Methods for the isolation and identification of triterpenes and sterols in medicinal plants

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ABSTRACT

Scientists around the world are constantly working on new drugs to fight many incurable diseases. Great hopes come with triterpene compounds and sterols. Refinement and selection of the appropriate techniques of isolation and identification, allows for a more satisfactory recovery sought from plant matter. This paper presents the most commonly used techniques for the isolation and identification of triterpenes and sterols.

Keywords: triterpene compounds, sterols, HPLC metod, TLC method

GENERAL CHARACTERISTIC OF TRITERPENES AND STEROLS

Triterpenes are a large group of natural substances occurring in the plant environment. They have a cyclic structure composed of a carbon skeleton based on six isoprene units. Except for squalene, all of them have a cyclic structure. They can be constructed from four (tetracyclic) or more, often five circles (pentacyclic structure). These compounds usually have one double bond and a secondary alcohol group. They differ in their distribution, number and type of oxygen functional groups. Triterpenes are two basic systems: prostane and dammarane. These systems differ in configuration at C–8 carbon atoms, C–14, C–17 and C–20 [23]. The most common triterpenes are in the form of acid, less common in alcohol and ketone compounds [51].

Up until recently more than 3000 of these compounds were noted. Cyclic triterpenes compounds are fixed, non-volatile, lipophilic and soluble in organic solvents. After the addition of acetic anhydride and concentrated sulfuric acid they exhibit a colour change (Lieberman-Burchard reaction). In particular, one can often find them in juice products, resins and the cortical tissues of trees (mainly acids and triterpene alcohols). They are also found in other plants such as fungi and lichenes [25]. One plant may contain up to 30-40 triterpene structurally different compounds. This usually depends on the amount of sugar, which can be as high as 8-10, and the types of compound connections [57]. The best known compounds are triterpene: α- and β-amyrin, ursolic acid, oleanolic acid, betulin. Recent studies indicate that the triterpene compounds appear in the family *Rosaceae*, *Potentilla erecta* in the species, *Rubus fruticosus*, mushrooms *Ganoderma* and *Inonotus* type, of which their are more than 50 compounds [25].

Triterpene saponins are the most valued in terms of pharmacology. They have anti-inflammatory, hypoglycemic and most important anti-cancer activity [21].

Plant sterols (phytosterols) have a hydroxyl group at position C3 or a double bond and the presence of an aliphatic chain at position C17. These are tricyclic compounds with varying degrees of saturation of the primary chain with a different number and type of side substituents [43]. Naturally-occurring sterols are derived from hydrocarbon systems: ergostane, stigmastane and cholestane. Sterols are present in both free and bound-in form of glycosides and esters. Especially prevalent in the world of taller plants are sitosterols (mainly β-sitosterol, less common are α - and γ-sitosterol). These are such as ergosterol (ergot fungus *Claviceps purpurea* spore), stigmasterol (*Glycine soya*, *Physostigma venenosum*), sitosterol (essential germinating cereal).

Plant sterols are precursors for the synthesis of steroid hormones, including estrogen and testosterone [27,54].

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Sterols have many activities, including cholesterol-lowering effect, anti-thrombotic, anti-atherosclerotic, anti-cancer, anti-ulcer and they also inhibit allergic reactions [1,7].

Triterpenes and sterols certainly give a much needed hope for medicinal advances and new drugs. The development of science and technology allows for even better opportunities for separation and isolation of these substances. Extracts are normally mixtures of many compounds having different polarities. Consequently, the purification step is very important [4].

METHODS OF TRITERPENES AND STEROLS EXTRACTION

One of the most important goals for each phytochemist is finding the proper extraction method, as well as the solvent. Thus, in order to optimize separation of the triterpene acids (ursolic acid, oleanolic acid, barbinervic acid, rotungenic acid and 24-hydroxy ursolic acid) in *Diospyros kaki* leaves, Fan et al. compared several extraction methods such as: Soxhlet extraction, reflux extraction, ultrasound assisted extraction, microwave assisted extraction. As the most effective microwave assisted extraction was chosen. Further experiments showed extraction time and solvent to solid ratio were statistically most significant factors on total triterpenoid extraction. The final extraction was performed under microwave power of 365.3 W for 13 min, with 60.27% ethanol as a solvent to solid ratio 22.26 [10]. The solution of methanol (70%) in water was used for choosing the proper extraction method of triterpenoid saponins from *Pulsatilla turczaninovii*. Microwave-assisted extraction (MAE), ultrasonic extraction (USE) and heat reflux extraction (HRE) were compared and MAE with a solid/liquid ratio of 1:20 (g/ml), a microwave power of 500 W, an extraction temperature of 80°C and an irradiation time of 3 min was the most effective [65]. Similarly MAE in a closed system was, compared with the classic techniques (maceration, Soxhlet and heat reflux extraction) and other modern techniques (ultrasonic extraction and accelerated solvent extraction), the most effective technique for extraction of two triterpenic acids: ursolic and oleanolic from *Lamii albi* flos. Ursolic acid were extracted in highest yield with 100% of generator power for 10 min, whereas for oleanolic acid longer time but lower energy was needed (30% generator power and 30 min) [60].

Some experiments showed that for those compounds ultrasound extraction was much better. Both urosloic and oleanolic acids were extracted from *Ligustrum lucidum* when ultrasonic irradiation was applied and less polar solvent was used (95% ethanol, 10 min at 40°C with the ratio of material to liquid at 1:20) [62]. Oleanolic acid, ursolic acid, hydroxyursolic acid, hydroxyoleanolic acid and euscaphic acid were also extracted by sonication in 100%

ethanol from *Prunella vulgaris*. Before, mixtures of alcohols with water were tested, but interfering compounds were also extracted, whereas pure ethanol reduces the extraction of polar interfering molecules [28].

Steroids and triterpenoids from stems, leaves and flowers of *Chresta exsucca*, *C. scapigera* and *C. sphaerocephala* were extracted by maceration (24 h, room temperature) and ultrasonic extraction (30 min, 30°C) using organic solvents (hexane, dichloromethane and methanol) and similar results were obtained. Stigmasterol, Δ7-stigmastenol, β-amyrin, β-amyrin acetate, α-amyrin, α-amyrin acetate, lupeol, lupeol acetate, β-amyrinonil acetate and α-amyrinonil acetate were extracted by both extraction methods, whereas the triterpenes: β-amyrin acetate, α-amyrin acetate, and lupeol acetate were more extracted by the ultrasound-assisted process, the triterpenes 11-oxoolean-12-ene, 11-oxours-12-ene, β-amyrinonil acetate, and α-amyrinonil acetate were better extracted by maceration [9].

Efficient and selective supercritical fluid extraction was compared to Soxhlet extraction with *n*-hexane and ethanol. Those methods were applied for isolation of β-amyrin and β-sitosterol from *Taraxacum officinale*. The effects of the pressure (150-450 bar) and temperature (35–65°C) were tested. Extraction under 450 bar at 60°C were the most effective. The yield obtained by SFE under mentioned conditions was slightly lower than that by hexane extraction but the concentrations of target compounds was higher. The yield of alcohol extraction was the highest but it is worth noticing that ethanol extracted unwanted, ballast materials from the plant and the final concentrations of target active compounds were very low [48].

Conventional Soxhlet extraction using a variety of solvents and supercritical fluid extraction were also compared in order to check the extraction efficiency of oleanolic and ursolic acid from seeds and leaves of *Plantago major* L. When diethyl ether was used in first tested method, the obtained results were similar to SFE. Simple SFE-CO_2 extraction without polar modifier was found not to be a suitable due to the low content of lipophilic and volatile compounds [52]. Ursolic acid was also isolated from *Ocimum sanctum* leaves and a stirred batch extraction was performed. The influence of various parameters has been studied and extraction for 40 min with methanol as a solvent at the temperature 50°C, speed of agitation 1000 r/min and solute to solvent ratio of 1:120 were optimum extraction conditions [55].

In order to quantitative triterpene distribution in various plant materials eg. *Aesculus hippocastanum, Aloe vera, Lavandula angustifolia, Thymus vulgaris, Olea europeae* accelerated solvent extraction (ASE) with ethyl acetale was used [20]. Triterpenes were also extracted for 2 h under the reflux from *Ganoderma lucidum*. The solu-

tion of ethanol and water (9:1) was used as extraction solvent and temperature was kept at about 80°C [15].

THIN LAYER CHROMATOGRAPHY IN IDENTIFICATION AND SEPARATION OF TRITERPENES AND STEROLS

TLC (thin layer chromatography) is an analysis technique of identification and separation. The most widely used adsorbent in TLC technique is silica gel. Complementary to silica gel plates are reversed phase C8 and C18. The combination of both techniques (silica gel and RP18 adsorbent) can improve the separation of analytes. The most commonly used solvents are prepared for TLC on silica gel and contain different proportions of chloroform-methanol-water (see Table 2). The use of other alcoholic solvents such as ethanol or isopropanol can significantly improve the separation. In case of more polar compounds, solvents should be acidified with acetic acid. Solvents should be selected depending on the type of the analyzed substances. Nonpolar and middle-polar solvents (including ethyl acetate and chloroform) are designed for simple extraction of derivative triterpenes, the aglycones or monoglucosides. For more complex compounds such as saponins referred type, aqueous methanol or ethanol are used. Triterpene compounds exhibiting high polarity are better separated after using a mixture of n-butanol-acetic acid-water (BAW) [2]. The separation of sterols is quite difficult and is carried out using a mixture of hexane-diethyl ether (97:3) [22]. Separation of triterpenes, depending on the occurring double bonds is possible by using a spray with concentrated aqueous methanolic $AgNO_3$ on the silica gel and activated for 30 min at 120°C [19].

All triterpenes (with the exceptions of glycyrrhizic acid and cucurbitacines) developed in silica gel are visible under UV light as black spots. To eliminate this, it is recommended to use different types of reagent spray. There are 50 different reagent sprays currently know (see Table 1) [31].

Table 1. Spray reagents used for triterpenes and steroids TLC detection [58].

REAGENT	COLOUR OF SPOTS
Blood reagent	White zones on a reddish background
Ehrlich reagent	Red coloration of furostanol derivatives, spirostanols do not react
Komarowsky reagent	Blue, yellow and red
Liebermann-Burchard	Blue, pink, green, brown, yellow in visible light, also seen under UV
Water	Sterols give white, not wetted spots
Vanillin-sulfuric acid	Blue, blue-violet and yellowish
Anisaldehyde-sulfuric acid	Blue red-violet in visible light and reddish or blue in UV-365

For the development of colour spots, it is necessary to heat the sprayed areas at 105°C-110°C for a few minutes. It should be noted that overheating can ruin the results, also that the coloured dots are not very stable [58]. Separation and determination of saponins in plant extracts is performed using 1D and 2D dimensional modes of TLC.

This method is very efficient, but there are several disadvantages associated with the parallel running of these applicable standards, variations between plates and colour reactions with reagent sprays [58].

An additional method of determining qualitative and quantitative composition of triterpenes is the TLC-colorimetry method. Test substances are subjected to the colorimetric determination in a crude extract and referred to their presence in the sample or the TLC separated bands are translated, extracted with ethanol and treated in an appropriate reagent (usually Earlich or vanilin reagents). Any misstatements may be a result of the presence of these components in the extract, for example sterols and acids with hydroxyl groups. The clean-up stage (SPE) of an inverted phase conducted before the final determination, effectively eliminates the disadvantages of this method [58].

Table 2. TLC methods of triterpenes and sterols determination.

Plant species	Compounds	TLC solvent	Ref.
Brassica oleracea L. (*Brassicaceae*)	α-Amyrin β-Amyrin Lupeol	C18 MeOH-CHCl₃ (1:1)	[33]
Hieracium pilosella L. (*Asteraceae*)	α-Amyrin β-Amyrin	n-C₇H₁₆-C₆H₆-EtOH (50:50:0,5)	[16]
Arnica montana L. (*Asteraceae*)	Arnidiol, Faradiol	CH₂CL₂-EtOAc (9:1)	[47]
Betula pendula Roth. (*Betulaceae*)	Betulin	n-C₆H₁₄- C₂H₄- C₂H₂ (2:1)	[11]
Cucurbita pepo L. (*Cucurbitaceae*)	Cucurbitacin	Diethyl ether-hexane-MeOH (70:30:5) CHCl₃-MeOH (95:5)	[35]
	Cucurbitacin B, D, E, I	MeOH-H₂O (45:55; 70:30)	
	Cucurbitacin C	EtOAc-C₆H₆ (75:25) Spray reagent: anilin-orthophosphate in EtOH	
Glycyrrhiza glabra L. (*Fabaceae*)	Glycyrrizin	BuOH-OHAc-H₂O (5:1:4) Spray reagent: 10% H₂SO₄	[38]
Gypsophila repens L. (*Caryophyllaceae*)	Gypenosides	BuOH-OHAc-H₂O (4:1:5) Spray reagent: phosphortungstic acid	
Olea Europea L. (*Oleaceae*)	Oleanolic acid	CHCl₃-Et₂O-MeOH (30:10:1) Spray reagent: anisaldehyde 10% H₂SO₄	
		C₆H₆-Me₂CO (36:13) Spray reagent:10% H₂SO₄	
Panax ginseng C.A. Meyer (*Araliaceae*) Protopanaksadiol	Proto-panaksatriol	CHCl₃-MeOH-H₂O (13:17:1)	[66]
		CHCl₃-MeOH-H₂O (10:3:1)	[36]
		C18 MeOH-H₂O (7:3)	
		CHCl₃-MeOH-H₂) Spray reagents: NH₄HSO₄ in 15% H₂SO₄ BuOH-EtOAc-H₂O (4:1:2)	[38]
		C₂H₄Cl₂-BuOH-MeOH-H₂O (30:40:15:25)	
		CHCl₃-MeOH-H₂O (65:35:10) Spray reagents: 10% H₂SO₄	
		CHCl₃-MeOH-H₂O (21:11:4) Spray reagents: Vaniline in H₂SO₄	

None of the current chromatographic techniques provides the optimum quality that
is required for the registration of medicinal plants but the TLC methods are still recommended by U.S. Pharmacopeial Forum if confirmation of peak identity is required.

The phytochemical work aimed at isolation and identification of natural products, the TLC is becoming preferably a supporting technique in the analysis of triterpene fractions, obtained from the column chromato-

Table 3. HPLC methods of triterpenes and sterols determination.

Plant species	Standard	HPLC solvent	Column	Ref.
Brassica oleracea L. (Brassicaceae)	α-Amyrin β-Amyrin Lupeol	ACN: 6.5% H₂O in ACN 0.8 mL/min Total time 35 min	Hypersil BDS C18 3 μm, 250 mm x 3 mm, 20°C	[33]
Betula pendula Roth. (Betulaceae)	Betulin	ACN: H₂O (80:20) isocratic mobile phase 1 mL/min	Supelcosil C18 (Sigma-Aldrich) 5 μm, 250 mm x 4.6 mm, 20°C	[11]
Lantana camara L. (Verbenaceae)	Betulinic acid	ACN: H₂O (80:20 v/v) 1 mL/min.	Hypersil BDS C18 250 mm x 4.6 mm	[49]
Boswellia serrata Flueck (Burseraceae)	Boswellic acid	Gradient from hexane to EtOAc and finally MeOH. Eluted with EtOAc-hexane (1:1)	C18 Superspher 100 250 mm x 4mm	[6]
Wilbrandia ebracteata Cogn. (Cucurbitaceae)	Cucurbitacin	ACN: H₂O (40:60) 1.2 mL/min	Supelco C18 2-5 μm, 150 mm x 4.6 mm	[39]
Ganoderma lucidum P. Karst. (Ganodermataceae)	Ergosterol	ACN 1.0 mL/min	C18 Diamonsil, 40°C	[30]
Glycyrrhizae glabra L. (Fabaceae)	Glycyrrizin	ACN: H₃PO₄ 1.0 mL/min.	Kromasil C18 4 μm, 4.6 mm x 250 mm, 35°C	[63]
		ACN:0.05% H₃PO₄ 1.0 mL/min. Time: 11 min	Agilent Eclipse Plus C18 1,8 μm, 4.6mm x 50 mm	[40]
		MeOH-perchlorate buffer (pH 7,5-7,7) from 1:9 to 10:0 in 120 min	LiChrospher C18 5 μm	[53]
		Phosphate buffer (pH 2.5): ACN 0 min 100:0 10 min 80:20 50 min 70:30 73 min 50:50 110 min 50:50 125 min 20:80 140 min 20:80	Mightysil C18 5 μm, 4.6 mm x 250 mm, 40°C	[59]
Panax ginseng C.A. Meyer (Araliaceae)	Ginsenosides	0.2% formic acid water (A): ACN(B) 20-30% B 0-3 min 30% B 3-10 min 30-50% B 10-17 min 50-90% B 17-19,5 min 90% B 19,5-24 min 90-100% B 24-25 min	Agilent Zorbax Extend-C18 1.8 μm, 4.6 mm x 50 mm, 25°C	[41]
		MeOH- H₂O (440:560)	Porasil μBondapak C18	[50]
		water(A):ACN/ H₂O (80+20 v/v)(B) 24% 0 min 24% 8 min 32% 18 min 40% 25 min 48% 42 min 100% 43 min 100% 44 min 24% 45 min	Agilent 1100 series-C18 5 μm, 150 mm x 4 mm, 25°C	[3]
		ACN: H₂O 1.0 mL/min	Kromasil C18 5 μm, 250 mm x 4.6 mm, 25°C	[5]
		CH₃CN(A)-H₂O(B) 20-22% A 10 min 28% A 11 min 42% A 25 min 100% A 26-30 min	Phenomenex Synergi Hydro RP 2 μm, 150 mm x 2.0 mm, 20°C	[14]
Hedera helix L. (Araliaceae)	Hederagenin	CHCl₃-MeOH 21:1 → 2:1	Cosmosil 5SL-II 250 mm x 10 mm	[18]
		Sodium acetate (pH 6.5):ACN Concentration gradient 7-20%, 1.0 mL/min	Lichrospher ODS 100-5 RP-18 5 μm, 250 mm x 4.6 mm, 55°C	[17]
Viscum album L. (Loranthaceae)	Oleanolic acid	MeOH- H₂O (9:1) +0 .05% TFA	C18 YMC R & D ODS 4,6 mm x 250 mm	[34]
		Hexane/ethyl acetate (50:50)	LiChrosorb Diol C18 5 mμ , 4.0 mm x 250 mm, 25°C	[24]
Calendula officinalis L. (Asteraceae)	Oleanolic acid, Ursolic acid	0.05% acetic acid in ACN(A): 0.5% acetic acid in H₂O(B) 0.6 mL/min. Total time: 110 min 0.00-15 min. 20% A 45 min 46% A 50 min 55% A 50-90 min 55% A 95 min 90% A 105 min 20% A	Eurospher 100-C18 5 μm, 250 mm x 4 mm	[26]
Prunella vulgaris L. (Labiatae)		0.01M phosphate buffer: MeOH (12:88 v/v) 0.8 mL/min.	Phenomenex Luna C18 5 μm, 250 mm x 4.6 mm, 25°C	[28]
Lamium album L. (Labiatae)		ACN: H₂O:1% H₃PO₄ (85:15:0.5 v/v/v) 0.8 mL/min.	LiChrospher 100 C18 5 μm, 250 mm x 4 mm, 10°C	[60]
Ligustrum lucidum W.T. Aiton (Oleaceae)		ACN: 0.5% acetic acid in H₂O (90:10 v/v) 0.8 mL/min.	Agilent Zorbax Extend C18 5 μm, 250 mm x 4.6 mm, 27°C	[62]
Vaccinium macrocarpon L. (Ericaceae)	Ursolic acid	2% aqueous acetic acid (A): MeOH with 2% acetic acid (B) 100% A 0-15 min 100% B 15-45 min 100% B 45-60 min	Nova-Pak C18 RP 20-μL, 150 mm x 3,9 mm	[37]
Pulsatilla turczaninovii Krylov et Serg. (Ranunculaceae)	Pulsatilla saponin A3, pulsatilla saponin B4, 23-hydroxybetulinic acid, pulsatilloside B, pulsatilloside C, cirenshenoside S, oleanolic acid	eOH + 0.1% formic acid (A): 0.1% v/v aqueous formic acid 700 mL/min	Sapphire C18 5 μm, 250 mm x 4.6 mm, 30°C	[65]

graphy. This is a very good technique that presently cannot be replaced with any other method.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) IN IDENTIFICATION AND SEPARATION OF TRITERPENES AND STEROLS

Currently, there are many techniques for the identification and separation of triterpen compounds. HPLC is the best and most valued technique used for this purpose because HPLC can deal with nonvolatile, highly polar compounds. This method has been widely used for the determination of both intact aglycones and saponins. Compound selection is usually prepared as standard (silica gel) and reversed phase (C8, C18) column, of which C18 is more desirable, occasionally -NH$_2$ and diol additives are used. Research has shown that carbohydrate and NH$_2$-modified columns are more efficient when it comes to the separation of some steroidal saponins and glycoalcaloids [46,64].

The main problem within the HPLC method is the detection of triterpenes because a large group of triterpenes has no chromophore groups necessary for UV detection [63]. Only a few compounds show supramaximal absorption in the UV range-detection at 254 nm (containing saponins: glycyrrhizin and glycyrrhetinic acid) [45]. Cucurbitacines are the second group, which can be monitored with UV detectors (maximum absorption of 232 nm). The use of precolumn derivatization solved the problem of low-wavelength UV through the introduction of a chromophore to the triterpene molecule and allowed UV detection at a higher wave length. Substances used for derivatization include bromophenacyl bromide (triterpenes with a carboxyl group), benzoate esters (hydroxy steroids and saponins) and 2,4-dinitrophenylhydrazine (DNPH) [8,12,13]. Evaporative Light Scattering Detection (ELSD) introduced to the detection of saponins also eliminates the problem of UV detection [44].

The method being increasingly used to identify saponins in plant extracts in recent years is a mass spectrometry combined with liquid chromatography. This technique is most reliable, and it provides qualitative and quantitative composition. However, unfortunately, this method is not perfect, because the structural information concerning the composition is limited.

Betuline is one of the first substances extracted from plant material; its isolation from the bark of the birch tree was made in the way of sublimation. Currently, there is a possibility of direct extraction of betulin and its structural analogues of plant extracts using the MIP technology.

This method was developed by French scientists and is based on impronted polymers synthesized by thermal polymerization of betulin as the template, methacrylic acid (MAA) or acrylamide (AA)-functional monomer, ethylene glycol dimethacrylate as crosslinking agent and chloroform as porogen. The next step was to compare the AA- and MAA-MIPs of their non-imprinted polymers (NIPS). This enabled the assessment of selectivity verses betulin and its derivatives. Obtained triterpene compounds were analyzed by HPLC at the MIP-SPE protocol. Analysis showed that after using the SPE optimization, the MAA-imprinted polymer was characterized by high selectivity and recovery (above 70%) from betulin and the greatest affinity for its analogues. Selective washing with chloroform and acetonitrile removed redundant matrix compounds (fatty acids) from the SPE cartridge (methanol as the elution solvent). MAA-MIP method has been successfully used for the fractionation of a methanolic extract from plane bark of betulin and betulinic acid [4].

Another method is for simultaneous determination of triterpenes by HPLC and sample preparation with matrix solid phase dispersion (MSDP). Chinese scientists presented it on the basis of the analysis of Chinese herbs. The analyzed sample was placed in an agate mortar and mixed with silica gel to obtain a homogeneous mass. The next step was to transfer the mixture to a teflon cartridge and triterpenes fraction eluted from the cartridge with the solvents dichloromethane-acetone (85:15). After evaporation of the solvent, the residue was dissolved in methanol. Triterpene compounds were separated on a ZORBAX stable bound (4.6 mm x 100 mm, 1.8 µm) C18 column by gradient elution (with acetonitrile and water-mobile phase) and identified with evaporative light scattering detection. This method is simple, selective and sensitive, and provides good reproducibility and sensitivity for the determination of the seven major triterpenes (betulin, betulinic acid, acetyl ursolic acid, ursolic acid, oleanolic acid, pomolic acid and erythodiol) [29].

OTHER METHODS USED IN THE IDENTIFICATION AND SEPARATION OF TRITERPENES AND STEROLS

The hemolytic activity of triterpene compounds is another method of identification. Some saponins have hemolytic activity and they can be used for the development of semi quantitative tests for their determination [32]. Hemolytic properties can be used for spotting hemolytic saponins on TLC plates. Developed plates should be gently dried from the solvent residue. After this, they need to be covered with a layer of gelatine/blood solution. After a few hours, whitening spots on the plates can be noticed, showing the presence of saponins [56].

Latest techniques allow researchers to determine the sequence of monosaccharides in the sugar chain, the linkage of sugars or the location of the sugars on the aglycone. These include mass spectrometry (MS) and the nuclear

magnetic resonance spectroscopy (NMR). However, using these methods is limited due to the high costs of equipment.

Research is being conducted on improving extraction techniques. One of them is the use of a microwave oven, which has become a very useful method for the extraction of oleanolic acid and ursolic acid from plant materials [61].

The GC-MS technique is sensitive and specific, it can therefore be used for the simultaneous identification and determination of a wide range of triterpene compounds in a variety of plants, even in trace amounts [42].

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