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ANALYTICAL STUDY AND QUALITY CONTROL OF BULGARIAN DRUGS WITH ANTIOXIDANT ACTIVITY

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Summary. In current work, there are summarized the phytochemical studies for plants from Bulgarian flora: Tribulus terrestris, Silibum marianum, Clinopodium vulgare, Leucorus nivalis, and drugs, containing extracts from this plants. By HPLC, there are determined the quantities of water and fat soluble vitamines: C, B, A, beta-carotene, E and D in plants; protodioscin in preparations, containing extracts from Trubulus terrestris; Galanthamine in Nivalin. Recently, the efforts are orientated to the realization of the complete control of quality and safety of plant products in compliance with Pharmacopoeial requirements and also the investigation of antioxidant effect.

Key words: antioxidants, plants, quality control

АНАЛИТИЧНО ПРОУЧВАНЕ И КАЧЕСТВЕН КОНТРОЛ НА БЪЛГАРСКИ ПРОДУКТИ ОТ РАСТИТЕЛЕН ПРОИЗХОД С АНТИОКСИДАНТНА АКТИВНОСТ

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Резюме. Обобщени са резултати от фитохимичното проучване на растения от българската флора – Tribulus terrestris, Silibum marianum, Clinopodium vulgare, Leucorus nivalis, както и на лекарствени продукти, съдържащи техни екстракти. Чрез HPLC са определени количествата на водно- и мастноразтворимите витамини (C, B, A, beta-carotene, E, D) в посочените растения; protodioscin в лекарствени продукти, съдържащи екстракт от Tribulus terrestris; Galanthamine в Nivalin. През последните години нарастват усилията за контрол и осигуряване на безопасността на растителните продукти в съответствие с фармакопейните изисквания, както и проучване на антиоксидантно действие.

Ключови думи: антиоксиданти, растителни продукти, качествен контрол

Most of ingredients from medicine plants are biological active compounds. Functions of phytochemicals are antioxidant, antiinflammatory, antiestrogenic, antiallergic, anticholesterolemic, antihemorrhagic, antimutagenic, antineoplastic. The antioxidant activity is of great importance for human life.

There is a dynamic balance between the amount of free radical oxygen sourses (ROS), generated in the body due to physiological errors in the cells, and the amount of protective antioxidants, which are sufficient only to cope with the physiological rate of ROS generation. The additional formation of ROS from environment or produced within the body cause a disturbance in the prooxidant – antioxidant balance in favor of the prooxidant state, thereby leading to oxidative stress – the condition experienced by a biological system living in the presence of oxygen. Unless ROS are removed from biological systems, they cause damage to defferent targets: lipids (R – OO.), loss of function of proteins (– SH), mutation of DNA/RNA (– OH.) [25].

Types of free radicals include: reactive oxygen species (ROS) (hydrogen peroxide, superoxide, hydroxyl radical); reactive nitrogen species (RNS) (NO); reactive metabolites or intermediates (metabolic activation of drugs, toxins, air pollutants, cigarette). Free radical-induced oxidative damage of crucial cellular molecules is involved in the pathogenesis of many chronic and degenerative diseases: cardiovascular (atherosclerosis) [9], neurodegenerative [10] (Alzheimer) [74], diabetes [46], cancer [61] and aging [87].

Free radical defense system includes: antioxidant enzymes (superoxide dismutases, peroxidases, catalases, thioredoxin/glutaredoxin); antioxidant quenchers; antioxidant-nutrients: Vit. C, Vit. A, Vit. E,

beta-carotene [23]; phytochemicals – antioxidants from plants – flavonoids [73], vitamins [23], alkaloids [81], terpenoids [29].

The antioxidant properties of phenolic acids and flavonoids are due to their redox properties, ability to chelate metals and quenching of singlet oxygen [67]. Subclasses of flavonoid include: flavonols (quercetin) [3], flavones (luteolin) [88], isoflavones (genistein) [57], flavanones (naringenin) [40], flavanonols (taxifolin) [85], flavan – 3 – ols (proanthocyanidins, catechins: catechin, epicatechin, gallocatechin, epigallocathechin) [21] and anthocyanidins (cyanidin) [1].

Plant antioxidants have protective effects against: cardiovascular and renal disorders [4]; impairment of memory and cognitive function (Alzheimer's disease) [32], carcinogenesis [39], ulcers [66], diabetes [69] and age-related neurological dysfunction [89].

Oxygen Radical Absorbance Capacity (ORAC) is a test tube analysis that measures the total antioxidant activity. High ORAC help to prevent most of the common diseases of aging, including cancer and heart disease.

Big sourses of different plant species containing compounds with antioxidant activity are widely spread in many contries all over the world: Africa [7], Asia [30] (Curcuma longa L.): China (anticancer) [16], (antirrheumatic) [27], India [78] (neurodegenerative diseases) [8], North America: Canada [2], Cuba (Curcuma longa L.) [64], Latin America [65], Australia [38], Europe [60].

In our studies, there has been estimated the content of some water and fat soluble vitamines as C, B, A, beta-carotene, E and D in Bulgarian flora: Silibum marianum, Leucorus nivalis, Tribulus terrestris, Clinopodium vulgare.

In Clinopodium vulgare, L. the content of vitamins C, A and E, phenolcarbonic acids (caffeic, ferulic), triterpens have been proved.

Vitamin C (Ascorbic acid) is a free radical scavenger, it is considered to be one of the most important antioxidants in extracellular fluids. Its protective effects extend to cancer, coronary artery disease, arthritis and aging [23].

HPLC separation of ascorbic acid and dehydroascorbic acids is obtained by HPLC system:

1) column: Primesep SB, 50/4.6 mm
2) mobile phase: MeCN (10%):HCOOH (0.1%)
3) flow rate: 1 ml/min
4) detection: ELSD.

Water-soluble vitamins niacinamide, pyridoxine, riboflavine and thiamine are separated by HPLC on column μBondapak and mobile phase : methanol.

Vitamin E is a fat-soluble substance present in all cellular membranes and is mainly stored in adipose tissue, the liver and muscles [23].

Vitamin E is a principal antioxidant in the body and protects polyunsaturated fatty acids in cell membranes from peroxidation. Alpha-tocopherol is the most common and most active [23].

Carotenoids are a group of red, orange and yellow pigments found in plant foods, particularly fruits and vegetables. Some carotenoids like beta-carotene act as a precursor of vitamin A [23].

On Fig. 1, there is described HPLC analysis of fat-soluble vitamins with multiwavelength detection at $\lambda = 225$ nm, $\lambda = 264$ nm, $\lambda = 325$ nm – Vit. A (1), Vit. A acetate (2), Vit. D_2 (3), Vit. D_3 (4), Vit. E (5), Vit. K_1 (6). This method is useful for plant extracts.

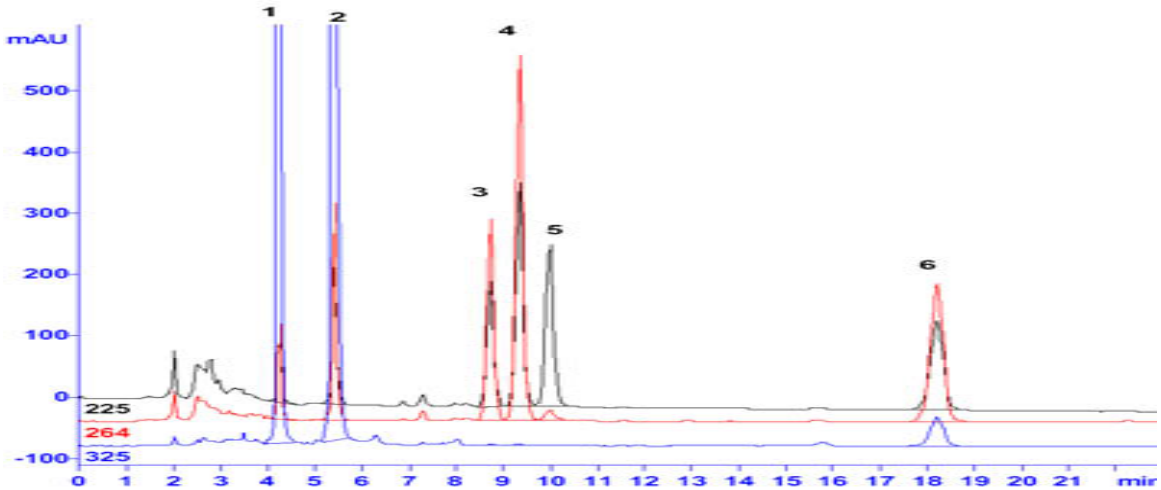


Fig. 1. *HPLC analysis of Vit. A (1), Vit. A acetate (2), Vit. D_2 (3), Vit. D_3 (4),Vit. E (5), Vit. K_1 (6)*

Tribestan (Tribulus terrestris L.) improves muscle growth and body strength, increases the number and motility of spermatozoa and the body's natural testosterone levels, helps in alleviating some symptoms associated with male menopause. Extracts from Tribulus terrestris [41, 55] and Tribulus alatus [36] show antioxidant potential.

Comparative analytical investigation of Tribulus terrestris preparations is presented [50]. Chromatograms of preparations with Tribulus terrestris from different manufacturers are shown on Fig 2.

One of the main compounds in Tribulus terrestris is protodioscin (Fig. 3).

The results for the content of protodioscin are obtained in different preparations using HPLC methods.

There are some natural products with content of Curcuma longa extracts on Bulgarian market.

Curcuma longa L. (turmeric) is one of the most popular species in tropical areas of Asia and Central America, containing natural antioxidants [18, 44, 72]. Curcuma longa is used in the prevention of pathologies associated with free radical damage [5]. Those effects have been attributed to curcuminoids, well-known hydroxyl radical scavengers and inhibitors of lipid peroxidation in vitro [68, 76].

Curcuminoids are polyphenol compounds and are responsible for the yellow color – curcumin, demethoxycurcumin and bis-demethoxycurcumin [34, 44].

Curcumin (1,7 – bis (4 – hydroxy – 3 – methoxyphenyl) – 1,6 – heptadiene – 3,5 – dione) (Fig. 4.) can exist in at least two tautomeric forms, keto and enol. The enol form is more energetically stable in the solid phase and in solution [11].

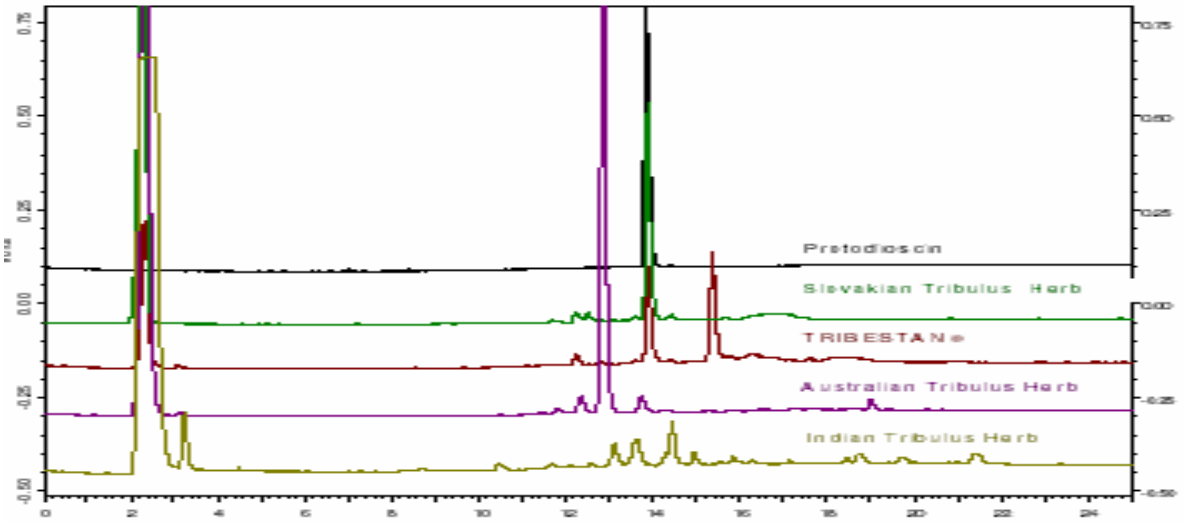


Fig. 2. *Chromatogram of preparation with Tribulus terretris*

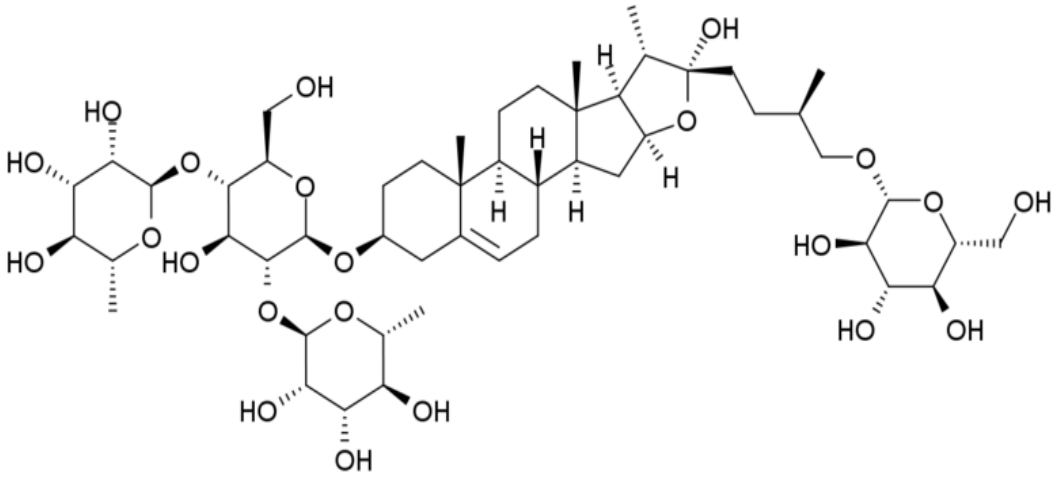


Fig. 3. *Srtructure of protodioscin*

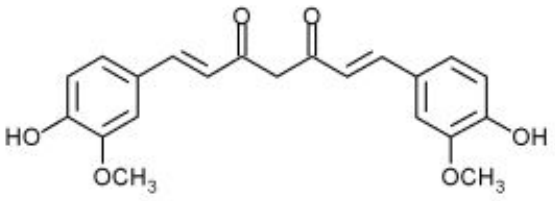


Fig. 4. *Structure of Curcumin*

Curcumin [72] scavenges superoxide radicals, hydrogen peroxide and nitricoxide from activated macrophages and as a singlet oxygen quencher [20]. H-atom donation from the β-diketone moiety to a lipid alkyl or a lipid peroxyl radical as a potentially more important antioxidant action of curcumin [35, 42]. In vitro and animal studies have suggested that curcumin may possess antioxidant, antitumor, antiarthritic, antiamyloid, antiinflammatory, antiprotozoal and antivenom activities [82]. Potential therapeutic effects of curcumin can help against neurodegenerative, cardiovascular (atherosclerosis) [63], pulmonary, metabolic, autoimmune and neoplastic diseases [56].

Because of its antioxidant activity [72], curcumin has been found to exhibit antimutagenic and anticarcinogenic properties [71]. Curcumin may suppress cancer development by helping inhibit enzymes that lead to tumor production and prevents cancer with inflammation by inducing production of enzymes used to detoxify electrophyllic species produced in lipid peroxidation [77].

Curcumin possesses immunomodulatory activity [33, 84] and protective effect on neuroinflammation and Alzheimer's disease [11].

A mixture of curcuminoids such as curcumin, demethoxycurcumin, bis–demethoxycurcumin protects normal human keratinocytes from xanthinehypoxanthine oxidase injury [13].

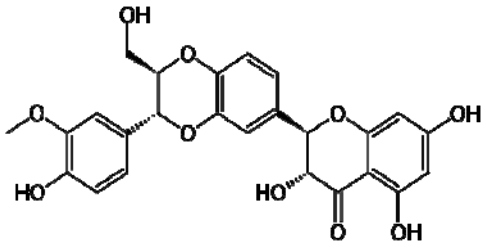
In addition to the curcuminoids, other compounds from rhizomes of Curcuma longa, possessing antioxidant capabilities include: α-terpinene, ascorbic acid, betacarotene, betasitosterol, caffeic acid, campestrol, camphene, dehydrocurdione, eugenol, p-coumaric acid, protocatechuic acid, stigmasterol, syringic acid, turmerin, α-turmeronol, β-turmeronol and vanillic acid [18].

Antioxidants are spread in: Curcuma amada Roxb. [59], Curcuma comosa Roxb. [14], Curcuma zedoaria [43].

For the registration of active oxygen forms, luminal-depending chemiluminescence is detected using chemiluminometer [58].

The main constituents of flavonolignan mixture silymarin from the seed and fruit extracts from Silibum marianum are silibinin, also known as silybin (the major active constituent, forming 70 – 80 % of silimarin) (Fig. 5.), isosibilinin, silicristin (Fig. 5.), silidianin (Fig. 6.) [19, 53].

Silibinin (silibin)



Silicristine

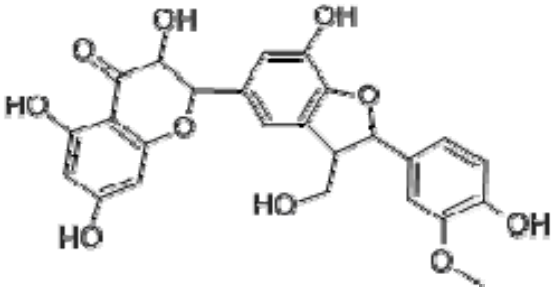


Fig. 5. *Structires of silibinin (silibin), silicristin*

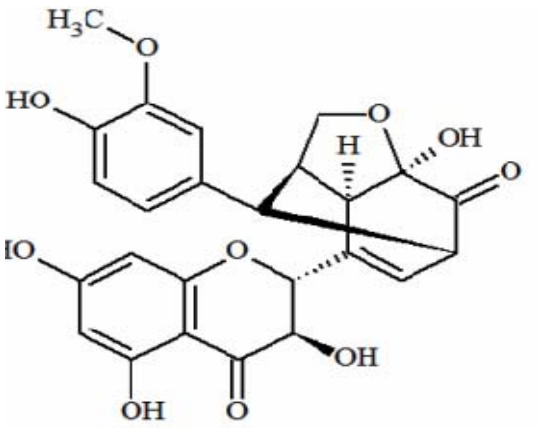


Fig. 6. *Structure of Silidianin*

Due to their phenolic structures, flavolignans have antioxidant effect and inhibit free radical – mediated processes [45]. Silimarin is known to possess different activities [26]: antioxidant, hepatoprotective [19, 70, 86], antiinflammatory, anticarcenogenic and immunomodulatory [37, 90]. Silimarin prevents sepsis-induced acute lung and brain injury [80], protects rat brain on oxydative stress [47]. Silibin and silicristin have stimulatory

effects of on kidney cells [75]. The oxidized derivatives of silibin have antiradical and antioxidant activity [28]. The extracts of the flowers and leaves of Silybum marianum have been used for centuries to treat liver, spleen and gallbladder disorders [62].

Clinopodium vulgare L. grows throughout Europe, Asia and North America. The herb is widely used in Bulgarian traditional medicine for treatment of skin irritation and swelling, and relieving the symptoms associated with mastitis and prostatitis. A gel, containing 20 % ethanolic extract of the plant (Clinogel TM) is developed by the Bulgarian pharmaceutical group Sopharma Ltd. for treatment of inflammatory-related skin conditions and prevention of skin aging. Clinopodium vulgare infusions are also used in traditional medicine to treat infirmities such as gastric ulcers, diabetes and cancer, that often exhibit gene expression alterations with a typical inflammatory signature. Plant is rich in phenolcarboxylic acids and flavonoids, which are classes of compounds well known for their antioxidant and antiinflammatory potential. The content of phenolcaboxylic acids is determined by GC [52]. Flavonoids and phenolcarbolic acids are identified by HPLC [48].

An aqueous extract of Clinopodium vulgare has a suppression effect of lipopolysaccharide-induced inflammatory responses [15]. Extracts in ethanol and propylene glycol have been proved to inhibit bacterial developmen [54]. Aqueous extracts have showed strong antitumourous activities [22]. Gentriacontan, isolated from Clinopoduim vulgare have been studied for anticancer properties [49].

The essential oil of Clinopoduim vulgare is found to possess remarkable radical-scavenging and antioxidant activities. The bioactive components from oil can act as primary and secondary antioxidants, scavenging free radicals, and can therefore inhibit the lipid peroxidation. Two of the main components of the Clinopodium vulgare essential oil fraction, oxygenated monoterpene thymol (39.8%) and monoterpene hydrocarbon γ-terpinene (29.6%) exhibit significant antioxidant activity. Other components in essential oil (%) with content higher than 0.6% are: α-Terpinene (3.7%), p-Cymene, (9.1%), Carvacrol (4.2), α-pinene (3.4%), β-pinene (3.1%), β-myrcene (2.3), α-thujene (1.4%) [79].

Flavanones from leaves of Inca muña (Clinopodium bolivianum) possess antioxidant capabilities [17].

Galantamine (Razadyne, Razadyne ER, Reminyl, Nivalin) is a drug developed and used for the treatment of mild to moderate Alzheimer's disease. It is an alkaloid that is obtained synthetically from the bulbs and flowers of the Caucasian snowdrop (Voronov'snowdrop), Lycoris radiata (Red Spider Lily), Galanthus woronowii (Amaryllidaceae) and related species [24].

Galantamine hydrobromide (4α S, 6R, 8a S) – 4a, 5, 9, 10, 11, 12 – hexahydro – 3 – methoxy – 11 – methyl – 6H – benzofuro [3a, 3, 2 – e, f] [2] benzazepin – 6 – ol hydrobromide) (Fig 7.), is a scavenger of ROS and exerts neuroprotection mainly by inhibition of the oxidative damage [24, 81].

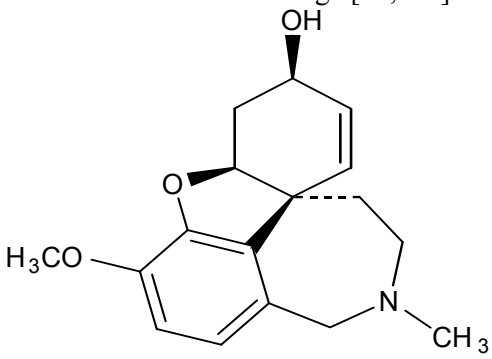


Fig. 7. *Structure of Galanthamine*

The latest clinical trials [83] and in vitro research [6] supported an increasing evidence that free radical-induced oxidative damage plays a role in the pathogenesis of Alzheimer's disease [31]. The brain is especially sensitive to oxidative damage because of its high content of easily oxidized fatty acids, high use of oxygen and low levels of endogenous antioxidants [12, 83].

Galanthamine and related alkaloids in nivalin (Licorine, Licorenine, Dihydrogalanthamine, Nivalidine) are determined by HPLC system: column Hibar LiChrosorb RP – 18, 25 cm/x 4 mm ID (10 μm), mobile phase: methanol : water (pH = 7.4), flow rate: 1 ml/min, t = 30°C, UV – detection at λ = 288 nm [51].

Bulgarian medicinal plants Tribulus terrestris, Silibum marianum, Clinopodium vulgare, Leucorus nivalis are rich sources of compounds with free radical scavenger activity.

Recently the efforts are orientated to the realization of the complete control of quality and safety of plant products in compliance with Eur. Pharmacopoeial requirements and also the investigation of antoxidant effect.

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