Chapter 10

Qualitative Analysis of Phytochemicals from Sea Buckthorn and Gooseberry

Ana-Alexandra Sorescu, Alexandrina Nuta, Rodica-Mariana Ion and Lorena Iancu

Additional information is available at the end of the chapter

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Abstract

This chapter describes in detail recent research results obtained from the qualitative screening of different phytochemicals found in aqueous extracts of sea buckthorn and gooseberry, fruits with important pharmacological effects due to their high content in vitamin C. Phytochemical investigations reveal the presence of active principles (e.g., saponins, flavonoids, alkaloids, carbohydrates, terpenoids, etc.) in sea buckthorn and gooseberry and are accomplished by using well-established standard methods. All these qualitative determinations rely on the visual color change reaction as a basic response to the presence of a specific phytochemical compound. The active principles from sea buckthorn and gooseberry are extracted according to a well-settled extraction method, which involves infusing the fruits in an aqueous medium, for 24 h, at a constant temperature of 4°C.

Keywords: phytochemicals, qualitative screening, sea buckthorn, gooseberry, aqueous extracts

1. Introduction

Phytochemistry, basically described as the chemistry of plants and different plant parts, is generally considered an early subdivision of organic chemistry and is very important in the identification of plant compounds with medicinal properties [1].

Phytochemistry is associated with numerous species of secondary metabolites produced in plants by biosynthesis and the natural combination of all these secondary metabolites gives the general beneficial therapeutic effects of that specific plant [2, 3].

Plants biosynthesize phytochemicals to protect themselves from insect attacks and plant diseases. Phytochemicals ("Phyto" is the Greek word for plant) are plant chemicals with no nutritional value, non-essential nutrients, and with disease preventive properties. Some of the most common phytochemicals are lycopene (found in tomatoes), flavonoids (found in fruits), and isoflavones (found in soy) [4, 5].

Species belonging even to the same genus can differ one from another in different proportions and sometimes these differences are subtle and extremely difficult to determine. Therefore, new phytochemical methods quickly developed coming in addition to those that were already known and applied [6, 7].

There are many known phytochemicals, and each has its own possible action [8–10]:

- *antioxidant*: protect human cells from oxidative stress thus considerably reducing the risk of developing numerous types of cancer;
- *hormonal action*: isoflavones are able to imitate human estrogens, reducing the symptoms of osteoporosis;
- *antibacterial*: can be used as alternative therapy against infections caused by different bacteria;
- *physical action*: many phytochemicals physically attach to cell walls thus preventing the adhesion of pathogens.

Sea buckthorn (*Hippophae rhamnoides* L.), an ancient plant with modern attributes, has numerous pharmacological effects: cardioprotective, inhibits platelet aggregation, lowers the levels of cholesterol and blood pressure, and provides antioxidant activity. The berries have an orange-yellowish color (see **Figure 1a**) and are an important source of vitamin C and A, phenolic compounds (especially flavonoids), and phytosterols [11, 12]. The mineral content (whether it's the fruit itself or the juice) is another important factor, which comes to complete all the beneficial properties of sea buckthorn: five essential minerals (calcium, iron, magnesium, sodium, and manganese) and four trace elements (chromium, vanadium, selenium, and cobalt) [13].

Gooseberries (*Ribes grossularia*) are generally divided into two groups, namely European (*Ribes grossularia* var. *uva-crispa*) and American (*Ribes hirtellum*) [14]. The fruits (**Figure 1b**) contain

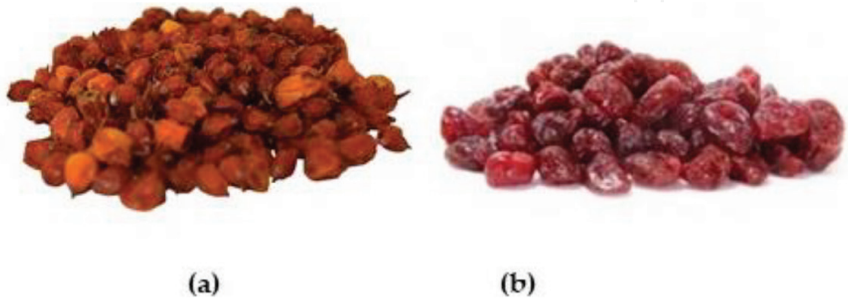

Figure 1. (a) Sea buckthorn and (b) gooseberry.

more than 80% water and important amounts of proteins, fibers, phenolic compounds, minerals, and vitamins [15]. Most species of the *Ribes* genus are rich in prodelphinidin, contain no ellagitannins, and are low in carotenoid content [16].

Although both sea buckthorn and gooseberry are used in traditional medicine for the treatment of various diseases, no clear scientific evidence exists to prove their therapeutic benefits and, therefore, it is very important to determine the qualitative content of these two fruits.

In this chapter, sea buckthorn and gooseberry dried fruits are used to prepare aqueous extracts using a method that involves the cold infusion at a constant temperature of 4°C for 24 h. The two aqueous extracts are further used for the qualitative screening of phytochemicals, and the most important bioactive chemical constituents that are studied are carbohydrates, flavonoids, alkaloids, glycosides, steroids, tannins, proteins, amino acids, and terpenoids. All these qualitative studies use standard analytical methods and the results are clearly detailed in the present chapter.

2. Preparation of aqueous extracts from sea buckthorn and gooseberry

Sea buckthorn (*Hippophae rhamnoides* L.) and gooseberry (*Ribes grossularia*) are bought readily dried from local natural shops and are further used to prepare aqueous extracts using a method that involves the following steps (**Figure 2**): grinding the dried fruits into a fine powder, weighting an exact amount of powder, and extracting it using a determined volume of distilled water at a constant temperature of 4°C.

The cold infusion takes place in sealed "French press" type coffee filters (**Figure 3**), one for every fruit involved in this research [17].

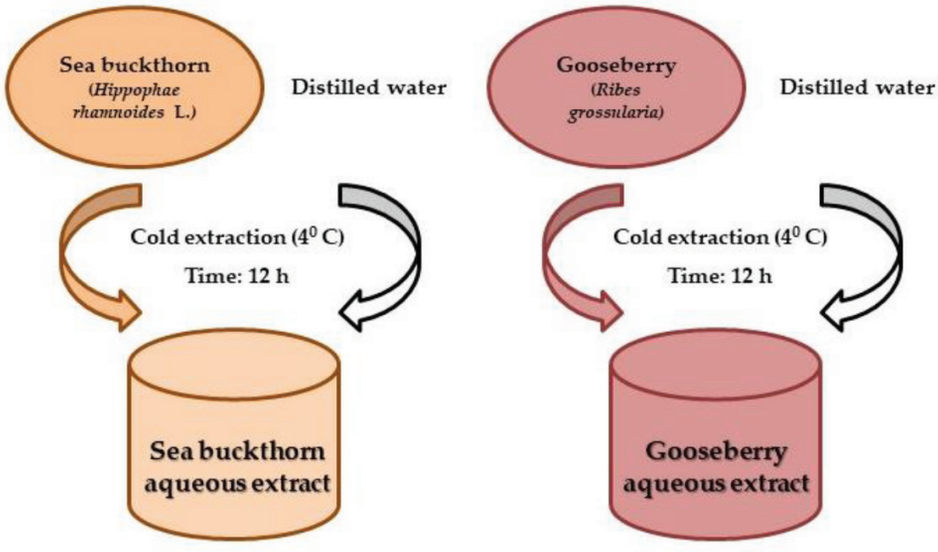

Figure 2. Preparation of sea buckthorn and gooseberry aqueous extracts.

Figure 3. "French press" type coffee filters used to prepare the aqueous extracts.

The two extracts were left to incubate for 24 h so that as much of sea buckthorn and gooseberry as possible could be transferred to the aqueous extracts. The aqueous extracts thus prepared were separated, filtered, and the volumes of the resulted aqueous extracts were measured. An additional vacuum filtration was carried out so that all debris were removed from the aqueous extracts.

The sea buckthorn aqueous extract and the gooseberry extract were kept in the refrigerator for more than 12 weeks for further use, without any alteration.

The extractive value (yield percentage) of the sea buckthorn and gooseberry samples were weighted before and after the preparation of the aqueous extracts and the results are presented in **Table 1** [18]:

$$\text{Extract yield } \% = \left[W_1/W_2\right] \times 100 \quad (1)$$

Crt. no.	Aqueous extract	Weight before extraction (g)	Weight after extraction (g)	Yield (%)
1	Sea buckthorn	25	18.66	74.64
2	Gooseberry	25	16.78	67.12

Table 1. Quantities of dry fruit before and after the aqueous extractions.

Crt. no.	Aqueous extract	Distilled water (mL)	Aqueous extract (mL)
1	Sea buckthorn	100	84
2	Gooseberry	100	92

The pH was measured for the two aqueous extracts and the value was 6.5 for sea buckthorn as well as for gooseberry aqueous extracts.

Table 2. Volume of resulted aqueous extracts.

where W_1 = net powder weight (g) resulted after the aqueous extraction and W_2 = total powder weight (g) used for the preparation of sea buckthorn and gooseberry aqueous extracts.

The volume of the resulted aqueous extracts was measured (mL) and compared to the initial volume of distilled water (**Table 2**).

3. Qualitative screening of phytochemicals from sea buckthorn and gooseberry

Different qualitative phytochemical analyses are known that allow, by using standard analytical techniques, the determination of chemical groups, or compounds in aqueous extracts from different plants. These qualitative tests are based on color or precipitation reactions as a positive response to the presence of those specific chemical compounds [19, 20]. All the color reactions allow only determining the presence or absence of various chemical groups and not the amount in which they are present in different aqueous extracts.

Standard qualitative methods are used to analyze qualitatively the aqueous extract prepared from sea buckthorn and gooseberry [21, 22].

3.1. Qualitative screening of carbohydrates

In nature, there are numerous carbohydrate materials that can be generally classified as follows [23]:

a. *Monosaccharides*: glucose, fructose, and galactose;

b. *Oligosaccharides*: sucrose, lactose, and maltose;

c. *Polysaccharides*: starch, glycogen, and dextrin.

Carbohydrates are usually neutral, water-soluble chemical compounds, but there are some exceptions and some, such as pectic acid, gluconic acid, or alginic acid, are acidic in the living world.

There are different standard phytochemical methods used for the qualitative screening of carbohydrates found in aqueous extracts [24]. The results obtained for sea buckthorn and gooseberry aqueous extracts are fully described in **Table 3**.

3.1.1. General screening of carbohydrates

Experimental: 1 ml Molisch reagent (a solution of α-naphthol in ethanol) is added to 2 ml aqueous extract and few drops of concentrated sulfuric acid are slowly dripped and the resulted solution is shaken carefully. The appearance of a violet ring at the interface of the two liquids indicates the presence of carbohydrates in the aqueous extracts.

In the case of sea buckthorn aqueous extract, the solution turns purple-red and a brown precipitate is obtained from gooseberry aqueous extract.

Phytochemical test	Sea buckthorn	Gooseberry
Carbohydrates (general)—Molisch	Purple red solution	Purple coloration
Carbohydrates (reducing sugars)—Benedict	Brick-red precipitate	Brick-red precipitate
Carbohydrates (reducing sugars)–Fehling A	Khaki solution	Green-yellow solution
Carbohydrates (reducing sugars)—Fehling B	Brown-yellow solution	Brown solution
Carbohydrates (monosaccharides)—Barfoed	Blue-green solution	Brick-red precipitate
Carbohydrates (reducing sugars)—Trommer	Red precipitate	Red-brown precipitate
Carbohydrates (reducing sugars)—Tollens	Black precipitate	Silver mirror
Carbohydrates (reducing sugars)—Moore	Red-brown solution	Red-brown solution

Table 3. Qualitative screening of carbohydrates.

3.1.2. Detection of reducing sugars

The general definition of reducing sugars is any type of sugar that can act as a reducing agent due to the free aldehyde or ketone groups. All monosaccharides are reducing sugars, along with some di-, oil- and polysaccharides. Several tests are available for detecting reducing sugars in aqueous extracts (**Figures 4** and **5**) (**Table 3**) [25].

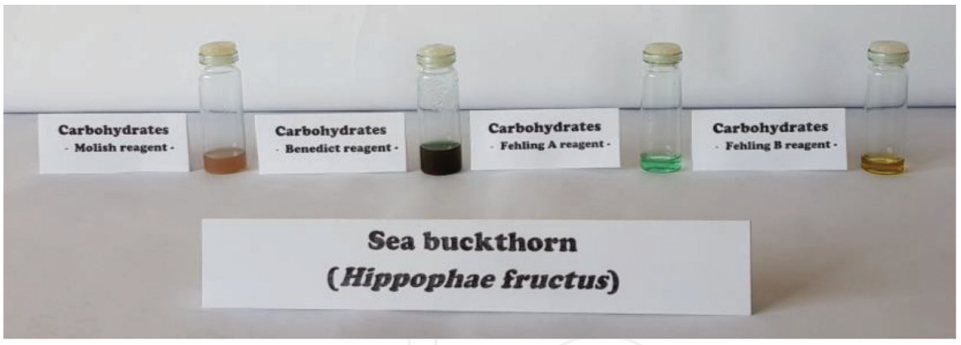

Figure 4. Carbohydrates in sea buckthorn aqueous extract.

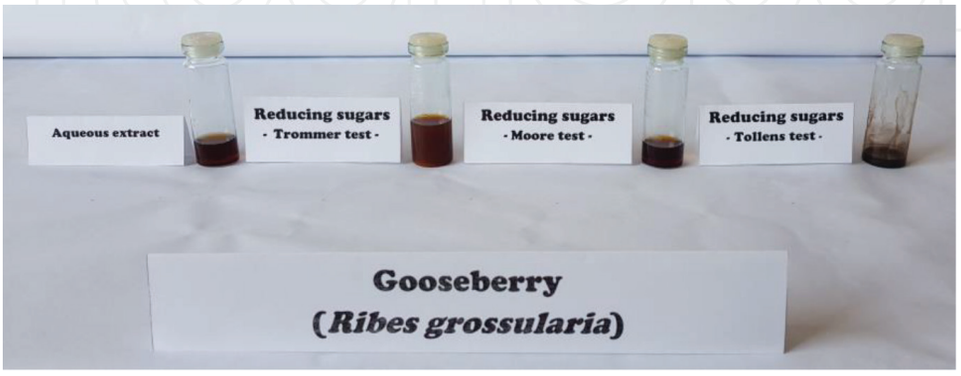

Figure 5. Reducing sugars from gooseberry aqueous extract.

a. *Benedict test*: to 1 ml of aqueous extract 5 ml Benedict's reagent (a complex solution of sodium carbonate, sodium citrate, and copper sulfate pentahydrate) was added and the resulted mixture is boiled for 5 min. Initially, the solution turns green and upon boiling a red, yellow, or green precipitate is formed.

b. *Fehling A test*: to 1 ml aqueous extract few drops of Fehling A reagent (aqueous solution of copper sulfate) are added; a green coloration indicates the presence of reducing sugars.

c. *Fehling B test*: to 1 ml aqueous extract few drops of reagent (a solution of potassium sodium tartrate in sodium hydroxide) are added and the formation of a brown coloration is a positive response.

d. *Barfoed test*: this test reveals the presence of reducing monosaccharides. To 1 ml aqueous extract, 3 ml Barfoed's reagent (solution of copper acetate) are added, boiled for 2 min and then cooled. A red precipitate is formed.

e. *Trommer test*: to 3 ml of aqueous extract an ml of 2.5% copper sulfate and 2 ml of 5% sodium hydroxide is added and the mixture is boiled for 3 min. Initially, a blue precipitate appears which turn red upon heating, thus indicating the presence of reducing sugars.

f. *Tollens test*: to 4 ml of aqueous extract a drop of dilute NH_4OH is added and then a solution of 0.1 M silver nitrate is poured to the resulted solution. After 5–10 min of boiling a silver mirror is formed (silver precipitates in the presence of reducing sugars).

g. *Moore test*: this test particularly reveals the presence of glucose. To 2 ml of aqueous extract an equal volume of 5% NaOH is added and the mixture is boiled for 5 min with. The solution has initially a yellow coloration that changer to reddish-brown.

By performing Molisch's test, it reveals that both aqueous extracts contain different classes of carbohydrates. Specific qualitative test for carbohydrates reveals the presence of monosaccharides in gooseberry aqueous extract and of di-, oil- and polysaccharides in both sea buckthorn and gooseberry extracts.

3.1.3. Detection of hexose sugars

Hexoses are monosaccharides that contain six carbon atoms and are divided into aldohexoses and ketohexoses depending on the functional group [26]. Three qualitative methods reveal the presence of hexose sugars and the results are presented in **Table 4**.

a. *Seliwanoff test*: to 1 ml of aqueous extract, 3 ml of Seliwanoff's reagent (a mixture of resorcinol in hydrochloric acid) is added and boiled for 2 min. A red solution is obtained indicated a positive reaction (**Figure 6**).

b. *Cobalt chloride test*: this test indicates the presence of either glucose or fructose or both. Three ml aqueous extract are mixed with 2 ml cobalt chloride and the solution in boiled. After cooling, few drops of 4% NaOH solution are added and the results are as follows: a greenish-blue solution (glucose), purplish-violet solution (fructose), or the upper layer turns greenish-blue, while the lower layer purplish (both glucose and fructose).

c. *Ammonium molybdate test*: this test reveals the presence of ketohexozes as follows: to 2 ml aqueous extract, 2 ml ammonium molybdate solution are added, the solution is then heated to form a bluish-green solution.

As it is clear from the **Table 4**, hexose sugars are present in both sea buckthorn aqueous extract as well as in gooseberry aqueous extract.

3.2. Qualitative screening of tannins and phlobatannins

Most of the tannins, a group of phenol compounds usually found in plants, are soluble in water. Phlobatannins are considered a novel class of ring-isomerized condensed tannins [17].

The test for tannins is generally described as [27]: to 1 ml aqueous extract 2 ml of 5% ferric chloride are added and a dark-blue or greenish-black color appears.

Phlobatannins are tested following a standardized method: to 1 ml aqueous extract of sea buckthorn and gooseberry few drops of diluted HCl (1%) is added and a red precipitate should appear (**Table 5**).

Tannins are present in both aqueous extracts, while small traces of phlobatannins can be found in gooseberry aqueous extract.

3.3. Qualitative screening of saponins

The general method involved in the qualitative analyze of saponins is: 2 ml of aqueous extract and 2 ml of distilled water are shaken for 15 min in a graduated cylinder. A 1 cm foam layer is a positive response to the presence of saponins (see **Table 6**).

Qualitative screening of saponins in aqueous extracts from sea buckthorn and gooseberry revealed that only the second one contains saponins.

3.4. Qualitative screening of flavonoids and phenolic flavonoids

Flavonoids have important functions in plants: attract pollinating insects, fight against different microbial infections, and control cell growth [28].

Flavonoids are tested according to the following method: 2 ml aqueous extract and 1 ml of 2N sodium hydroxide are mixed. A yellow color indicates the presence of flavonoids.

Phytochemical test	Sea buckthorn	Gooseberry
Carbohydrates (hexose sugars)—Seliwanoff	Cognac-red solution	Red solution
Carbohydrates (hexose sugars)—cobalt chloride	Lower layer-blue precipitate, upper layer-pink solution	Reddish solution, yellow-white precipitate
Carbohydrates (hexose sugars)—ammonium molybdate	Blue-green solution	Blue-green solution

Table 4. Qualitative screening of hexose sugars.

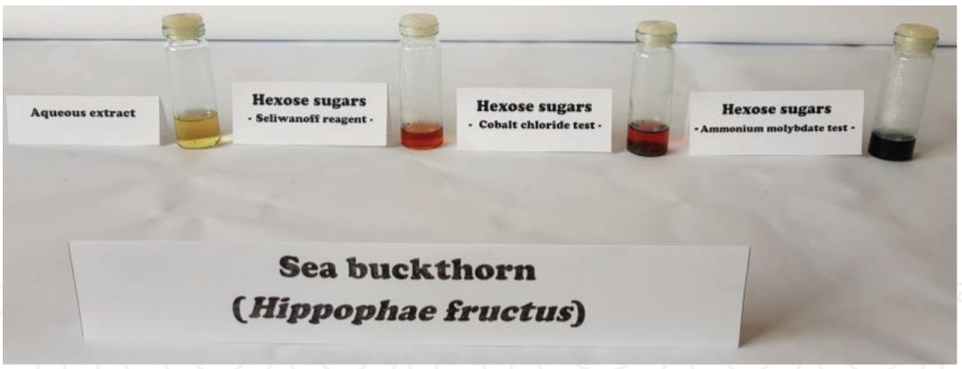

Figure 6. Hexose sugars in sea buckthorn aqueous extract.

Phytochemical test	Sea buckthorn	Gooseberry
Tannins	Green-black solution	Green-black solution
Phlobatannins	Pale pink solution	Red-orange solution

Table 5. Qualitative screening of tannins and phlobatannins.

Phytochemical test	Sea buckthorn	Gooseberry
Saponins	0.2 cm foam layer	1.5 cm foam layer

Table 6. Qualitative screening of saponins.

The test for phenolic flavonoids (**Figure 7**): 1 ml aqueous extract is mixed with 2 ml of 10% lead acetate solution and a brown precipitate indicates a positive response (see **Table 7**).

Flavonoids are present in both aqueous extracts (sea buckthorn and gooseberry), while phenolic flavonoids are present as small traces in gooseberry aqueous extract.

3.5. Qualitative screening of alkaloids

Alkaloids are a group of basic plant bioactive compounds that possess an N-containing heterocycle, are generally colorless, crystalline, insoluble in water but soluble in many organic solvents [29].

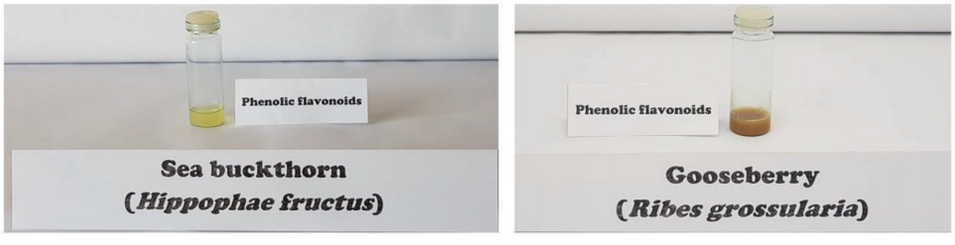

Figure 7. Phenolic flavonoids in sea buckthorn and gooseberry.

Phytochemical test	Sea buckthorn	Gooseberry
Flavonoids	Green-yellow solution	Light brown yellow solution
Phenolic flavonoids	Light yellow solution	Opalescent brown-yellow solution

Table 7. Qualitative screening of flavonoids and phenolic flavonoids.

There are three different standard phytochemical methods used to determine the presence of tannins in aqueous extracts from sea buckthorn and gooseberry:

a. *Wagner test*: 1 ml aqueous extract and 1 ml Wagner's reagent (iodine in potassium iodide solution) react and if a reddish-brown precipitate is formed it indicates a positive reaction.

b. *Mayer test*: to 1 ml aqueous extract, 2 ml concentrated HCl is added followed by few drops of Mayer's reagent (a solution of mercuric chloride and potassium iodide in water); a green color or white precipitate indicates the presence of alkaloids (the results are presented in **Table 8**).

c. *Hager test*: 2 ml aqueous extract and 2 ml Hager's reagent (a saturated aqueous solution of picric acid) are mixed together and a yellow precipitate indicates a positive test.

According to the results presented in **Table 8**, alkaloids are absent from all the aqueous extracts.

3.6. Qualitative screening of anthraquinones and anthocyanosides

The method used for the qualitative screening of anthraquinone compounds involves the reaction of 1 ml aqueous extract with a few drops of 10% ammonia solution with the formation of a pink precipitate.

Anthocyanosides are present when a pink color appears after the reaction between 1 ml aqueous extract with 5 ml dilute hydrochloric acid (1%). The results are detailed in **Table 9**.

Phytochemical test	Sea buckthorn	Gooseberry
Alkaloids—Wagner	Red-brown solution	Red-brown solution
Alkaloids—Mayer	Light-yellow solution	Red-brown solution
Alkaloids—Hager	Clear yellow solution	Red-brown solution

Table 8. Qualitative screening of alkaloids.

Phytochemical test	Sea buckthorn	Gooseberry
Anthraquinones	Green-yellow solution	Brown-yellow solution
Anthocyanosides	Light yellow solution	Brown-yellow solution

Table 9. Qualitative screening of anthraquinones and anthocyanosides.

According to the results presented in **Table 9**, anthraquinones and anthocyanosides are absent from both aqueous extracts.

3.7. Qualitative screening of proteins and amino acids

Proteins are involved in all physiological processes that take place in all living cells. Proteins are colloidal, do not diffuse through the plasma membrane, are irreversible coagulated upon heating and are insoluble in neutral salts [30].

Amino acids are amphoteric phytocompounds, highly reactive, with an amino and carboxylic acid moiety, therefore, being mostly water soluble.

3.7.1. General screening of proteins and amino acids

Experimental: 1 ml aqueous extract reacts with 5–6 drops of Millon's reagent (mixture of mercuric nitrate, mercurous nitrate, concentrated nitric acid, and distilled water) and a white precipitate is formed that changes its color to red upon heating. Millon's test is a non-specific test for detecting proteins and amino acids (tyrosine) and, therefore, it must be confirmed by other qualitative tests.

The results obtained after the two aqueous extracts react with Millon reagent are as follows: an opalescent orange solution in the case of Sea buckthorn and a red-brownish precipitate in the case of Gooseberry, therefore confirming the presence of small amounts of proteins and/or aminoacids in Gooseberry aqueous extract.

3.7.2. Detection of amino acids

There are two different standard methods used (see results in **Table 10**):

a. *Ninhydrin test*: take 3 ml aqueous extract and mix it with three drops of 5% lead acetate solution then heat the resulted solution. A purple or blue coloration indicates a positive reaction (**Figure 8**).

b. *Test for cysteine*: 5 ml aqueous extract is boiled with a small amount of 40% NaOH and few drops of 5% lead acetate solution are added. A black precipitate is formed.

The test for cysteine gives a positive reaction in the case of sea buckthorn, while ninhydrin test is negative for both aqueous extracts.

Phytochemical test	Sea buckthorn	Gooseberry
Proteins and amino acids—Millon	Opalescent orange solution	Red-brownish precipitate
Amino acids—ninhydrin test	Opalescent white-yellow solution	Opalescent orange solution, gray precipitate
Amino acids—test for cysteine	Red-brown solution, black precipitate	Opalescent dark-brown solution

Table 10. Qualitative screening of amino acids.

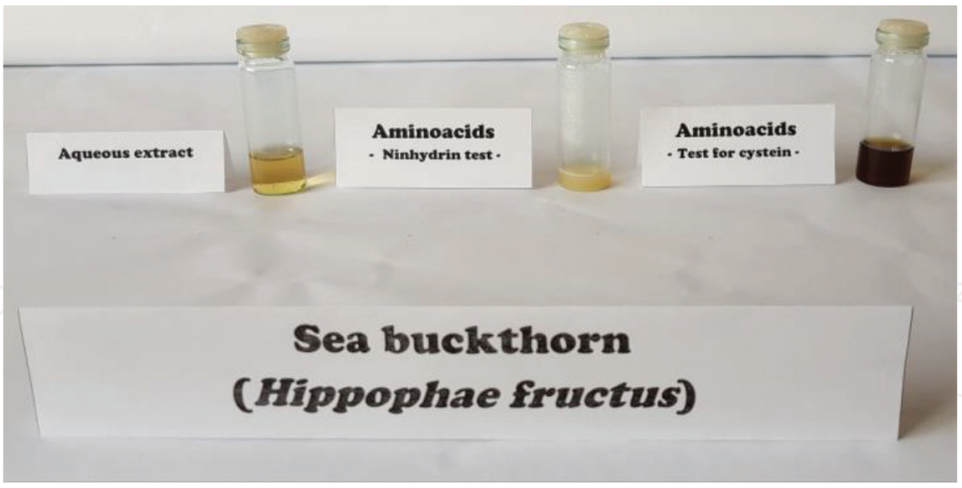

Figure 8. Amino acids in sea buckthorn.

3.7.3. Detection of proteins

There are two different standard methods used (see results in **Table 11**):

a. *Biuret test:* to 3 ml aqueous extract, 3 ml 4% sodium hydroxide solution, and few drops of 1% copper sulfate are added to form a purple solution.

b. *Xanthoproteic test*: to 3 ml aqueous extract, 1 ml of concentrated H_2SO_4 is slowly dropped. A white precipitate appears that turns yellow upon boiling and orange after 1 ml of NH_4OH solution is added.

3.8. Qualitative screening of steroids and terpenoids

The general procedure to test the presence of steroids is: to 1 ml aqueous extract, 10 ml chloroform is added and then slowly 10 ml sulfuric acid is dripped. Upper layer turns red and sulfuric acid layer turns yellow-green.

Terpenoids are analyzed by reacting 1 ml aqueous extract with 2 ml of chloroform and then, slowly, few drops of concentrated sulfuric acid. An interface with a reddish-brown coloration appears (**Table 12**). The change in color can be observed in **Figure 9**.

The qualitative screening of steroids revealed that these phytochemicals are absent from all the extracts while very small traces of terpenoids could be visually observed in gooseberry aqueous extract.

Phytochemical test	Sea buckthorn	Gooseberry
Proteins and amino acids—Millon	Opalescent orange solution	Red-brownish precipitate
Proteins—biuret test	Green solution	Brown solution
Proteins—xanthoproteic test	Opalescent brown solution	Dark-brown precipitate

Table 11. Qualitative screening of proteins.

Phytochemical test	Sea buckthorn	Gooseberry
Steroids	Colorless layer, brown ring, colorless upper layer	Pale-yellow layer, thick brown ring, pale-yellow upper layer
Terpenoids	Colorless	Brown interface

Table 12. Qualitative screening of steroids and terpenoids.

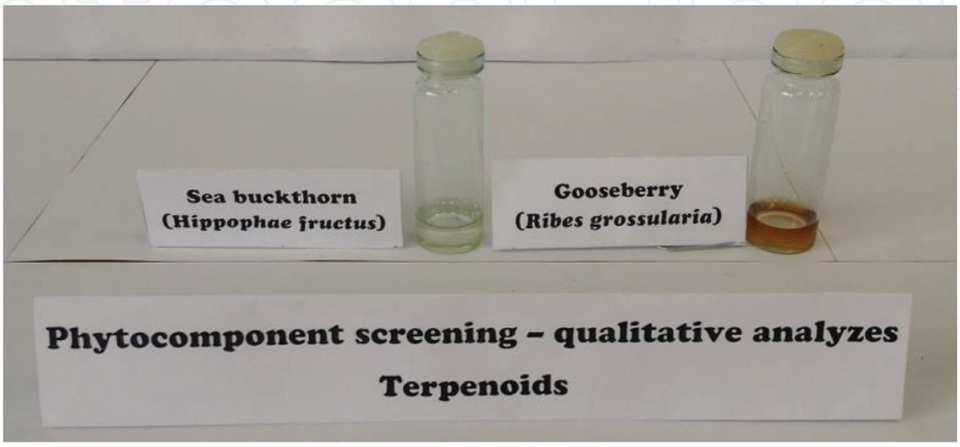

Figure 9. Terpenoids in sea buckthorn and gooseberry.

3.9. Qualitative screening of glycosides

There are three different standard phytochemical methods:

a. *$FeCl_3$ reagent*: the test is for cardiac glycosides: 1 ml aqueous extract, 1 ml $FeCl_3$ reagent (1 ml 5% $FeCl_3$ solution mixed with 99 ml glacial acetic acid) and few drops of concentrated H_2SO_4 gives a greenish-blue color that appears in time.

b. *Keller-Killiani test*: the test is for cardiac glycosides: 5 ml aqueous extract, 2 ml glacial acetic acid, a drop of $FeCl_3$ solution, and 1 ml concentrated H_2SO_4 form a brown ring and often a purple ring appears below (see results in **Table 13**).

c. *Borntrager test*: this test reveals the presence of anthraquinonic glycosides: 2 ml aqueous extract react upon boiling with 2 ml H_2SO_4. The solution is filtered, and equal volumes of chloroform are added and shaken vigorously, and two layers can be clearly observed. The organic layer is separated, and ammonia is added to form a pinkish-red color as a sign of positive reaction.

Phytochemical test	Sea buckthorn	Gooseberry
Glycosides (cardiac)—$FeCl_3$ reagent	Orange-yellow solution	Red-brown solution
Glycosides (cardiac)—Keller-Killiani test	Brown ring at the interface	Brown ring at the interface
Glycosides (anthraquinonic)—Borntrager test	Colorless lower layer, opalescent white upper layer	Colorless lower layer, light-yellow upper layer

Table 13. Qualitative screening of glycosides Keller-Killiani test is positive for both aqueous extracts.

4. Conclusions

This chapter describes the qualitative phytochemical screening of two aqueous extracts prepared from dried fruits of sea buckthorn and gooseberry, plants with the important pharmacological properties and rich in nutrients. The qualitative screening consists of standard methods that are able to determine whether a phytochemical is present or not in the aqueous extracts.

The two aqueous extracts are obtained after a cold infusion at a constant temperature of 4°C for 24 h and are kept at the refrigerator for more than 12 weeks without alteration.

The general screening of carbohydrates revealed that, in the case of sea buckthorn aqueous extract, the solution turns purple-red and a brown precipitate is obtained from gooseberry aqueous extract. Molisch's test revealed that both aqueous extracts contain different classes of carbohydrates. Specific qualitative test for carbohydrates reveal the presence of monosaccharides in gooseberry aqueous extract and of di-, oil- and polysaccharides in both sea buckthorn and gooseberry aqueous extracts.

Alkaloids are absent from both extracts, while cardiac glycosides are present. The test for cysteine gives a positive reaction in the case of sea buckthorn, while ninhydrin test is negative for both aqueous extracts.

The results obtained when aqueous extract from sea buckthorn reacts with Millon reagent is an opalescent orange solution and, in the case of gooseberry aqueous extracts, a red-brownish precipitate is formed, thus confirming that small amounts of proteins and/or amino acids are present in gooseberry.

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Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Author details

Ana-Alexandra Sorescu[1,2]*, Alexandrina Nuta[1,3], Rodica-Mariana Ion[1,2] and Lorena Iancu[1]

*Address all correspondence to: anaalexandrasorescu@yahoo.com

1 The National Research and Development Institute for Chemistry and Petrochemistry—ICECHIM, Evaluation and Conservation of Cultural Heritage, Bucharest, Romania

2 Materials Engineering Doctoral School, Valahia University, Târgoviște, Romania

3 The Romanian Academy, "Stefan S. Nicolau" Institute of Virology, Bucharest, Romania

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