EXTRACTION AND ANALYSIS OF CONDENSED TANNINS IN CASTANEA SATIVA MILL.
EKSTRAKCIJA I ANALIZA KONDENZIRANIH TANINA CASTANEA SATIVA MIIL.

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ABSTRACT

Proanthocyanidins, also known as condensed tannins are widespread in woody plants, but are also found in certain forages. Castanea sativa Mill. are exploited for various purposes, but a little is known about potential of this species and possible application in diet and therapy. The parts of chestnut such as: seed, peeled seed, brown seed shell, red internal seed shell, leaves, catkin, spiny bur, as well as the new and old chestnut bark were extracted with 50% ethanol as an extragents. Contents of total phenolics and total flavonoids were measured by application of the standard colorimetric assay. The total condensed tannins content estimated was highest in red internal seed shell 15.29%CE (vanillin assay) and 3.12%CT (acid butanol assay). Also high content of total phenolic compounds and condensed tannins had extracts of catkin, brown seed shell of chestnut and new chestnut bark. Extracts of C. sativa Mill. can be a potential resource of natural tannins with possible application in diet and therapy.

Keywords: Castanea sativa Mill., condensed tannins, extracts, total flavonoids, total phenolics

SAŽETAK

Proantocijanidini, poznati kao kondenzirani tanini su jako rasprostranjeni u drvenastim biljkama, a pronađeni su i u hrani. Castanea sativa Mill. se dosta koristi za različite namjene, međutim malo je poznat njegov potencijal i moguća primjena u dijeti i terapiji. Dijelovi kestena kao što su; cijeli plod, srž ploda, vanjska smeđa kora ploda, crvena unutrašnja kora ploda, list, resa, ježura, kao i nova i stara kora drveta su ekstrahirani primjenom 50% etanola kao ekstragensa. Sadržaj ukupnih fenola i flavonoida je određen primjenom standardnih kolorimetrijskih testova. Sadržaj ukupnih kondenziranih tanina je najviši u crvenoj unutrašnjoj kori 15,29%CE (vanilin test) i 3,12%CT (kiseli butanolni test). Visok sadržaj ukupnih fenolnih tvari i kondenziranih tanina je određen u ekstraktima rese, vanjske smeđe kore i nove kore drveta. Ekstrakti C. sativa Mill. mogu biti potencijalna sirovina koja sadrži prirodne tanine sa mogućom primjenom u dijeti i terapiji.

Ključne riječi: Castanea sativa Mill., kondenzirani tanini, ekstrakti, ukupni flavonoidi, ukupni fenoli

INTRODUCTION

European chestnut, Castanea sativa Mill. (Fagaceae), a deciduous tree growing in Southern Europe, specially in the Mediterranean region and the Balkans, produce wood and chestnuts that have considerable economical value. The utilization for chestnuts in Europe being emphasized the marron glace production; some references also relate their use in baking, mainly under economically difficult condition [2]. Thanks to their nutritional composition with a low fat content, completely free of cholesterol, with a low sodium and high potassium content, and a moderate but high quality protein content, chestnuts are balanced and quality food [1].

Seeds of C. sativa are used in paediatrics for treatment of gastroenteritis and as a gluten-free diet in cases of coeliac disease. In the Middle Ages the raw seeds were disclosed as useful in the treatment of heart disorders [4]. C. sativa leaves (CSL) used in folk medicine as a tea in France to treat hacking cough and diarrhe. It is known that leaves contain phenolic compounds, and is demonstrated that the aqueous, methanol, and ethyl acetate extracts of CSL had good antioxidant potential as compared to Vitis Vinifera [3].

There has been an increased interest in phenolics found in plant foods. The flavonoids have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities [9].

Plant polyphenols (tannins) constitute a complex group of naturally occurring polymers, and a rigorous chemical definition is difficult. Thus, tannins are considered to be polyphenolic metabolites of plants with a molecular weight larger than 500 and with the ability to precipitate gelatin and other proteins from solution [8]. Plant polyphenols have an astringent taste, also resulting from their interaction with protein and they are believed to be active agents in many traditional medicines and herbal teas, although high consumption of such teas is known to lead to medical problems [18]. Based on their structure, tannins can conveniently be divided into two groups, hydrolyzable and condensed tannins. Proanthocyanidins (PA) or condensed tannins are polymeric flavonoids [19]. Catechins and some low-molecular weight PA have received considerable attention owing to their various biological activities, in particular their effects on arteriosclerosis [7] and their oxygen free radical scavenger ability [15].

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. Based on numerous evidence of the strong biological activity of phenolics and on the lack of data on their content, the aim of this study was focused on determination of their content in differents parts of C. sativa Mill.

MATERIAL AND METHODS

Spetrophotometrical determinations were carried out using a spectrophotometer Hewlett Packard 8452. For extraction, the ultrasonic bathroom Branson model b-220 Smith-Kline Company (50/60 Hz, 125 W) was used. Folin-Ciocalteu reagent was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gallic acid and (+)-catechin hydrate (Fluka A.G., Buchs, Switzerland) were used. Vanillin was obtained from Merck (Darmstadt, Germany) and Lipophilic Sephadex 20 was obtained from Sigma Aldrich. All other used chemicals and solvents were of the high analytical grade and were obtained from commercial providers.

Sample Preparation

A volume of 250 mL of 50 % ethanol was added (the ratio sample:solvent was 1:5; w/v) to 50 g of the sample. The extraction by ultrasound was performed (30 min at 50 Hz and 125 W). After the determination of mass, and the addition of solvent, the liquid extract was obtained by filtration through Whatman Grade No. 4 filter paper. The aliquot of the extract was taken and the solvent was completely removed by evaporation under vacuum, at 40°C. In this way, the dry extract of the investigated chestnut samples was obtained. The yield of dry extract was calculated (Table 1).

Plant Material

Chestnut samples were collected in the area of the Una – the Sana canton (Bosnia & Herzegovina) in 2006, and included: leaves, catkins, seed, new and old chestnut bark and spiny burs. The seeds were without bur. Also, parts of the seed have been investigated, such as: peeled chestnut (hand-peeled), the brown seed shell and the red internal seed shell. After drying at room temperature, the samples were milled for further analysis in a laboratory homogenizer. The mean particle diameter of investigated samples was determined according to Pharmacopoea Jugoslavica IV.

Determination of total phenolic compounds

Total phenolics were determined in dry extracts by Folin - Ciocalteu procedure [17, 6]. The absorbance was measured at 765 nm and content of total phenolic compounds in investigated plant methanol extracts, was expressed as grams of gallic acid equivalents (GAE) per 100 g of the dry extract sample (%; w/w), i.e. %GAE.

Determination of total flavonoids

Total flavonoids content was measured by the aluminium chloride colorimetric assay [20]. The absorbance

was measured against prepared blank at 510 nm and total flavonoids were expressed as grams of catechin equivalents (CE) per 100 g of the dry extract sample (%; w/w), i.e. %CE.

Determination of total condensed phenolics – Vanillin assay

Total condensed phenolics content were determined in dry extracts by vanillin assay [13]. The absorbance was measured at 500 nm and content of total condensed phenolics in investigated samples was expressed as grams of catechin equivalents (CE) per 100 g of the dry extract sample (%; w/w), i.e. %CE.

Determination of total condensed phenolics – Acid butanol assay

Total condensed phenolics content also was analysed by butanol-HCl assay [12]. After separation tannins from non-tannin phenolics of chestnut leaves dry extracts by Sephadex LH 20 column chromatography, internal standards of condensed tannins was made. The absorbance was measured at 550 nm and content of total condensed phenolics in investigated extracts was expressed as grams of conndensed tannins (CT) per 100 g of the dry extract sample (%; w/w), i.e. %CT.

Statistical analysis

All determinations were made in the triplicate and the values were averaged and reported along with the standard deviation (± Standard Deviation). Statistical analysis was carried out by using Microsoft Excel 2000 software (CORREL statistical function). Correlation coefficients (r) determining the relationship between investigated variables and Pearson correlation test was conducted to determine the correlation among variables. Significant levels were defined using $P \leq 0.05$.

RESULTS AND DISCUSSION

Parts of sweet chestnut such as peeled seed, brown seed shell, red internal seed shell seeds, leaves, catkin, spiny bur, as well as the new and old chestnut bark were extracted by 50% ethanol as an extragents. The plant material was milled to the mean particle size from (0.18 ± 0.008) to (0.40 ± 0.021) mm.

Phenolics are not uniformly distributed in plants within the tissues. Insoluble phenolics are the components of cell walls, while soluble phenolics are fit in the plant cell vacuoles [14]. The outer layers of plants contain higher levels of phenolics than those located in their inner parts [10]. The effect of ultrasound accelerate the extraction of organic compounds from plant matterials due to disruption of cell walls and enchanced mass transfer of cell content [5], and we thus used ultrasound extraction in our investigation.

The yield of dry extract was from (1.82 ± 0.066)% for spiny burs to (12.79 ± 0.092)% for peeled seed of sweet chestnut (Table 1).

The highest content of total phenolic compounds (3.28 ± 0.154)% GAE was found in dry extract of catkin, while the lowest content (0.42 ± 0.067)% GAE was obtained for the dry extract of the seeds (Table 2). The total flavonoid content was ranged from (0.09 ± 0.003)% CE for peeled chestnut to (1.44 ± 0.096)% CE for the red internal seed shell.

The vanillin reaction of an aromatic aldehyde, vanillin with meta-substituted ring of flavonols yields a red adduct. The reaction is not specific for condensed tannins. Any appropriately substituted monomeric flavanol reacts in the assay. The most commonly used standard in the vanillin assay is catechin under normal reaction condition [16].

The total condensed tannins content estimated by vanillin

Table 1: Mean particle size and yield of dry extract.Values are presented as means ± S.D.
Tablica 1: Srednji promjer čestica i prinos suhog ekstrakta *Castanea sativa* Mill. Vrijednosti su predstavljene kao srednja vrednost ± standardna devijacija

Sample	Mean particle size (mm)	Yield of dry extract %(w/w)
Seeds	0.27 ± 0.007	7.09 ± 0.091
Peeled seed	0.26 ± 0.009	12.79 ± 0.092
Brown seed shell	0.40 ± 0.021	3.30 ± 0.083
Red internal seed shell	0.24 ± 0.017	6.79 ± 0.077
Catkin	0.24 ± 0.023	10.04 ± 0.046
Leaf	0.18 ± 0.008	4.94 ± 0.038
New chestnut bark	0.26 ± 0.013	7.84 ± 0.073
Old chestnut bark	0.27 ± 0.005	3.40 ± 0.089
Spiny burs	0.28 ± 0.008	1.82 ± 0.066

Table 2 : Total phenolics content and total flavonoids content
Tablica 2: Sadržaj ukupnih fenola i flavonoida

Sample	Total phenolics content (%GAE)	Total flavonoids content (%CE)
Seeds	0.42 ± 0.067	0.17 ± 0.008
Peeled chestnut	0.59 ± 0.029	0.09 ± 0.003
Brown seed shell	1.19 ± 0.126	0.65 ± 0.021
Red internal seed shell	2.82 ± 0.063	1.44 ± 0.096
Catkin	3.28 ± 0.154	0.60 ± 0.031
Leaf	1.40 ± 0.011	1.40 ± 0.011
New chestnut bark	3.00 ± 0.078	0.75 ± 0.069
Old chestnut bark	1.70 ± 0.097	0.69 ± 0.056
Spiny burs	0.49 ± 0.023	0.13± 0.017

Table 3: Total condensed tannins content
Tablica 3: Sadržaj ukupnih kondenzovanih tanina

Sample	Condensed tannins contents	
	Vanillin assay (%CE)	Acid butanol assay (%CT)
Seeds	0.39 ± 0.022	0.88 ± 0,023
Peeled chestnut	0	0
Brown seed shell	2.78± 0.093	1.67 ± 0.058
Red internal seed shell	15.29 ± 0.282	3.12 ± 0.085
Catkin	0.49 ± 0.042	0.95 ± 0.082
Leaf	0	0.08 ± 0.007
New chestnut bark	3.91 ± 0.063	1.89 ± 0.081
Old chestnut bark	0.76 ± 0.045	0.58 ± 0.034
Spiny burs	0	0.08 ± 0.008

assay was highest in red internal seed shell (15.29 ± 0.282)% CE (Table 3). Also high value of condensed tannins was observed in extracts of new chestnut bark and brown seed shell of sweet chestnut. In the other hand, extracts of peeled chestnut, leves and spiny burs did not contain condensed tannins.

The other assay, butanol-HCl method has greatest strength on confirmation of presence of a polymeric interflavan structure. Hydrolyzable tannins do not react in the assay. To minimize the problems from use of inappropriate standards, the use of internal standards derivates from the plant materials under study has been proposed. Internal standard in our investigation gained by purification of chestnut tannins on Sephadex LH 20.

In the same manner as with vanillin assay, red internal seed shell had maximum of condensed tannins (3.12 ± 0.085) % CT. Rich in condensed tannins were extracts of new chestnut bark and brown seed shell. Peeled chestnut did not hold condensed tannins, and a very low contents had leaves and spiny burs (0,08 % CT).

In correlation analysis, highly significant correlations ($P < 0.01$) existed between yield of dry extract and total phenolics ($r = 0.78$), total phenolics and total flavonoids ($r = 0.80$) as well as total phenolics and condensed tannins determined by acid butanol assay ($r = 0.79$).

CONCLUSIONS

Tannins have been reported to exert physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immunoresponses. The dosage and type of tannins are critical to these effects. It was determined that the high content of both total phenolic compounds and condensed tannins had catkin, red internal and brown seed shell of sweet chestnut and new chestnut bark. Consequently, extracts of C. sativa Mill. can be a potential resource of natural tannins with possible application as a part of diet in prevention of some diseases, such as cardiovascular disease.

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