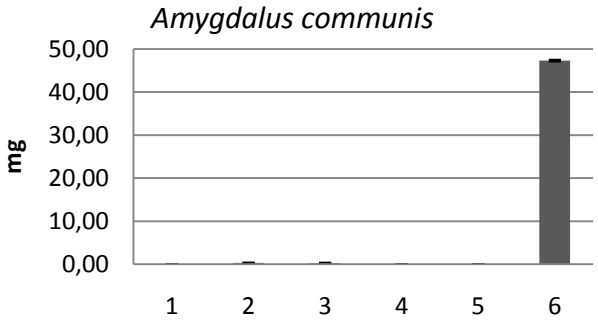
#	Time	Area	Height	Width	Area%	Symmetry
1	2.636	81	4.6	0.2497	4.795	0.858
2	3.68	124.8	1.9	0.7609	7.387	2.01
3	8.732	91	8.1	0.1755	5.382	1.381
4	9.604	51.8	12.8	0.0612	3.065	1.253
5	9.909	1058.7	268.5	0.06	62.647	1.052
6	10.523	18.7	3.4	0.0798	1.107	1.038
7	14.359	72.1	2.3	0.3761	4.268	11.348
8	14.528	23.8	2.1	0.1524	1.411	2.056
9	14.648	11.9	2.1	0.0792	0.705	1.303
10	14.787	30.1	2.5	0.1579	1.779	0.692
11	15.046	75.1	2.2	0.4218	4.444	0.228
12	18.283	31.8	5.6	0.0823	1.883	1.644

Fig.25 HPLC *Armeniaca vulgaris.* sample 3. RT = 9.9 (retention time)

Amygdalin determination in samples

Retention time Rt in HPLC analysis were about 9.9 min (Fig.24, Fig.25). The Amygdalin content in 1 g of the different genus of stone fruits kernels is shown in tables below (Tab.3-9).

Amygdalus	AMG/seeds (mg /g)	SD	CV
1	0.00	0.00	
2	0.26	0.07	25.80
3	0.23	0.07	31.69
4	0.00	0.00	
5	0.00	0.00	
6	47.30	0.13	0.28
Median	0.12	mg AMG in 1 g seeds	

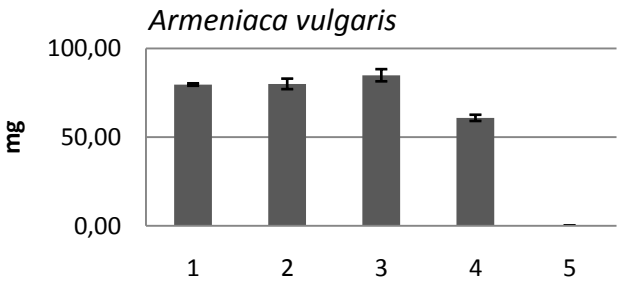


Tab.3 HPLC *Amygdalus communis* var. *dulcis*; 1.' Moncayo' ripe (Mallorca); 2. unripe (Mallorca);
3. ripe (Mallorca); 4. overripe (Mallorca); 5. trade sort (CZE);
(SD) standard deviation is represented by vertical bars in graph; (CV) coefficient of variation; values are means from two measurements

APRICARC®	AMG/seeds (mg /g)	SD	CV
AP	11.87	0.04	0.33

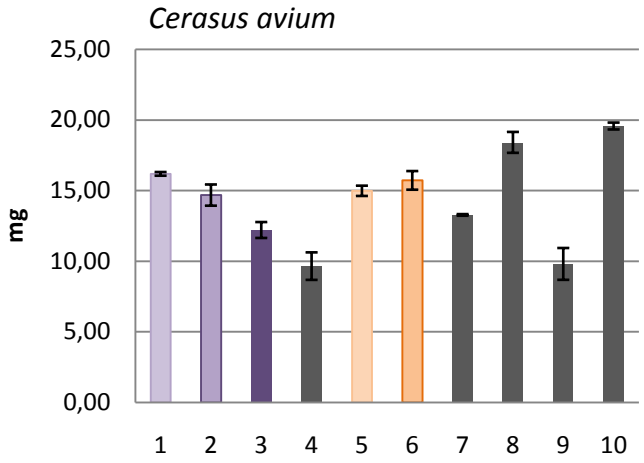
Tab.4 HPLC B17 APRICARC®*;* AP. B17 APRICARC®;
(SD) standard deviation is represented by vertical bars in graph; (CV) coefficient of variation; values are means from two measurements

Armeniaca	AMG/seeds (mg /g)	SD	CV
1	79.59	0.68	0.86
2	79.97	2.96	3.71
3	84.84	3.42	4.04
4	60.82	1.75	2.88
5	0.00	0.00	
Median	79.59	mg AMG in 1 g seeds	



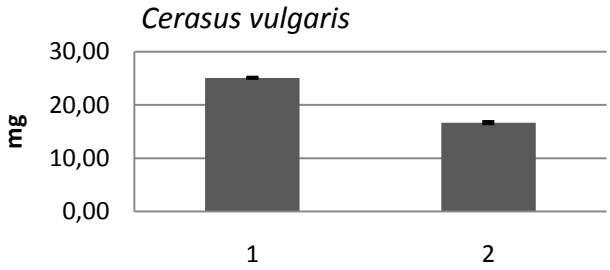
Tab.5 HPLC *Armeniaca vulgaris;* 1. trade sort (S. M., CZE); 2. trade sort (CZE); 3. trade sort (ESP); 4. trade sort (unknown); 5. trade sort (PK);
(SD) standard deviation is represented by vertical bars in graph; (CV) coefficient of variation; values are means from two measurements

Cerasus a.	AMG/seeds (mg /g)	SD	CV
1	16.18	0.13	0.80
2	14.68	0.75	5.11
3	12.21	0.56	4.61
4	9.65	0.97	10.09
5	14.98	0.36	2.42
6	15.72	0.66	4.20
7	13.27	0.06	0.43
8	18.41	0.74	4.02
9	9.80	1.13	11.49
10	19.57	0.24	1.24
Median	14.83	mg AMG in 1 g seeds	



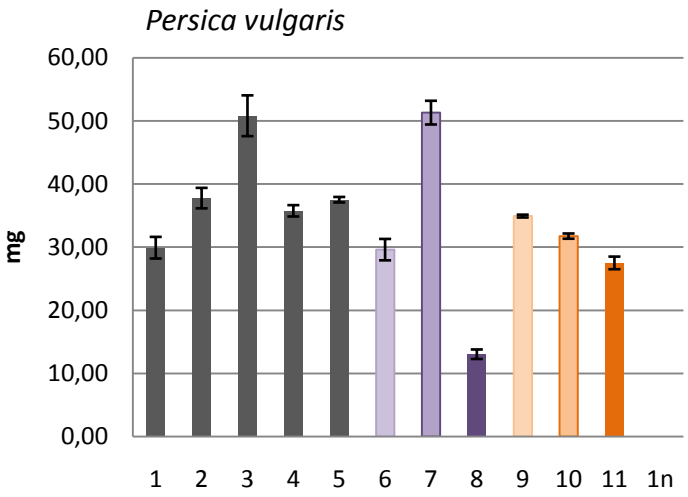
Tab.6 HPLC *Cerasus avium;* 1. *dur.*, unripe (Světlá p. J.); 2. *dur.*, ripe (Světlá p. J.), 3. *dur.*, overripe (Světlá p.J.); 4. *jul.*, ripe (Světlá p. J.); 5. *dur.,* unripe (Postřelmov); 6. *dur.*, ripe (Postřelmov); 7. ripe (S.B., CZE); 8. wild, ripe (S.B., CZE); 9. *jul.*, ripe (S.B., CZE); 10. ripe (Jablůnka);
(SD) standard deviation is represented by vertical bars in graph; (CV) coefficient of variation; values are means from two experiments

Cerasus v.	AMG/seeds (mg /g)	SD	CV
1	25.08	0.14	0.56
2	16.67	0.23	1.40
Median	20.88	mg AMG in 1 g seeds	



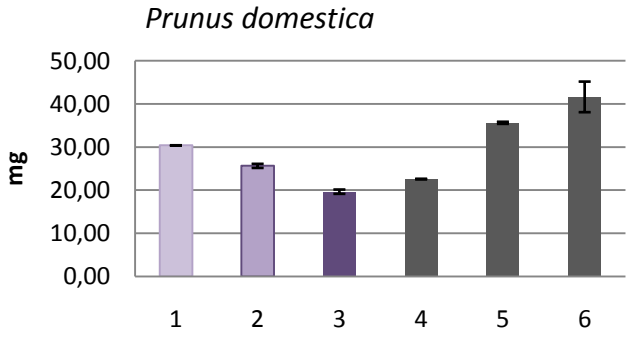
Tab.7 HPLC *Cerasus vulgaris;* 1. ripe (S.M., CZE); 2. ripe (Dobruška)
(SD) standard deviation is represented by vertical bars in graph; (CV) coefficient of variation; values are means from two experiments

Persica	AMG/seeds (mg /g)	SD	CV
1	29.91	1.72	5.74
2	37.77	1.62	4.30
3	50.82	3.23	6.36
4	35.75	0.90	2.52
5	37.52	0.44	1.16
6	29.60	1.70	5.73
7	51.31	1.88	3.66
8	13.03	0.76	5.80
9	34.92	0.22	0.64
10	31.75	0.41	1.30
11	27.50	1.00	3.63
1n	0.17		
Median	34.92	mg AMG in 1 g seeds	



Tab.8 HPLC *Persica vulgaris;* 1. ripe (Mallorca); 2. ripe (Světlá p. J.); 3. ripe (Roudnice n. L.); 4. ripe (ESP); 5. wild, ripe (Světlá p.J.); 6. 'Redhaven', unripe (Turnov); 7.. 'Redhaven', ripe (Turnov); 8. 'Redhaven', overripe (Turnov); 9. unripe (Plzeň); 10. ripe (Plzeň); 11. overripe (Plzeň); 1n. *nucipersica, trade* sort (unknown); (SD) standard deviation is represented by vertical bars in graph; (CV) coefficient of variation; values are means from two experiments

Prunus	AMG/seeds (mg /g)	SD	CV
1	30.40	0.04	0.14
2	25.67	0.47	1.84
3	19.68	0.50	2.53
4	22.61	0.06	0.28
5	35.66	0.24	0.67
6	41.67	3.55	8.52
Median	28.04	mg AMG in 1 g seeds	



Tab.9 HPLC *Prunus domestica;* 1. unripe (Světlá p. J.); 2. ripe (Světlá p. J.); 3. overripe (Světlá p. J.); 4. ripe (Jablůnka); 5. ripe (Příbram); 6. ripe (Dobruška);
(SD) standard deviation is represented by vertical bars in graph; (CV) coefficient of variation; values are means from two experiments

2. BIOLOGICAL ACTIVITY OF AMYGDALIN IN CULTURED CANCER CELLS

2.1. BIOLOGICAL MATERIAL

Breast cancer cells MCF-7 (Cell Culture Unit for CAI Medicine Biology, University of Alcalá, Alcalá de Henares, ESP)

Cervical cancer cells HeLa (Cell Culture Unit for CAI Medicine Biology, University of Alcalá, Alcalá de Henares, ESP)

Prostate cancer cells PC-3 (Cell Culture Unit for CAI Medicine Biology, University of Alcalá, Alcalá de Henares, ESP)

2.2. CHEMICALS

2.2.1. Chemicals

- acetonitrile HPLC grade (Scharlau, Barcelona, ESP) - ACN
- benzaldehyde p.a. - BA
- benzyl alcohol p.a. - BOH
- dimethyl sulfoxide PA-ACS (Panreac, Barcelona, ESP) - DMSO
- Dulbecco`s Phosphate Buffered Saline without Ca^{2+}/Mg^{2+} (Sigma-Aldrich Co., Saint Louis, USA) - DPBS
- methanol HPLC-supergradient (Scharlau, Barcelona, ESP) - MeOH
- purified water (Milli-Q, type I.)
- trypan blue 0.4% (Sigma-Aldrich Co., Saint Louis, USA)
- trypsin-EDTA solution 0.25% without Ca^{2+}/Mg^{2+} (Sigma-Aldrich Co., Saint Louis, USA)
- thiazolyl tetrazolium bromide - 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl-tetrazolium bromide (Sigma-Aldrich Co., Saint Louis, USA) - MTT

- phosphate buffer system, pH=7.4 (potassium di-hydrogen phosphate (Sigma-Aldrich Co., Saint Louis, USA), sodium hydroxide p.a. (Merck, Darmstadt, GER)) - PBS
- sodium phosphate buffer 10 mM, pH=3.1 (sodium hydroxide p.a. (Merck, Darmstadt, GER), phosphoric acid 85% p.a.) - NaPB

2.2.2. Cell grow mediums

- Duibeco`s Modified Eagle`s Medium with 4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate (D6429- Sigma-Aldrich Co., Saint Louis, USA) - DMEM
- RPMI-1640 Medium with L-glutamine and sodium bicarbonate (R8758- Sigma-Aldrich Co., Saint Louis, USA) - RPMI
- Foetal Bovine Serum (Sigma-Aldrich Co., Saint Louis, USA) - FBS
- Antibiotic Antimycotic Solution (100x) with 10.000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL (Sigma-Aldrich Co., Saint Louis, USA) - ATB

2.2.3. Standards

- Amygdalin from apricot kernel ≥99%, (Sigma-Aldrich Co., Saint Luis, USA) [(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy](phenyl)acetonitrile, CAS 29883-15-6
- β-D-Glucosidase from almonds (w.a. 135 000 Da, 7.7 U/mg) (Sigma-Aldrich Co., Saint Luis, USA) CAS 9001-22-3

2.2.4. Chromatographic mobile phase (MP)

HPLC: 10 mM sodium phosphate buffer (pH 3.1) containing 6% acetonitrile.

2.3. INSTRUMENTS

centrifuge KUBOTA 5100 - 5 min, 14 speed

CO_2 incubator Revco RC0300T-5-VBC - 37°C, 5% CO_2

laminar box Telstar PV 30/70 (vertical flow)

microscope Nikon Eclipse TS 100

Neubauer counting chamber

pH meter

spectrophotometer Bio-Tek instruments ELx800 Absorbance Reader

shaking water bath incubator

HPLC hp 1090 Series II

pump: Ternary pump

detector: 79883AX UV/VIS

column: Col KROMAPHASE C18 5.0 µm, 250 mm x 4.6 mm

2.4. METHODOLOGY

2.4.1. Buffer systems preparation

Phosphate buffer system, pH=7.4 (PBS)

27.22 g of potassium di-hydrogen phosphate was dissolved in purified water to 1000.0 mL. 250.0 mL of this 0.2 mol/L solution was added into 393.4 mL sodium hydroxide 0.1 mol/L prepared by dissolving 0.42 g of sodium hydroxide in purified water added to 1000.0 mL. Appropriate pH was measured by pH meter. (44)

Sodium phosphate buffer system 10 mM, pH=3.1 (NaPB)

3.58 g of disodium hydrogen phosphate dodecahydrate was dissolved in 100.0 mL and its pH was adjust by 85% phosphoric acid into pH=3.1. This solution was dissolved in purified water to 1000.0 mL. This preparation was modified according to Czech Pharmacopoeia. (44)

2.4.2. Samples preparation

Samples from purchased standards of Amygdalin and enzyme β-glucosidase were always prepared prior to use.

2.4.2.1. Calibration curve samples

Three stocks of amygdalin solution (5.0 mg) and three stocks of benzaldehyde solution (10.0 µL) for calibration curves were prepared. 5.0 mg of amygdalin was dissolved in 200.0 µL of MeOH and mobile phase containing NaPS and 6% of acetonitrile was added to 10.0 mL in a volumetric flask. 10.0 µL of benzaldehyde was mixed with 200.0 µL of MeOH and mobile phase containing NaPS and 6% of acetonitrile was added to 10.0 mL in a volumetric flask. Every stock solution was analysed for duplicate.

2.4.2.2. MTT assay samples

Amygdalin stock solution for MTT assay was prepared in concentration of 33.33 mg/mL by dissolving amygdalin in DMEM or RPMI medium. This was then diluted to 3/4, 1/2, 1/4 and 1/10 of stock solution.

Concentration of β-glucosidase stock solution with its enzymatic activity 7.7 U/mg was counted for to have the same activity like 250 nM of β-glucosidase (22 - 37 U/mg) used in Syrigos *et al.* (45) Calculated molar concentration was approximately 1200 nM (0.16 mg/mL). β-glucosidase stock solution for MTT assay was prepared in concentration of 3.24 mg/mL for 1200 nM samples and 4.86 mg/mL for 1800 nM samples by dissolving β-glucosidase in DMEM or RPMI medium. 1800 nM sample was then diluted to 2/3 and 1/3 of stock solution.

2.4.2.3. Samples for degradation *in vitro* studies

Amygdalin stock solution for degradation *in vitro* studies was prepared in concentration of 5.97 mg/ mL by diluting this mass portion in 1 mL PBS (pH 7.4).

β-glucosidase stock solution for degradation *in vitro* studies was prepared in concentration of 1.07 mg/mL for 1200 nM samples by diluting mass portion in 1 mL PBS (pH 7.4).

Benzyl alcohol was analysed with HPLC in concentration of 0.1 mg/ mL of MP.

2.4.3. Cell culture tests

2.4.2.4. Cells cultivation

Cervical cancer cells HeLa and breast cancer cells MCF-7 were routinely cultured in DMEM medium supplemented with 10% (v/v) foetal bovine serum (FBS) and 10% Antibiotic Antimycotic Solution.

Prostate cancer cells PC-3 were routinely cultured in RPMI medium supplemented with 10% (v/v) foetal bovine serum (FBS) and 10% Antibiotic Antimycotic Solution.

Cultivation of all type of cells took place in T-75 flasks. The medium was routinely renewed by 10 mL of tempered new medium every second day except weekends and cells were grown in a humidified atmosphere of 95% air - 5% CO_2 at 37°C. Along the experiments, cells were observed by microscope in order to detect any morphological changes.

2.4.2.5. Cells passage

Cell passage was done when needed, mostly once a week. For all types of cells the same method was used. The difference was only in medium used.

After medium was removed, cells were washed with 10 mL DPBS twice. 2 mL of Trypsin-EDTA was added into flask for detaching adherent cells. After a half of a minute of actuation, Trypsin-EDTA was removed and cells in a flask were cultivated in incubator for roughly 3 minutes. 5 mL of medium was added to floating cells and cell suspension homogenized by pipetting.

To form a new passage, 1 mL of cell suspension was pipetted in a new T-75 flask which had been already prefilled with 9 mL of medium. To prepare 24-well microplates the cell suspension was centrifuged 5 min., 14 speed and resuspended in 5.0 mL of new medium.

2.4.2.6. 24-well microplates seeding

A Neubauer counting chamber was filled by 10.0 µL of cell suspension mixed with 10.0 µL of Trypan blue. Cells were counted in four large counter squares. If the cell overlapped a top or a right ruling it was counted in whereas if it overlapped a bottom or a left ruling it was not. Trypan blue coloured dead cells were not involved into calculation. The cell concentration in cell suspension was counted according to formula:

$$c = 2\bar{x}10^4 \ (\text{mL}^{-1})$$

c ..... cell amount in 1 mL

$\bar{x}$ ..... average of viable cell number in four counted squares

2 ..... dilution by Trypan blue

10^4 ..... conversion to mL

All the cells were cultured in 24-well microplates in concentration of 20 x 10^3 cells/well in a total volume of 500 µL. The cells were left to stabilize in an incubator for 48 hours. After stabilization, all medium was removed and replaced with a new one and with amygdalin and β-glucosidase samples in a total volume of 1 mL.

2.4.4. Degradation of amygdalin - *in vitro* studies

Studies, analyzed by HPLC, were done with the samples of amygdalin with β-glucosidase for degradation *in vitro* studies. 100.0 µL of mixed amygdalin solution (0.5 mg in 83.8 µL of PBS) with 1200 nM β-D-glucosidase solution (16.2 mg in 16.2 µL of PBS) were incubated at 37°C for 0; 10; 20; 30; 60 and 120 minutes. The hydrolysis reaction was stopped

by adding 900.0 µL of mobile phase with 6% of acetonitrile containing 20.0 µL of MeOH and by freezing with liquid nitrogen. Study was performed two times and final samples were stored in a freezer and defrosted prior to HPLC analysis.

2.4.5. Analysis

2.4.5.1. MTT assay

Cell viability was determined by a colorimetric assay, using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The MTT assay is based on the conversion of yellow MTT into insoluble blue formazan crystals by living cells, which determines mitochondrial activity. Since for the most cell population the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs on cell lines. (46) The cells were seeded in a triplicate at a concentration of 20 x 10^3 cells/well on a 24-well plate. Hela, MCF-7, and PC-3 cells were treated with

- 300 µL amygdalin samples at concentrations of 1; 5; 10 mg/mL alone and with 50 µL of β-glucosidase samples at concentration of 1200 nM, (24 h, 48 h; and 4 h for HeLa)
- 300 µL amygdalin samples at concentrations of 1; 2.5; 5; 7.5; 10 mg/ml alone and with 50 µL of β-glucosidase samples at concentrations of 600 nM, 1200 nM, 1800 nM, (24 h)

and cultivated next 24 or 48 hours. After adding 100 µL of MTT labelling reagent containing 5 mg/mL of MTT in phosphate-buffered saline (PBS) to each well, the cells were incubated in a chamber for another 3.5 hours. Supernatant was removed from wells after incubation and 500 µL of DMSO was added to each well to dissolve formazan crystals formed in adherent viable cells. The viability was measured by spectrophotometr Bio-Tek-instruments ELx800 at a test wavelength of 595 nm with a reference wavelength of 690 nm. Optical density was calculated as the difference between the reference wavelength and the test wavelength. Percentage of viability was calculated as (optical density of drug treated sample/optical density of untreated sample)x100. All the experiments were performed triplicate and repeated twice. The manner of sample adding on 24-well plates is shown in tables (Tab.10, Tab.11).

Plate 0.

Control 1 mL	Control 1 mL	Control 1 mL	AMG1 300 µL	AMG1 300 µl	AMG1 300 µl
AMG2 300 µL	AMG2 300 µL	AMG2 300 µL	AMG3 300 µL	AMG3 300 µL	AMG3 300 µL
AMG1 300 µL, B-G 50 µL	AMG1 300 µL, B-G 50 µL	AMG1 300 µL, B-G 50 µL	AMG2 300 µL, B-G 50 µL	AMG2 300 µL, B-G 50 µL	AMG2 300 µL, B-G 50 µL
AMG3 300 µL, B-G 50 µL	AMG3 300 µL, B-G 50 µL	AMG3 300 µL, B-G 50 µL	B-G 50 µL	B-G 50 µL	B-G 50 µL

Tab.10 Samples application on plates. (AMG1) amygdalin in c=1 mg/mL, (AMG2) amygdalin in c=5 mg/mL, (AMG3) amygdalin in c=10 mg/mL, (B-G) 1200 nM β-glucosidase

Plate 1.

Control 1 mL	Control 1 mL	Control 1 mL	AMG1 300 µL	AMG1 300 µl	AMG1 300 µl
AMG2 300 µL	AMG2 300 µL	AMG2 300 µL	AMG3 300 µL	AMG3 300 µL	AMG3 300 µL
AMG4 300 µL	AMG4 300 µL	AMG4 300 µL	AMG5 300 µL	AMG5 300 µL	AMG5 300 µL
AMG1 300 µL, B-G1 50 µL	AMG1 300 µL, B-G1 50 µL	AMG1 300 µL, B-G1 50 µL	AMG2 300 µL, B-G1 50 µL	AMG2 300 µL, B-G1 50 µL	AMG2 300 µL, B-G1 50 µL

Plate 2.

AMG3 300 µL, B-G1 50 µL	AMG3 300 µL, B-G1 50 µL	AMG3 300 µL, B-G1 50 µL	AMG4 300 µL, B-G1 50 µL	AMG4 300 µL, B-G1 50 µL	AMG4 300 µL, B-G1 50 µL
AMG5 300 µL, B-G1 50 µL	AMG5 300 µL, B-G1 50 µL	AMG5 300 µL, B-G1 50 µL	AMG1 300 µL, B-G2 50 µL	AMG1 300 µL, B-G2 50 µL	AMG1 300 µL, B-G2 50 µL
AMG2 300 µL, B-G2 50 µL	AMG2 300 µL, B-G2 50 µL	AMG2 300 µL, B-G2 50 µL	AMG3 300 µL, B-G2 50 µL	AMG3 300 µL, B-G2 50 µL	AMG3 300 µL, B-G2 50 µL
AMG4 300 µL, B-G2 50 µL	AMG4 300 µL, B-G2 50 µL	AMG4 300 µL, B-G2 50 µL	AMG5 300 µL, B-G2 50 µL	AMG5 300 µL, B-G2 50 µL	AMG5 300 µL, B-G2 50 µL

Plate 3.

AMG1 300 µL, B-G3 50 µL	AMG1 300 µL, B-G3 50 µL	AMG1 300 µL, B-G3 50 µL	AMG2 300 µL, B-G3 50 µL	AMG2 300 µL, B-G3 50 µL	AMG2 300 µL, B-G3 50 µL
AMG3 300 µL, B-G3 50 µL	AMG3 300 µL, B-G3 50 µL	AMG3 300 µL, B-G3 50 µL	AMG4 300 µL, B-G3 50 µL	AMG4 300 µL, B-G3 50 µL	AMG4 300 µL, B-G3 50 µL
AMG5 300 µL, B-G3 50 µL	AMG5 300 µL, B-G3 50 µL	AMG5 300 µL, B-G3 50 µL	B-G1 50 µL	B-G2 50 µL	B-G2 50 µL
B-G2 50 µL	B-G2 50 µL	B-G2 50 µL	B-G3 50 µL	B-G3 50 µL	B-G3 50 µL

Tab.11 Samples application on plates. (AMG1) amygdalin in c=1 mg/mL, (AMG2) amygdalin in c= 2.5 mg/mL, (AMG3) amygdalin in c=5 mg/mL, (AMG4) amygdalin in c=7.5 mg/mL, (AMG5) amygdalin in c=10 mg/mL, (B-G1) 600 nM β-glucosidase, (B-G2) 1200 nM β-glucosidase, (B-G3) 1800 nM β-glucosidase.

2.4.5.2. Determination by HPLC

Calibration curve was constructed using six different concentrations of standard solution containing 12.5; 25; 50; 100; 250 and 500 µg/mL of amygdalin. Calibration curve of benzaldehyde was constructed using five different concentrations of standard solution containing 10; 25; 50; 75; 100 µg/mL of benzaldehyde. These were diluted with mobile phase from stock solution of amygdalin (0.5 mg/mL) and used as the HPLC samples. The calibration curve was constructed according to the peak areas and the concentrations of amygdalin from six measurements.

Chromatographic condition

Chromatograph:	HPLC HP 1090 Series II
Pump:	MCO-REV-E3321
Autosampler:	MCO-REV-E3321
Detector:	UV/VIS λ=214 nm
Column:	Col KROMAPHASE C18 (4.6 mm x 250 mm, 5 µm)
Mobile phase:	10 nM NaPS (pH 3.1) containing 6% ACN
Injection volume:	10 µL
Flow rate:	1 mL/min
Temperature:	30°C

2.4.5.3. Statistical analysis

All the MTT tests were performed in triplicate and repeated two times. Results were expressed graphically as means with calculated standard deviations (SD) represented by vertical bars. Statistical significance was applied using Microsoft office Excel (2007). The difference between a control and one group was analyzed by one way ANOVA. Duncan`s multiple range test was used to identify significant differences. Results with different letters were considered statistically significant ($p < 0.05$).

2.5. RESULTS

2.5.1. Calibration curves

Calibration curve - Amygdalin

214 nm	c (µg/mL)	Area ø	SD	CV
1	12.5	928.1	42.7	4.60
2	25	2027.7	73.5	3.62
3	50	3808.1	92.6	2.43
4	100	7449.7	304.7	4.09
5	250	18716.2	489.0	2.61
6	500	37415.5	1145.4	3.06

Tab.12 Amygdalin calibration curve results. (SD) standard deviation; (CV) coefficient of variation; values are means of six measurements

$$y = 74.698x + 52.743$$

$$R^2 = 0.999$$

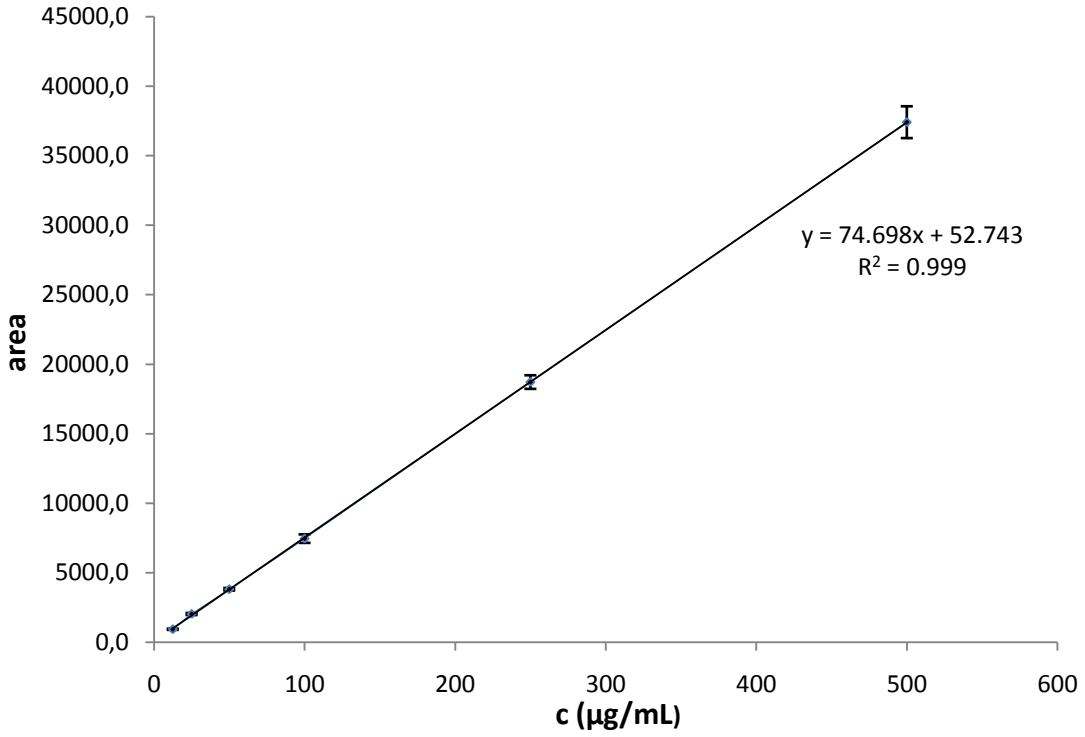


Fig.16 Calibration curve - Amygdalin standard. Standard deviation is represented by vertical bars in graph; (R^2) coeficient of determination; values are means from six measurements

Calibration curve - Benzaldehyde

214 nm	c (µg/mL)	Area ø	SD	CV
1	10	1581.6	314.7	19.90
2	25	4512.4	389.1	8.62
3	50	9272.4	554.1	5.98
4	75	13524.8	1304.4	9.64
5	100	16376.9	798.7	4.35

Tab.13 Benzaldehyde - Calibration curve results. (SD) standard deviation; (CV) coefficient of variation; values are means of six measurements

$$y = 185.06x - 169.35$$

$$R^2 = 0.999$$

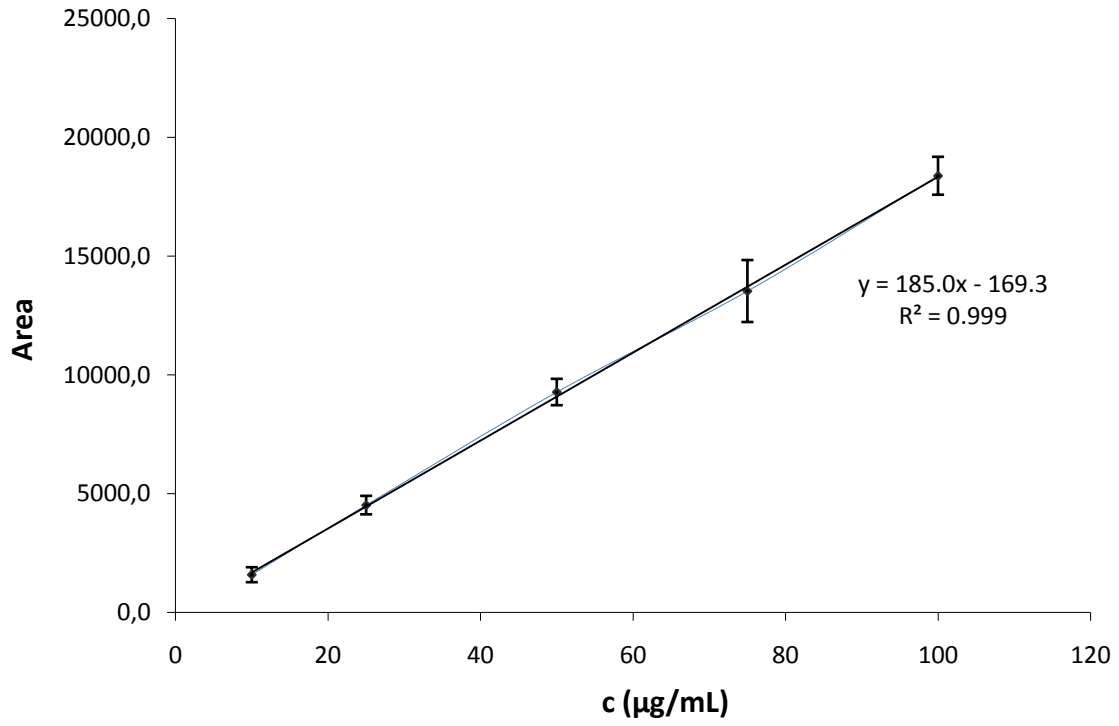


Fig.27 Calibration curve - Benzaldehyde standard. Standard deviation is represented by vertical bars in graph; (R^2) coeficient of determination; values are means from six measurements

2.5.2. MTT assay

Results of cell viability of three types of cell - HeLa, MCF-7 and PC-3 are represented by graphs (Fig.28 - Fig.38) depicting incubation with active substances for 24 h, 48 h, 24 h for more concentrations of amygdalin and β-glucosidase and for 4 h at HeLa cells to see the main time of cytotoxic activity. Compared with the control, inhibition of the cell proliferation increased significantly with increasing amygdalin concentration and incubation time in the range of 0 - 10 mg/mL, and 4 - 48 h, respectively. The presence of the β-glucosidase hydrolysis on amygdalin resulted in a significant increase in growth inhibition.

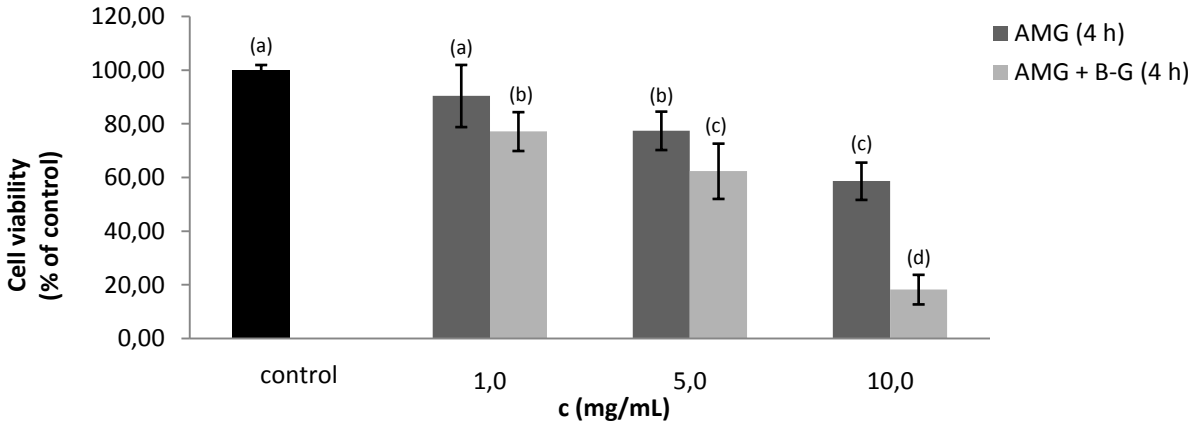


Fig.28 Viability of HeLa cells after treatment for 4 h. Concentration of β-glucosidase was 1200 nM. First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase letters (a-d) are significantly different ($p < 0.05$) among different groups.

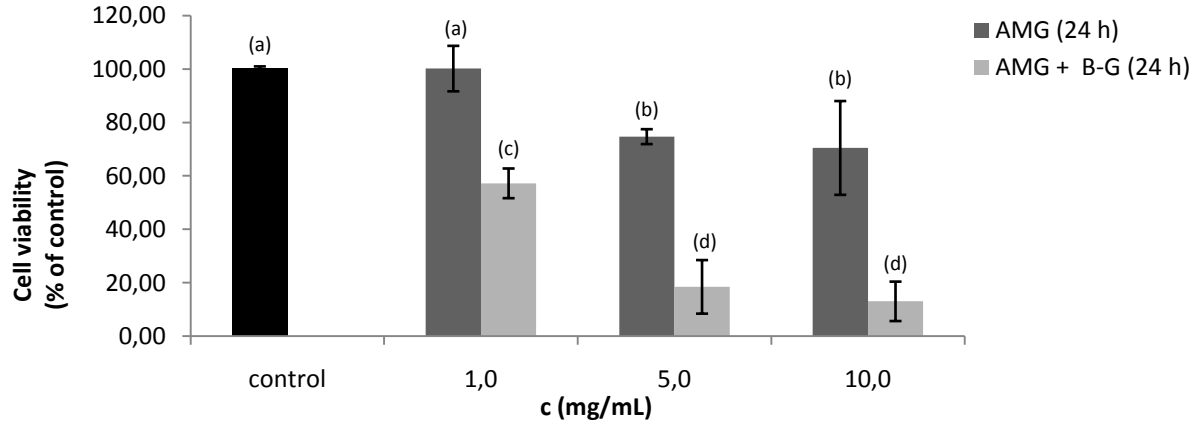


Fig.29 Viability of HeLa cells after treatment for 24 h. Concentration of β-glucosidase was 1200 nM. First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase letters (a-d) are significantly different ($p < 0.05$) among different groups.

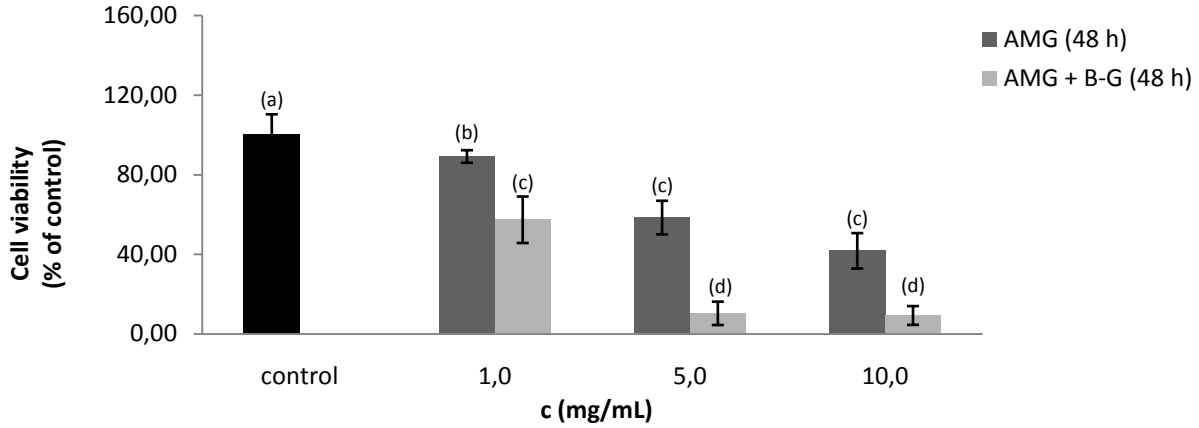


Fig.30 Viability of HeLa cells after treatment for 48 h. Concentration of β-glucosidase was 1200 nM. First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase letters (a-d) are significantly different ($p < 0.05$) among different groups.

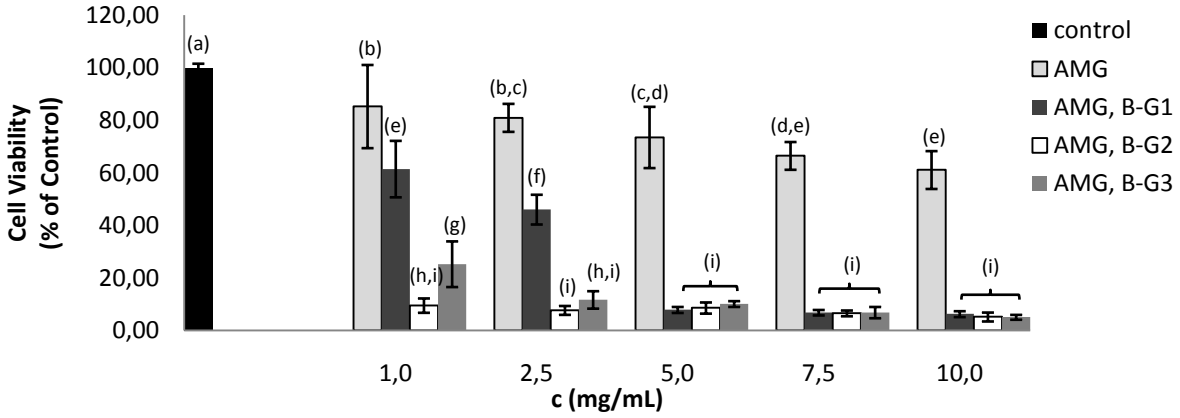


Fig.31 Viability of HeLa cells after treatment for 24 h. Concentration of β-glucosidase was 600 nM (B-G1), 1200 nM (B-G2), 1800 nM (B-G3). First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase letters (a-i) are significantly different ($p < 0.05$) among different groups.

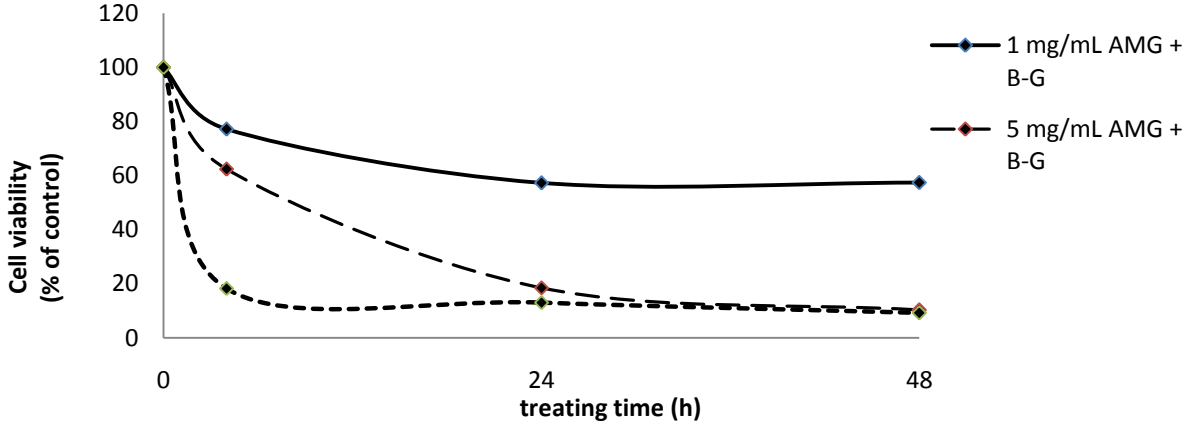


Fig.32 Enzymatic activation of amygdalin according to cell viability of HeLa cells in time.

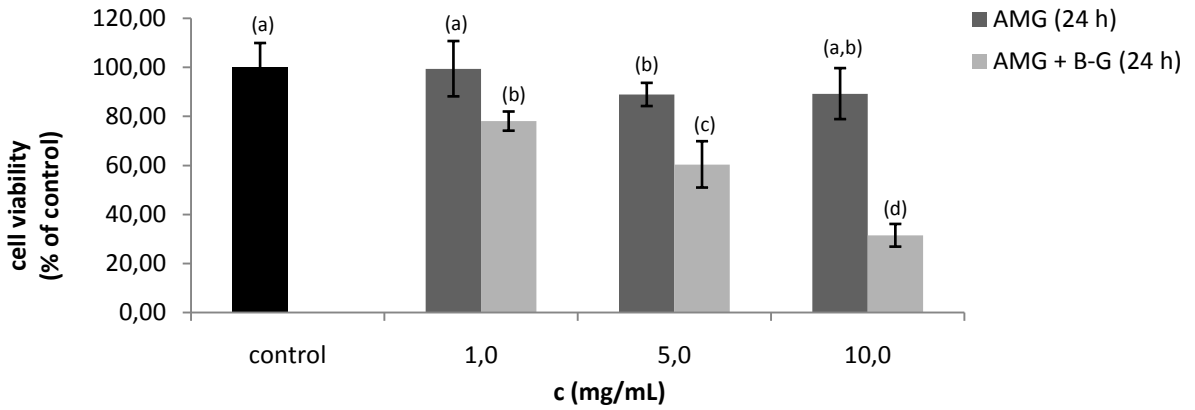


Fig.33 Viability of PC-3 cells after treatment for 24 h. Concentration of β-glucosidase was 1200 nM. First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase letters (a-d) are significantly different ($p < 0.05$) among different groups.

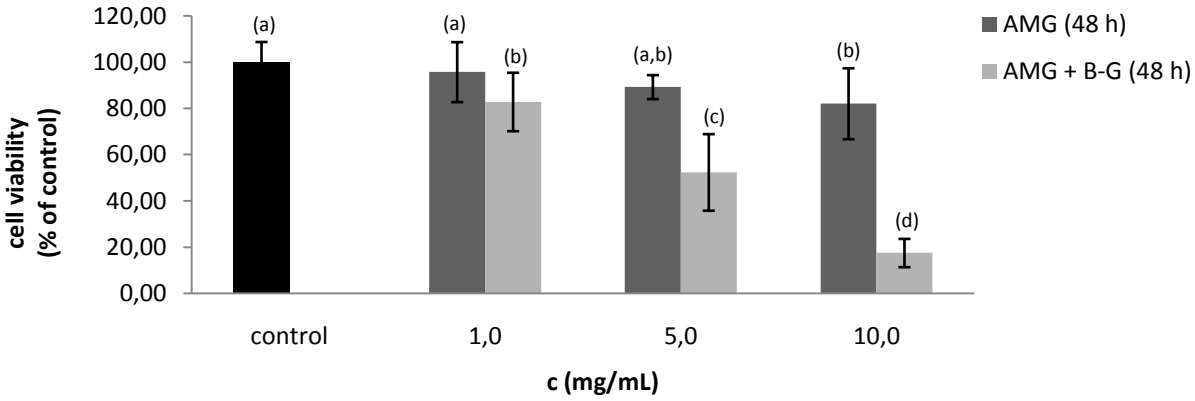


Fig.34 Viability of PC-3 cells after treatment for 48 h. Concentration of β-glucosidase was 1200 nM. First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase letters (a-d) are significantly different ($p < 0.05$) among different groups.

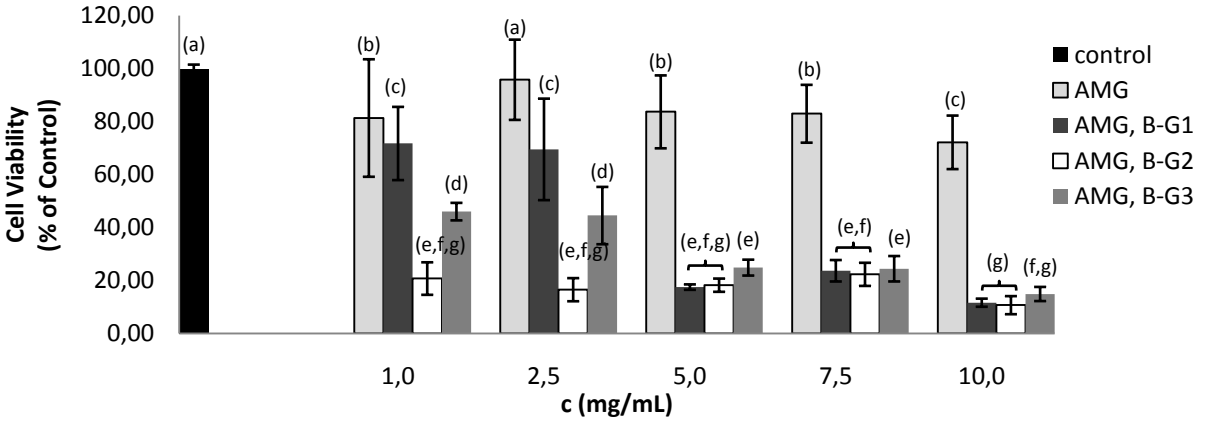


Fig.35 Viability of PC-3 cells after treatment for 24 h. Concentration of β-glucosidase was 600 nM (B-G1), 1200 nM (B-G2), 1800 nM (B-G3). First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase letters (a-g) are significantly different ($p < 0.05$) among different groups.

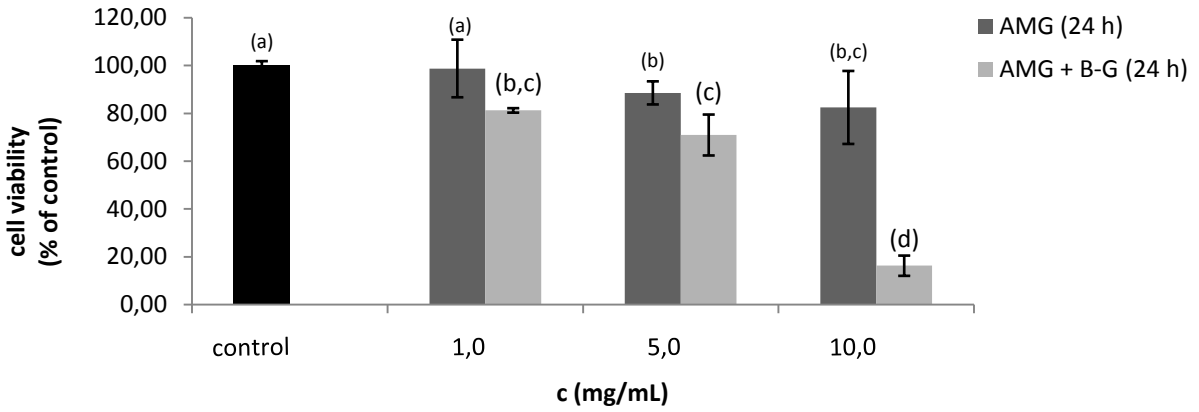


Fig.36 Viability of MCF-7 cells after treatment for 24 h. Concentration of β-glucosidase was 1200 nM. First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase letters (a-d) are significantly different ($p < 0.05$) among different groups. (*) represents $p < 0.05$ compared to control.

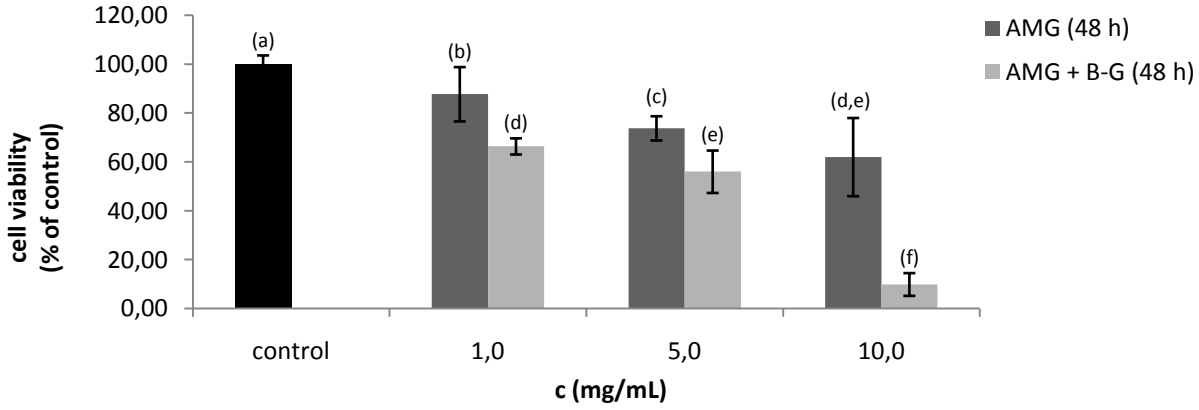


Fig.37 Viability of MCF-7 cells after treatment for 48 h. Concentration of β-glucosidase was 1200 nM. First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase letters (a-f) are significantly different ($p < 0.05$) among different groups.

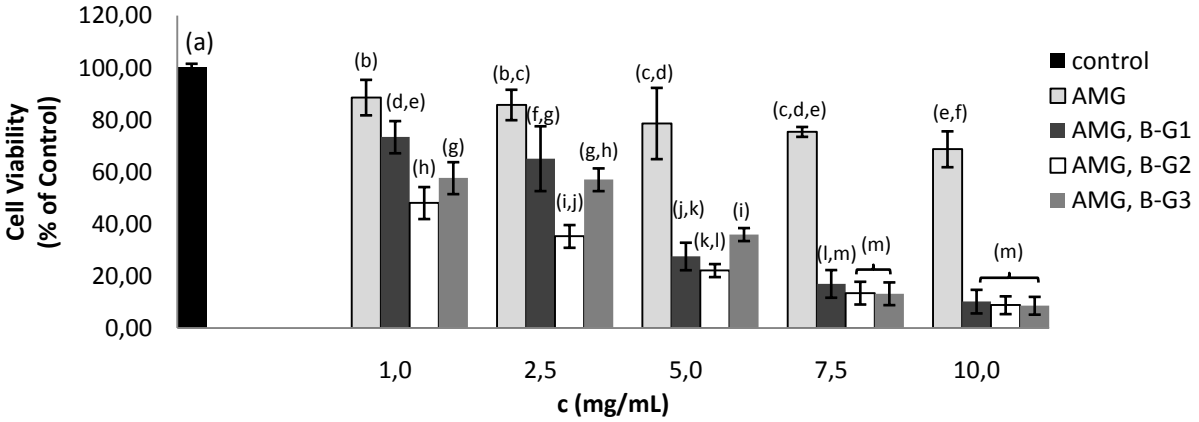


Fig.38 Viability of MCF-7 cells after treatment for 24 h. Concentration of β-glucosidase was 600 nM (B-G1), 1200 nM (B-G2), 1800 nM (B-G3). First column represents controls contained test cells and culture medium, but no test compound. Data are expressed as mean (n=6). Standard deviation is represented by vertical bars in graph. Values in column with different lowercase (a-m) letters are significantly different ($p < 0.05$) among different groups.

2.5.3. Amygdalin degradation - *in vitro* studies

In vitro study of enzymatic degradation of amygdalin showed that β-glucosidase hydrolyzes amygdalin during the first hour of process into benzaldehyde and other compounds that could be prunasin, mandelonitrile or benzyl alcohol. (47) HPLC chromatographs with amygdalin, benzaldehyde and benzyl alcohol standards and degradation of amygdalin were analyzed after 10 min., 20 min., 30 min., 1 h. and 2 h. The results are presented below. (Fig.51 - Fig.58)

	Rt (min)	Area ø	SD	CV	c (µg/mL)
10 min	5.8	19427.75	267.55	1.38	259.38
	9.9	3943.10	301.10	7.64	
	22.5	4211.70	100.00	2.37	23.67
20 min	5.8	10962.10	457.20	4.17	145.05
	9.9	6780.40	308.30	4.55	
	22.5	6146.90	330.70	5.38	34.13
30 min	5.8	3448.65	765.05	22.18	45.46
	9.9	9398.90	162.40	1.73	
	22.5	6926.05	81.85	1.18	38.34
1 h	5.8	194.95	20.75	10.64	1.90
	9.9	7345.10	571.30	7.78	
	22.5	5339.45	27.85	0.52	29.77
2 h	5.8	62.40	13.60	21.79	0.13
	9.9	4919.45	1696.35	34.48	
	22.5	2772.65	806.65	29.09	15.90

Tab.13 HPLC analysis of amygdalin degradation. Concentration of amygdalin and benzaldehyde was calculated from calibration curves. (RT) retention time; (Area ø) mean of peak area value; (SD) standard deviation; (CV) coefficient of variability; means are values from two measurements

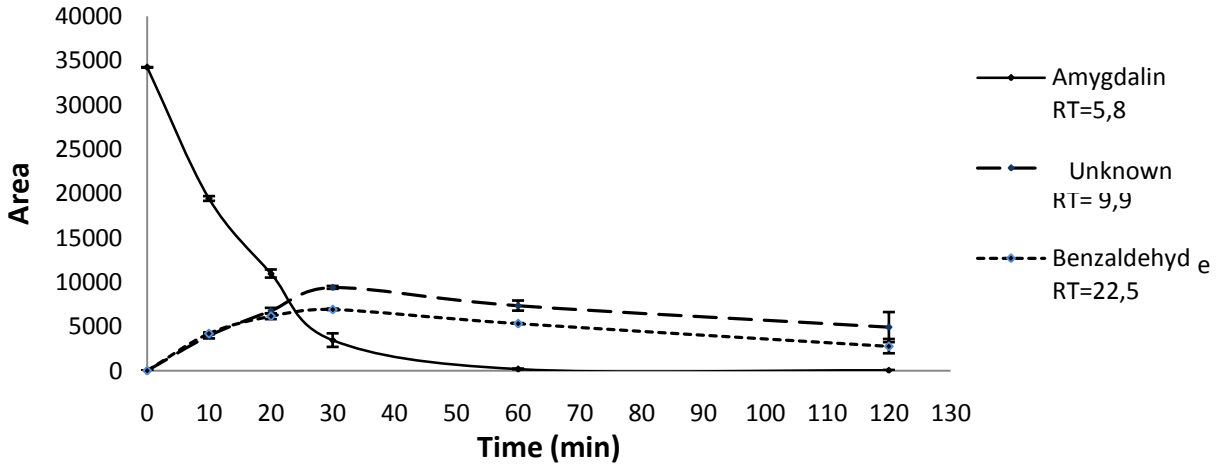


Fig.39 Amygdalin hydrolysis by β-glucosidase in time. (RT) retention time. Standtard deviations is represented by vertical bars in graph.

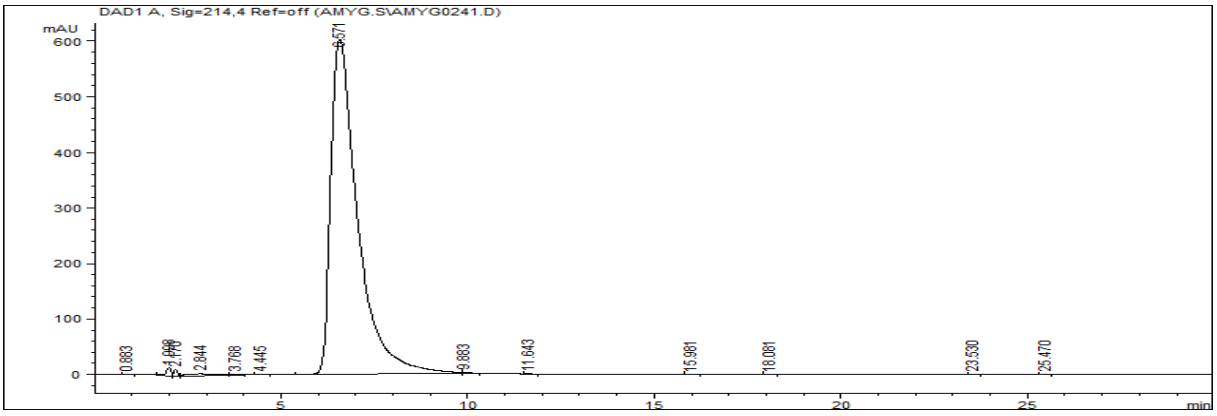


Fig.40 Amygdalin standard

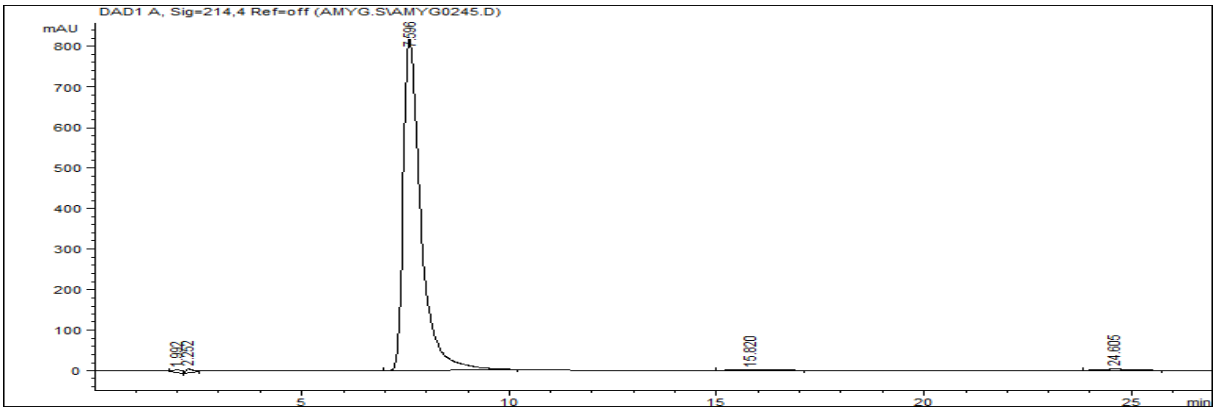


Fig.41 Benzyl alcohol standard

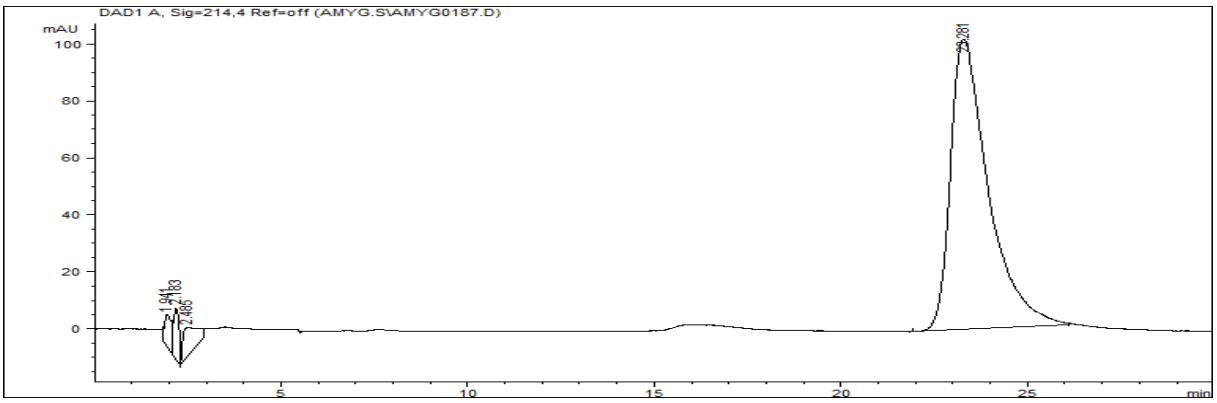


Fig.42 Benzaldehyde standard

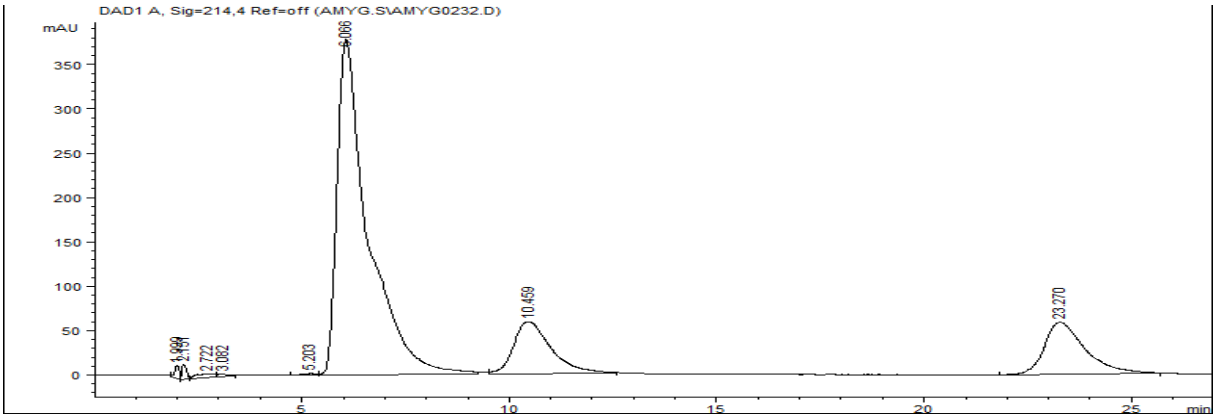


Fig.43 Amygdalin degradation after 10 min.

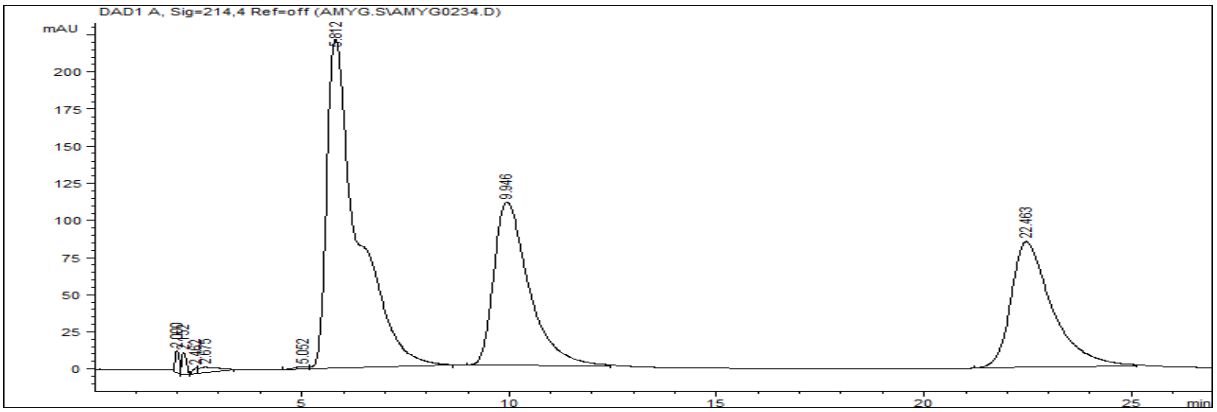


Fig.44 Amygdalin degradation after 20 min.

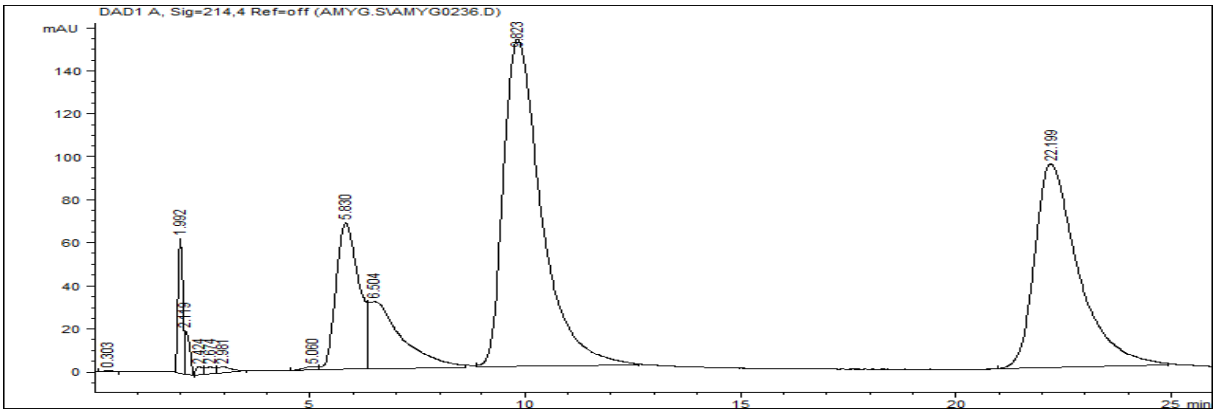


Fig.45 Amygdalin degradation after 30 min.

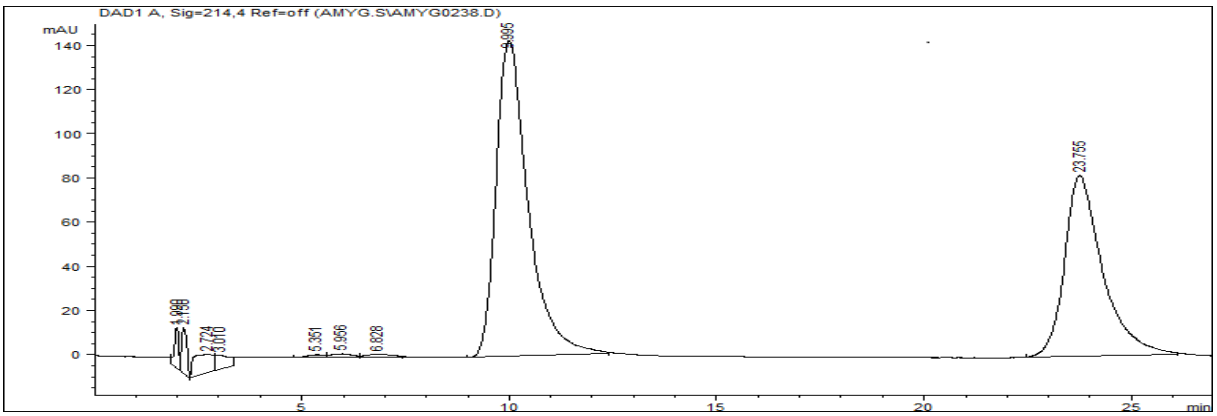


Fig.46 Amygdalin degradation after 1 h.

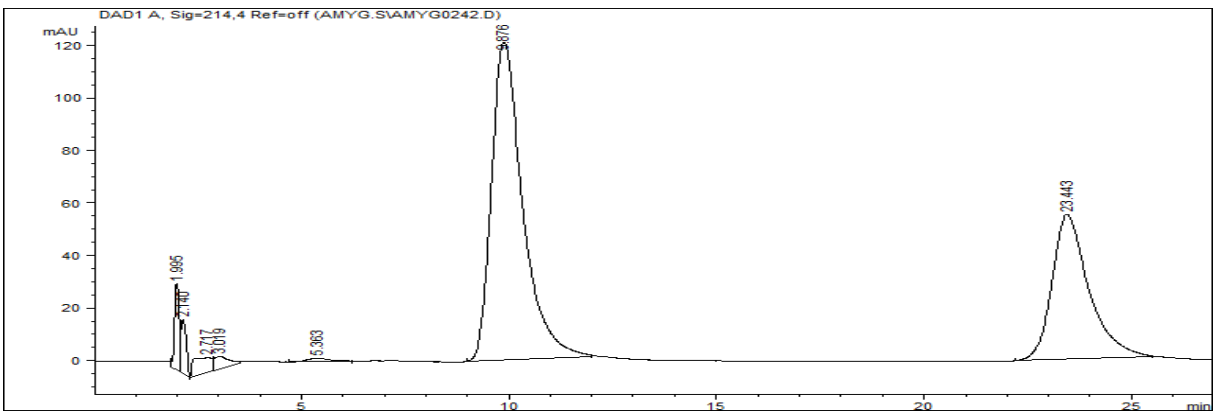


Fig.47 Amygdalin degradation after 2 h.

VI. DISCUSSION

Amygdalin is a natural substance, a cyanogenic glycoside, that can be found in a variety of plant species. It is present in high concentration in seeds of rosaceous fruits. Even in the ancient times, Egyptians used "aqua amigdalarum" for the treatment of some skin tumors. (48) Nowadays, there is conflicting evidence (Milazzo *et al.* (1)) discussing amygdalin`s role as a tumor anti-proliferator. However, amygdalin as a cyanogenic glycoside is considered to be a toxic component which can cause lethal cyanide poisoning. (36) The LD_{50} of amygdalin with β-glucosidase taking orally in rats is approximately 522 - 600 mg/kg of body weight and LD_{50} for amygdalin alone is about 800 mg/kg of body weight. (49) (50) Severe cyanide poisoning can be dangerous even more in this time of popularity of Amygdalin/Laetrile complementary and alternative medicine (CAM). Such cases were already documented: one of them was 4-years old boy from Germany with CAM treatment of cancer by vitamin B17 (51). For other examples please see Sauer *et al.*(51)

In spite of the great advances in the diagnosis and treatment of malignant tumors, cancer continues to be one of the principal causes of death in developed counties. Although it is true that surgery and radiotherapy are capable of curing some patients with localized tumors and that chemotherapy has achieved significant improvements in treatment of some types of malignant tumors, the general mortality rate of cancer has not improved much (1.8% per year in men and 1.6% per year in women (52)). Many patients find that their disease is so widespread that the chemotherapy drugs cannot be given in sufficient dosages (due to their high toxicity) to destroy extensive tumoral mass. Many patients cannot be exposed to therapy, surgery or radiotherapy because of the undesirable effects. (48)

This work is focused on determination of amount of amygdalin in trade stone fruits of tribe Amygdaleae, its changes during the ripening and local differences in content of amygdalin. Kernels from apricots, almonds, cherries, plums and peaches were collected during the year 2012 mostly in the Czech Republic but also in Spain and Pakistan. Other were bought in common markets. The amount of amygdalin was also measured in dietary supplement called B17 APRICARC®.

Some problems had to be avoided during process of amygdalin content determination in seeds. One of them was epimerization of D-amygdalin to neoamygdalin (L-mandelonitrile-β-D-gentiobioside) which forms a new peak in High performance liquid chromatography and has no antitumor activity.

This epimerization takes place in water. (25) (53) It was reported that D-amygdalin in kernels undergoes a hydrolytic reaction because of enzyme emulsin (β-gluco-sidase) when placed in water and that it is almost decomposed when attempts are made to its extraction from a powder form. (53) The third problem was whether to extract from whole seeds or from seeds crushed into crude powder. According to Hwang *et al.* (24), sizes larger than a half of the seed should be used while extracting in water in order to eliminate enzymatic hydrolysis. The second option is to use crude powder of kernels without inner shell where the highest concentration of β-glucosidase is present. To prevent epimerization and hydrolysis by β-glucosidase extraction in a 3 - 5% aqueous citric acid or in methanol can be used. (25) (53) Because enzymatic hydrolysis during the extraction in citric acid or methanol does not happen, it is preferable to extract from a crude powder where the extraction yield and extraction rate is better than during the extraction from whole pieces. (24)

Extraction of a crude powder with inner shell in methanol was chosen for simplicity.

Amygdalin determination from extracts was performed by TLC and HPLC with use of amygdalin standard. Methanol extracts were separated on silica gel in pre-saturated chamber using ascending technique (see chapter 1.4.4.1.). This process is inspired by Harborne (54). For spots detection UV lamp, Bromcresol green followed by 0.5% $KMnO_4$, in NaOH (42), Dragendorff reagent, 2, 4-dinitrophenylhydrazine, and vanillin in sulphuric acid followed by heating on 100°C were tested. The vanillin/sulphuric acid appeared as the only applicable visualization reagent. It was used further. Visible spots in TLC analysis, with $R_F = 0.53 \pm 0.02$, corresponded to amygdalin standard. There is a possibility that some part of amygdalin in extracted samples decomposed during the chromatography development, due to the emulsin (β-glucosidase) contented in seeds and water presented in mobile phase. Degradation products appeared in chromatograms. If this is the case then, as Sánchez-Pérez *et al.* (20) and Haisman *et al.* (21) marked, possible products of amygdalin hydrolysis are prunasin, glucose, gentiobiose, mandelonitrile, benzaldehyde and hydrocyanic acid. The three components mentioned last are volatile and difficult to detect chromatographically. (21) Because of water in mobile phase, prunasin, as a molecule less polar then amygdalin could be detected with lower R_F than amygdalin meanwhile glucose or other glucosides with higher ones. But In comparison with R_F values by Haisman *et al.* (21) spots with $R_F = 0.27 \pm 0.01$ could examine glucose and uncertain spots with $R_F = 0.80 \pm 0.02$

prunasin (Fig.16 - 22). To confirm which spot means what, TLC with prunasin, amygdalin and glucose standards in the same chromatographic conditions may be done.

Determination by high performance liquid chromatography with reverse-phase HPLC in the first experimental part was carried out in accordance with Lv *et al.* (22). Their setup with gradient elution by mixture of acetonitrile and water was used. The second experimental part followed methodology of Hwang *et al.* (23), concretely their method avoiding epimerization and hydrolysis of amygdalin because of the possibility of occurrence in mediums used in cell cultures. Every sample from the first part of experimental work was measured twice from one extract. The highest content of amygdalin was detected in seeds of apricots. From un-official source, people in Pakistan eat apricots with its seed too without any undesirable effect. Surprisingly amygdain was not detected in apricot seeds from Pakistan. Probably it was a sweet variety with any amygdalin production. The amygdalin amount increases during the ripening but in really old seeds the concentration of amygdalin was lower. This was also mention by Sánchez-Pérez *et al.* (20), by Zhao (19) and by Dicenta *et al.* (55). Sweet almonds do not contain detectable concentration of amygdalin but the bitter ones do. HPLC analysis of nectarines did not show any amount of amygdalin. We detected following amygdalin content in kernels of seven species: 0.01% in sweet almonds, 4.7% in bitter almonds, 8.0% in apricots, 1.5% in sweet cherries, 2.1% in sour cherries, 3.5% in peaches, 2.8% in plums and 1.2% in dietary supplement B17 APRICARC®.

Our study on cancer cell inhibition demonstrate that amygdalin alone and also amygdalin activated by β-glucosidase, inhibit proliferation and induces apoptosis in human cervical HeLa, breast MCF-7, and prostatic PC-3 cancer cells. Results (Fig.28 - Fig.38) showed that stand-alone amygdalin had no strong inhibition effect, however amygdalin hydrolyzed by β-glucosidase had significant anticancer effect on cells. The apoptotic effect of amygdalin with displayed morphological traits, such as nuclear condensation and fragmentation consistent with the apoptotic program cell death, was confirmed by Zhou *et al.* (56) and Kwon *et al.* (57). The apoptotic cell death by caspase-3 activation through the up-regulation of pro-apoptotic Bax protein and the down-regulation of anti-apoptotic Bcl-2 protein in DU145 and LNCaP prostate cancer cells was shown in Chang *et al.* (58).

IC_{50} values (50% cytotoxic concentration) were calculated using GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA) (see Tab.14). In table (Tab.14) can be seen that the highest inhibition activity by amygdalin and amygdalin activated by β-glucosidase occurred in HeLa cells with enzymatic activation after 48 h. MCF-7 and PC-3 cells were less sensitive to amygdalin and amygdalin activated by β-glucosidase. For further research may be useful to test this also on non-cancer cells to find out the selectivity rate.

IC_{50} (mg/mL)	24 h		48 h	
	AMG	AMG + B-G	AMG	AMG + B-G
HeLa	15.27	1.04	7.52	0.59
MCF-7	27.69	6.17	13.97	4.41
PC-3	44.71	6.61	31.06	5.46

Tab.14 IC_{50} values from MTT tests on HeLa, MCF-7 and PC-3 cancer cells.
AMG (amygdalin stand alone), AMG + B-G (amygdalin with β-glycosidase)

Control wells with cells treated with vehicle alone or with β-glucosidase (one concentration) alone in 24-well plates (Tab.9) showed no changes in cell proliferation or viability. When more concentrations of β-glucosidase were tested (Fig.31, Fig.35, Fig.38), all results of control wells containing only enzymes in plates 3. (Tab.10) showed slight cell viability decrease. There was also an observation of increased inhibition effect in plates 2. (Tab.10) of these tests and so the highest inhibition effect in wells with the middle concentration value (1200 nM) of β-glucosidase. The reason for the highest activity of β-glucosidase in concentration 1200 nM could not be in enzymatic saturation or interfering space limitation, because the results of using β-glucosidase in concentration 1200 nM in plates 2. did not correspond with the results of the same concentrations made in plates 0. (Tab.9) (Fig.29, Fig.31). Possible explanation could be in the essence of the toxic matter cyanide, which is volatile and could cause cross-contamination in wells. (59) In plates 0. (Tab.9), there were 9 wells containing amygdalin with β-glucosidase where hydrolysis and cyanide release could take place. Of these, 3 wells contained amygdalin in the highest used concentration (10 mg/mL). In plates 2., there were 24 wells with the possibility of hydrolysis of which 6 wells with the 10 mg/mL amygdalin. The concentration of released cyanide in plates 2. was much more intense and could influenced cells in wells reciprocally. The number of hydrolysed wells in plates 3. was 15 of which 6 wells with 10 mg/mL amygdalin. Cell

viability in control wells treated only with β-glucosidase without amygdalin was influenced in plates 3.(Fig.31, Fig.35, Fig.38)

Amygdalin extracted from seeds in aqueous solution is epimerized to neoamygdalin (L-mandelonitrile- β-gentiobioside), which is inactive against cancer cells whereas extracted in methanol remains active. Because the plant extract contains not only amygdalin but also β-glucosidase, MTT tests with this extract and extract with β-glucosidase should be more effective. It should inhibit more the proliferation of cell lines. (57) Anti-cancer activity of bitter almond extract and extract with β-glucosidase was also tested in this work but, because of the unpredictable interaction between some compounds of extract and MTT solution forming artefacts, false positive results occurred. MTT assay for next viability testing of extract samples should be modified by washing out technique by which the extract is washed out from wells after the incubation time before adding the MTT solution. (59)

MTT assay on HeLa cell line was carried out for three incubation times (4 h., 24 h., and 48 h.) to find the time dependence of enzymatic hydrolysis and cell affecting. HPLC degradation studies showed complete enzymatic hydrolysis of amygdalin during the first hour of incubation amygdalin with β-glucosidase. Amygdalin degraded to benzaldehyde with Rt = 23 min. and another substance with Rt = 10 min. The unknown substance is presumably prunasin. Its standard may be analyzed for confirmation. Unknown peak and peak for benzaldehyde did not increase equally. This could mean that hydrolysis runs in two steps and is caused by one enzyme with two different activities or two enzymes. In the first step, amygdalin hydrolysis to prunasin might have been faster than the hydrolysis from prunasin to benzaldehyde in the second step. This hypothesis is supported by findings of Haisman *et al.* (21) who measured chromatographic products and calculated kinetics constants. Their results confirmed the two enzyme mechanism theory. During the enzymatic reaction gentiobiose is not produced. Amygdalin is hydrolysed to prunasin and glucose and prunasin is consequently hydrolyzed to mandelonitrile and glucose. Mandelonitrile is hydrolyzed to benzaldehyde and hydrocyanic acid in the end. Different effect can be achieved by using acid hydrolysis. In such case the glycosidic bonds are attacked randomly. This causes that in addition to prunasin, glucose and mandelonitrile, gentiobiose is also produced. (21)

VII. CONCLUSION

Methanol extracts from stone fruit seeds of *Amygdalus c., Armeniaca v., Cerasus a., Cerasus v., Persica v., Prunus d*. and dietary supplement B17 APRICARC® prepared by Soxhlet extraction were analyzed by TLC and HPLC for content of cyanogenic glycoside amygdalin. All samples except sweet almonds (*Amygdalus c. var. dulcis*) and Pakistan variety of apricots (*Armeniaca v.*) contained measurable value of this compound. The highest median value of amygdalin was found in apricot seeds (except those from Pakistan). The highest concentration of amygdalin in seeds was present during the ripeness. Afterwards the amygdalin concentration decreased.

In the tests of anti-proliferative activity on different types of cancer cells (HeLa, MCF-7, PC-3), stand-alone amygdalin showed only slight dose dependant inhibitory effect on proliferation rate in all types of tested cells with the highest activity after 48 hour treatment on HeLa cells. Stand-alone enzyme β-glucosidase did not show any toxic effect.

Amygdalin hydrolyzed by enzyme β-glucosidase from almonds showed significant inhibitory effect in all types of cells in time and dose dependant manner.

In vitro amygdalin degradation occurred during the first hour of enzymatic hydrolysis when the HPLC analysis demonstrated benzaldehyde and probably also prunasin or mandelonitrile as degradation products.

This research shows that cyanogenic glycoside amygdalin is present in almost every trade stone fruits of Amygdaleae except the sweet varieties of almonds and that it has inhibition potential on different types of cancer cells. Moreover, positive effect of amygdalin activated by β-glucosidase is even stronger. Investigation of proliferating effect on healthy cells may create an interesting follow up to the research done in this thesis and it is also a great further research possibility.

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ABSTRAKT

Janatová, M.: Stanovení obsahu amygdalinu v tržních peckovinách a jeho biologická aktivita v buněčných kulturách karcinomu. Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové, Katedra Farmaceutické botaniky a ekologie, Hradec Králové, 2015, 74 s.

Peckovice podtřídy Amygdaleae čeledi Rosaceae jsou známy pro své antioxidační účinky, výživové látky a vitaminy. Jejich semena jsou ale hlavně spojována s obsahem kyanogenního glykosidu amygdalinu a jeho potenciálnímu inhibičnímu efektu na rakovinné buňky.

Cílem této práce byla kvantitativní analýza obsahu amygdalinu v extraktech semen tržních peckovin (meruněk, mandlí, švestek, třešní, višní, broskví) a jednoho výživového doplňku B17 APRICARC®. Extrakce byla provedena metanolem v Soxhletově extraktoru a analyzována pomocí TLC a reverzní HPLC s UV detekcí o vlnové délce 214 - 215 nm. Nejvyšší koncentrace amygdalinu byla naměřena ve zralých semenech meruněk se střední hodnotou 79,59 mg/g semen. Nedetekovatelné množství se ukázalo u všech sladkých mandlí a meruněk přivezených z Pákistánu. Enzymatická hydrolýza a degradace amygdalinu β-glukosidázou byla provedena *in vitro* a analyzována reverzní vysokoúčinnou kapalinovou chromatografií (rp-HPLC).

Dále byly provedeny biologické testy účinku amygdalinu a enzymu β-glukosidázy z mandlí na rakovinné buňky. Komerční amygdalin a jeho kombinace s komerčně získanou β-glukosidázou byly zkoušeny *in vitro* na třech lidských rakovinných koloniích buněk: HeLa (děložního hrdla), MCF-7 (prsu) a PC-3 (prostaty) pomocí MTT testu viability/cytotoxicity. Testy ukázaly inhibiční účinek na všech koloniích závislý na dávce i době expozice. Samotný amygdalin projevil jen mírné inhibiční účinky. Ve spojení s β-glukosidázou byl inhibiční efekt značný.

Klíčová slova: Amygdalin, β-glukosidáza, MTT test, HPLC, protirakovinný, kyanogenní glykosid

ABSTRACT

Janatová, M.: Determination of amygdalin content in trade stone fruits and its biological activity in cultured cancer cells. Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Department of Pharmaceutical Botany and Ecology, Hradec Králové 2015, pp.74

Stone fruits from tribe Amygdaleae of Rosaceae family are known for their antioxidant activity and amount of nutrients and vitamins. Their seeds are connected with content of cyanogenic glycoside amygdalin and their possible effect on inhibition of cancer cells growing.

The aim of study is to quantitatively analyze amount of amygdalin in extracts of trade stone fruit seeds (apricots, almonds, plums, cherries, sour cherries, peaches) and one dietary supplement B17 APRICARC®. Kernels were extracted in methanol by Soxhlet extractor and subsequently analyzed by TLC and by reverse phase HPLC with UV detection on 214-215 nm wavelength. The highest concentration of amygdalin was found in apricot seeds during their ripeness with the median value 79.59 mg/g of seeds. On the other hand, in sweet almonds and apricots from Pakistan was not detected any amount. The enzymatic hydrolysis by β-glucosidase and degradation of amygdalin was studied *in vitro* and analyzed by reverse-phase High performance liquid chromatography (rp-HPLC).

Anticancer effect of amygdalin and enzyme β-glucosidase from almonds was tested. The effect of commercial amygdalin and its combination with commercial β-glucosidase was tested on three human tumor cell lines HeLa (cervical adenocarcinoma), MCF-7 (breast) and PC-3 (prostatic) *in vitro* using MTT assay for cell grow inhibition/cytotoxicity. The MTT viability assay showed that all samples inhibited growth of all types of cells. The effect was dependant on dosage and exposition time. Stand-alone amygdalin had only slight effect but in a combination with β-glucosidase, the inhibition of proliferation was significant. Results further show that the combination of amygdalin with β-glucosidase increases the inhibition effect significantly.

Keywords: Amygdalin, β-glucosidase, MTT assay, HPLC, anticancer, cyanogenic glycoside