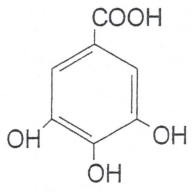
Practice No. 8

ANALYSIS OF DRUGS CONTAINING TANNINS

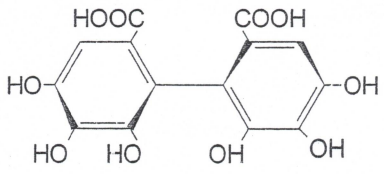
Tannins are a chemically heterogenous group of compounds which have the common feature of tanning hides into leather. Chemically, they are multivalent phenol compounds of different molecular weight. True tannins have a molecular weight from 1000 to 5000. Many tannins are glycosides. Tannins are divided into two main groups:
a) hydrolysable tannins
b) condensed tannins

Hydrolysable tannins

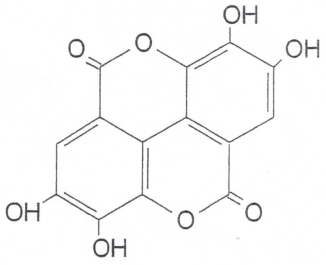
They are subject to hydrolysis by acids or enzymes. They are composed of several molecules of phenolic acids as gallic acid, hexahydroxydiphenic acid, which are associated with a glucose molecule through an ester bond. Gallic acid produces blue color with salts of iron. They are commonly known as pyrogallic tannins. The group of hydrolysable tannins is divided into two types – gallotannins and ellagitannins. Ellagic acid can occur through the lactonization of hexahydroxydiphenic acid during chemical hydrolysis of tannins, the term ellagitannins is considered incorrect, but nonetheless it is frequently used.



gallic acid



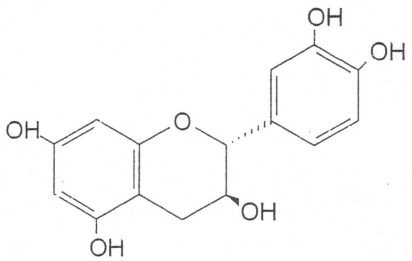
hexadihydroxyphenic acid



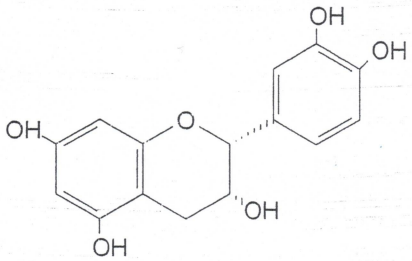
ellagic acid

Condensed tannins

Not subject to the effect of acids and enzymes, not hydrolyzed into simple molecules and do not contain a sugar component. They are close to flavonoid pigments and have polymeric structure of flavan-3-ol. They are converted into red insoluble compounds called phlobaphens. Condensed tannins produce green coloration with ferric chloride. They occur almost in all plant organs.



(2R,3S)-(+)-catechin



(2R,3R)-(-)-epicatechin

There are many external features common to the entire group, which are used for the characterization of tannins:

- astringent taste
- precipitate aqueous solutions of proteins and alkaloids, causing agglutination of erythrocytes

- give color reaction and precipitates with salts of heavy metals
- their solutions oxidize and darken when in contact with air

Due to the fact that tannins are not chemically uniform substances, we cannot count on the result of a single reaction while performing their qualitative analysis.
1% aqueous extract of tannin drugs, which is prepared by boiling the drug with water for 15 minutes and filtering, is usually used for qualitative tests of tannins.

Qualitative responses to tannins can be divided into two groups:

Precipitation reactions

They are using the ability of different substances to precipitate tannins from solutions (solutions of proteins, salts of alkaloids, salts of heavy metals, some dyes, oxygen, etc.). The particularly important one is the reaction of tannins with gelatin and the reactions with metal salts, especially Cu2+, Pb2+, Ag+, Cd2+, etc

Reactions of tannins with gelatin
Add the gelatin solution to 5 ml of clear analyzed solution dropwise and examine, whether the clot is formed. Gelatin must be added cautiously, because the clot can be dissolved in an excess of reagent. It is recommended to always use fresh solution of gelatin. All the tannins provide precipitate or turbidity, but not all the species are equally susceptible to this reaction (pseudotannins). The chemical structure of precipitates produced during the reaction is still controversial. (Also some organic substances, such as salicylic acid, p-oxybenzaldehyde, respond positively)

Reactions of tannins with formaldehyde
By boiling with formaldehyde and hydrogen chloride solutions, pyrocatechin tannins precipitate quantitatively, while the pyrogallol tannins remain largely in solution. Reaction is carried out so that 5 ml of concentrated HCL and 10 ml of 40% formaldehyde solution are added to 50 ml of the extract in the boiling flask (250 ml). The mixture is boiled for 30 minutes under a reflux condenser. Potential precipitate (pyrocatechin tannins) is filtered. First portion of the filtrate is discarded, 10 ml of the filtrate are then mixed with 1 ml of 1% ferric alum solution and 5 g of sodium acetate. Blue-violet color occurs with pyrogallol tannins.
Explanation:
Phenols and their derivatives polycondensate with aldehydes. First, condensation occurs, while hydroxymethylphenols are formed. Additional condensation takes place in the presence of acidic catalysts. The OH group controls the access to ortho and para positions, compounds which have these positions vacant react preferably.

Reactions of tannins with bromine water
Pyrocatechin tannins precipitate within 5 minutes through short boiling and bromine water exposure.
Explanation:
This is the bromination (electrophilic substitution), via which tetrabromo derivatives are created. Vacant ortho and para positions are required for substitution of bromine.

Reactions of tannins with heavy metals
Precipitates of tannins with metals usually don't have a constant composition. The ratio of metals and tannins in them varies and is dependent on the reaction conditions (degree of dilution, temperature, method of solution adding, etc.). They are not mostly stoichiometrically created salts, but compounds of variable composition.

Reaction with lead acetane

All tannins quantitatively precipitate by alkaline lead acetate. In addition to tannins, it is also used to precipitate the pseudotannins of phenolic nature. Normal lead acetate precipitates some pyrogallol tannins quantitatively (e.g. in the extract of chestnut and oak wood), but with most of the tannins, the precipitation is not quantitative and the solution gives a positive reaction with ammonium-ferric sulfate after the removal of the precipitate. By adding acetic acid, the precipitation of pyrocatechin tannins is prevented by normal lead acetate, while pyrogallol tannins are partly or completely precipitated. Based on these properties, we can separate both of these groups of tannins, although not quantitatively, by adding 10 ml of 10% acetic acid solution and 5 ml of 10% normal lead acetate solution to 5 ml of filtered aqueous extract of the drug.. After 5 minutes, the evaluation is carried out. If there is no precipitate, there are only pyrocatechin tannins present in the drug, otherwise there are pyrogallol tannins.

Explanation:

Both types of tannins are acidic in nature, but in comparison with acetic acid, the pyrogallol tannins, which precipitate with lead acetate in acidic environment and probably create salts, are the stronger acids. With pyrocatechin tannins, tannin salts do not occur.

There are other solutions, besides lead acetate, used to prove the presence of tannins, with which tannins form precipitate: 10% silver nitrate solution, 5% alkaline zinc acetate solution.

Color reactions

They result from the reaction of tannins with salts of some metals. The most important of these are the reactions of tannins with ferric salt solutions. The basis of this color reaction is the formation of a strong acidic complex compound of ferric ion with phenol. Therefore this reaction is also given by many non-tannin compounds of phenolic nature. Another color reaction, used for the evidence of tannins is the reaction with phosphowolframic acid in an alkaline environment, when an intense blue color occurs (tungsten blue).

Reactions of tannins with ferric salts

Adding few drops of 1% ferric alum solution to 2 to 3 ml of neutral solution of tannins gives greenish tint in the presence of pyrocatechin tannins, while pyrogallol tannins are colored blue. With the same amount of both types of tannins in the mixture, the blue tint is more intense.

The reaction of tannins with ferric salts is very sensitive in a neutral environment. In an acidic environment, even with pyrogallol tannins the color turns purple. The solution of ferric chloride is always acidic due to hydrolysis, so it is better to use the ferric alum solution for the reaction.

Excess of ferric ions that have the oxidative ability, change the color to brown (during time and also due to the influence of atmospheric oxygen).

Reactions of tannins with concentrated sulfuric acid

Add 1 ml of concentrated sulfuric acid to 2 ml of tannin solution cautiously so that the solutions don't mix. Colored ring forms at the contact surface, whose color is notable for many tannins. When the solution is shaken and diluted with distilled water, the color of the ring goes into the solution in most cases. Solutions of tannins in 96% alcohol are the best suited for this reaction.

Fluorescence analysis

Extracts of tannins drugs typically fluoresce, especially if wool of filter paper is moistened by them.

Determination of content of tannins by colorimetric method (CSL 4)

A colorimetric method is used for the content determination of these compounds, tannins precipitate by alkaline zinc acetate and form colored products with Folin-Ciocalteu reagent. Based on the reduction effect of tannins the blue colored solution of polywolframate occurs, whose absorbance is measured at 720 nm.

Determination method:
0,2000 g of the powdered drug is weighed into a 100 ml flask, 40,0 ml of water is added and boiled under a reflux cooler for 10 minutes. Then the extract is filtered through filter paper into a 100 ml volumetric flask. The residue on the filter is placed in a flask and the extraction is repeated once more with 30,0 ml of water. Flasks and filter are washed with hot water and then the filtrate is merged with the previous extracts. The volume is filled up by water to the mark. 5,0 ml of well-mixed extract is placed into a spin tube, then 2,0 ml of alkaline zinc acetane solution is added, mixed and the resulting precipitate is centrifuged at 2000/min for 5 minutes. Clear liquid above the precipitate is removed, then 7,5 ml of water is added, mixed thoroughly and centrifuged again in the same way. After removing the liquid above the precipitate, the precipitate is dissolved in 2,5 ml of 5% sulfuric acid solution. The solution is transferred into a volumetric 50 ml flask and water is added up to the mark.
3,0 ml of Folip-Ciocalteu reagent are added to 1,0 ml of this solution (equivalent to 0,10 mg of drug) and left for 15 minutes. Then 3,0 ml of 10% sodium carbonate solution are added, everything is mixed well and after 1 hour, the absorbance is measured at 720 nm in 10 m layer against the blank solution consisting of 1,0 ml of water, 3,0 ml of Folin-Ciocalteu reagent and 3,0 ml of 10% sodium carbonate solution.

The content is calculated from the calibration curve.

Quercus cortex

Quercus robur a *Quercus petraea, Fagaceae*
Content compounds: condensed tannins (catechins and partially ellagitannins), flavonoids and purpurogallin – red of oak bark
The drug must contain at least 9,0% of tannins.

Identification:
1- 10 ml of methanol is added to 0,2 g of the powdered drug and heated to boiling. The solution is filtered after cooling. 0,5 ml of vanillin solution and 1,0 ml of fuming hydrochloric acid is added to 1,0 ml of the filtrate, while the solution turns red (Catechin tannins). (CsL 4)
2- 0,1 ml of the solution from step 1 is mixed with 100 ml of water and 0,1 ml of ferric chloride solution in 96% alcohol (100 g/l), the solution turns blue-green, later blue-black (tannins). (CL 97)
3- About 30 ml of 1% drug infusion is prepared by boiling for 10 minutes and filtering through cotton wool. Approximately 5 ml of the filtrate are measured into five tubes and following agents are added dropwise: 10% silver nitrate solution, 5% potassium dichromate solution, 4% copper sulphate solution, 3% lead acetate solution and 0,5% gelatin solution. The results are recorded (preferably in tables) and compared with the results that were given by other tannin drugs with those agents.

Hamamelidis folium

Hamamelis virginiana, Hamamelidaceae

Content compounds: hydrolysable tannins - hamamelitannin, digalloyl-hexons, flavonoids, saponins, essential oils. The sugar component of hamamelitannins is hydroxymethylribose
Drug must contain at least 3,5% of tannins.

Identification:

1- 5 ml of water is added to 0,1 g of the powdered leaves, boiled and the infusion is filtered after cooling. 1 drop of ferric chloride solution is added to the filtrate, black and green precipitate appears (tannins) (CsL 2).
2- By heating with potassium hydroxide, leaf fragments turn blue to blue-green (CsL 2).
3- See test 3 in *Quercus Cortex* drug.

Bistortae radix

Bistorta major, Polygonaceae

Content compounds: Catechin tannins, which can oxidize into phlobaphens (rattan red), carbohydrates, starch, N-methyl tyrosine.
Drug must contain at least 8% of tannins.

Identification:

1- 0,5 g of the powdered drug is boiled with 5 ml of water and the infusion is filtered after cooling. 1 drop of ferric chloride solution is added to the filtrate, the solution turns brown-green to black and green (tannins) (CsL 2).
2- See test 3 in *Quercus Cortex* drug.

Tormentillae radix

Potentilla erecta, Rosaceae

Content compounds: tannins of catechin type, which can be oxidized into phlobaphens, traces of essential oil
Drug must contain at least 12,0% of tannins.

Identification:

1- 1,0 g of the powdered drug is boiled with 15 ml of water and is filtered after cooling. The filtrate is divided into two parts.
   a. 1 drop of ferric chloride solution is added to 5 ml of the filtrate, greenish precipitate appears (tannins). (CsL 3)
   b. 5 ml of the filtrate is acidified with concentrated hydrochloric acid, khaki color should appear. If excess of diluted potassium hydroxide solution is added, the liquid turns clear red (oxidation products of tannins). (CsL 3)
2- See test 3 in *Quercus Cortex* drug.
3- About 0,1 g of powdered drug (IV) is mixed with 10,0 ml of water and is left to stand for 1 hour with occasional shaking. The liquid is then filtered. 2,0 ml of ferrous ammonium sulfate (100 g/l) are added to the filtrate, and opacity and dark gray color appears, after prolonged stall, black precipitate settles and the liquid above it is colored blue-green (catechin tannins) (CsL 4).

Agrimoniae herba

Agrimonia eupatoria a *Agrimonia procera, Rosaceae*

Content compounds: catechin tannins, triterpenes, silicic acid, traces of essential oils, amides of nicotinic acid, traces of iron
Drug must contain at least 3,5% of tannins.

Identification:

1- See test 1. in *Folium hamamelidis* drug. 2- See test 3 in *Quercus Cortex* drug.

Color reaction	Cortex quercus	Folium hamamelidis	Radix bistortae	Radix tormentilae	Herba agrimoniae
No. 18 Reaction with alum					
Reaction with conc. sulfuric acid					
Precipitate reaction					
10% $AgNO_3$					
5% $K_2Cr_2O_7$					
4% $CuSO_4$					
3% $(CH_3COOH)_2Pb$					
Gelatin solution					

Identification of all polyphenols by colorimetric methods

0,500 g of the drug is mixed with 150 ml of water in the boiling flask. It is boiled for 30 more minutes in a water bath under a reflux condenser. The solution is filtered. The first 50 ml of the filtrate is removed.

All the polyphenols: 5,0 ml of the filtrate is diluted with water to 25 ml. 2,0 ml of this solution is mixed with 1,0 ml of phosphowolframic acid and 17,0 ml of 20% sodium carbonate solution. After exactly 2 minutes the absorbance is measured at 750 nm (A) using water as a blank. The content in percent is calculated using the formula:

$$\frac{3{,}125 \cdot A}{0{,}316 \cdot m}$$

m – weight of the drug in grams